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Thyroid nodules and nodular goitre: a stem cell disease?*

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INTRODUCTION

The occurrence of nodules by age is a hallmark of the human thyroid gland and some other glands (1). Indeed 20 to more than 50% of the normal population develop one or more thyroid nodules during life-time (2). The majority of these nodules are non-functioning (more than 80%) but only 5% of these scintigraphically "cold" lesions are malignant tumours (3, 4). In 20% of these cases, a follicular neoplasia is cytologically diagnosed by fine needle biopsy, which makes a subsequent surgery obligatory (5). But only in a few percent of these patients histological diagnosis confirms malignancy. Unfortunately, despite many efforts, up to now there is no pre-operative approach to clearly differentiate between benign and malignant tumours in these patients. One reason for this is our limited knowledge of the molecular aetiology of benign non-functioning nodules. Although many pathogenetic factors such as iodine deficiency, mutagenesis, overexpression of growth factors and their related receptors, altered signalling, recent data on gene expression profiles and a genetic predisposition are known a comprehensive concept for the pathogenesis of thyroid nodules and nodular goitres is still missing (6, 7).

Histologically, thyroid nodules are classified as adenoma (with a characteristic capsule), as adenomatous or hyperplastic nodule or lesion (8). However, independent of their histology, molecular biology analysis has revealed that a majority of these nodules are clonal, i.e. derived from a single cell or a naturally occurring clonal cell patch, and thus meet the criteria usually applied to characterize true benign neoplasias (reviewed in 6, 1).

Classical theory considers a differentiated follicular thyroid cell, altered by a sequence of molecular aberrations achieved during cell cycling, as the primary source of thyroid tumourigenesis (9). With this respect the high frequency of tumour formation in the thyroid gland seems to be surprising, since, compared to highly proliferating tissues such as the colon, the growth rate of human thyroid cells is rather low. It has been estimated that human thyrocytes divide only about five times during adulthood which corresponds to a turnover time of about 8.5 years for the follicular thyroid cell (10). Furthermore, tissues with high cell turnover such as the colon are more sensitive to mutagenesis and other molecular mechanisms that initiate tumour formation, whereas in resting tissues such as the thyroid these mechanisms should be less operative.

Adult stem and progenitor cell in thyroid tissue

Stem cells are classified as embryonic or as adult stem cells. An embryonic stem cell is derived from inner cell mass, which is part of the early (d 4 to 5) embryo called the blastocyst, whereas an adult stem cell is an undifferentiated, quiescent or slow cycling cell that occurs in a differentiated tissue (11, 12). Adult stem cells are capable of making identical copies of themselves throughout the organism's lifetime, which is referred to as "self-renewal". By "asymmetric" cell division they generate one self-copy and one precursor or progenitor cell, a partly differentiated cell that further divides and gives rise to differentiated cells. Adult stem cells have been detected in different tissues such as colon, skin, pancreas, liver and brain (13-

17). Recently, we have identified stem cells in the human thyroid gland (18).

Due to their pluripotency and undifferentiated state stem cells are widely believed to be involved in the pathogenesis of tumours since these cells share many properties with cancer cells such as self-renewal and indefinite growth (11, 19, 20). The hypothesis of thyroid cancer as a stem cell disease has also been proposed by several authors (21, 22). Proof for this assumption comes from the very recent finding of cancer stem cells in some thyroid carcinoma cell lines by us and by other (23, 24). In colorectal cancer, the concept of a stem cell disease has also been extended to the benign tumour precursors, the low-grade adenoma (25). In this benign neoplasia, stemness- and proliferation-associated genes are already activated.

Thyroid nodules, stem cells and their niches

Very recently, evidence has been provided for a stem cell hierarchy in the adult human breast (26). In contrast to the essentially quiescent stem cells, progenitor cells were found to be more actively dividing. If such a hierarchy also exists in the thyroid gland, one may speculate that (analogous to colorectal adenoma) such progenitor cells are the source for the generation of thyroid nodules.

Only about 1-2 of 10⁵ cells of nodular goitres are stem or progenitor cells (18, own unpublished data). Surprisingly, a relative resistance to growth stimulation was observed by us and by others (in mouse thyroid glands) when stem and progenitor cells were maintained in mono-culture (27, 28). Such observations that were also made with other stem cells may be explained by the interaction between stem cells and micro-environmental cells (niche cells) (29). Niche cells provide a sheltering environment that protects stem cells from uncontrolled differentiation stimuli, apoptotic stimuli and on the other hand from excessive proliferation that could lead to cancer (29). The lack of these regulatory cells may explain why adult stem cells when isolated as single cells do not proliferate despite intense growth stimulation.

However, which mechanism renders a quiescent stem or progenitor cell to proliferate and grow out to form a tumour? In vitro, induction of apoptosis reduces the strict control of niche cells (30). Under these conditions, stem cells in thyroid cell cultures gradually escaped niche control and grew out to form three-dimensional spheres, designated thyro-spheres, when stimulated with EGF and basic FGF (27). These stem cell-derived spheres were composed of 5% stem cells and 95% progenitor cells which may indirectly prove the much higher cycling activity of the progenitors as mentioned above. When growth stimulation of fast-cycling progenitor cells was terminated and TSH-enriched medium was added, proliferation rate of progenitor cells slowed down and the differentiation process was initiated.

Thyroid nodules and nodular goitres as a stem cell disease

Can the above described experiment serve as a model for the role of some fast-cycling progenitor cells in the pathogenesis of thyroid nodules? Are there any arguments that support the concept of thyroid nodules and nodular goitres as a stem cell disease?

There is some evidence from different studies that

1. stem cells reside in thyroid tissue (as in all other tissues) for life-time of the organism (29).
2. stem cells and their progeny are under the control of niches that limit proliferation of these undifferentiated cells (29).
3. induction of apoptosis and (excessive) growth stimulation can overcome strict niche control (30).
4. under these conditions actively cycling, more or less differentiated progenitor cells (some of them so far without a (fully) developed iodine metabolism) may grow faster than the surrounding differentiated thyrocytes.

Based on this evidence, epidemiological data, and the general concept of stem cells as a source of benign and malignant tumours we hypothesise a role of stem cells and their progeny in the pathogenesis of thyroid nodules and nodular goitres, as follows (figure 1): population studies have demonstrated that nodular transformation is increasing with age whereas the goitre size is decreasing (31-33). Throughout the aging thyroid gland, adult stem cells are detectable that maintain the capacity of proliferation and differentiation (18, 27). Experimental studies revealed that growth factors, their related receptors and growth-related signalling peptides are highly expressed or even overexpressed in thyroid nodules and nodular goitres (6, 34). Some of the growth factors are potent stimulators of thyroid stem cell growth in vitro (27). The proliferation of quiescent stem cells is controlled by signals from putative niche cells. In vitro, malnutrition can limit or even overcome the control which results in an outgrowth of stem cells as thyro-spheres. Histological and immunohistochemical studies demonstrated hypofunction, destruction and necrosis of normal thyroid tissue in goitre tissues (6), conditions that may be equivalent to in vivo focal malnutrition thereby affecting the control of niches on thyroid cell growth in vivo. In addition, there is some experimental evidence that apoptosis of thyrocytes is a main factor of cell loss during goitre formation (35). Apoptosis of

thyrocytes is, however, a prerequisite for thyro-sphere formation and therefore the proliferation of stem and progenitor cells in vitro (27). Thus, the short but intense stimulation of stem cells by growth factors in vitro may correspond to processes of nodular transformation in vivo that last for months, years or even decades. During this time, some cells may additionally accomplish molecular aberrations that provide a second growth advantage, for example ras mutations in few non-functioning thyroid nodules (reviewed in 7).

More than 20 years ago, transplantation of nodular goitre tissues on nude mice demonstrated autonomous growth of some thyroid cells with a constitutively higher growth potential (36). In addition, transplantation of autonomously growing embryonic human thyroids suggested that "the autonomously replicating cells that initiate nodule formation in human multinodular goitres reflect the persistence in the adult gland of cells with fetal growth potential" (37). Further studies are necessary to confirm the concept of thyroid nodules and nodular goitre as a stem cell disease and thus the putative role of cells with a "fetal growth potential".

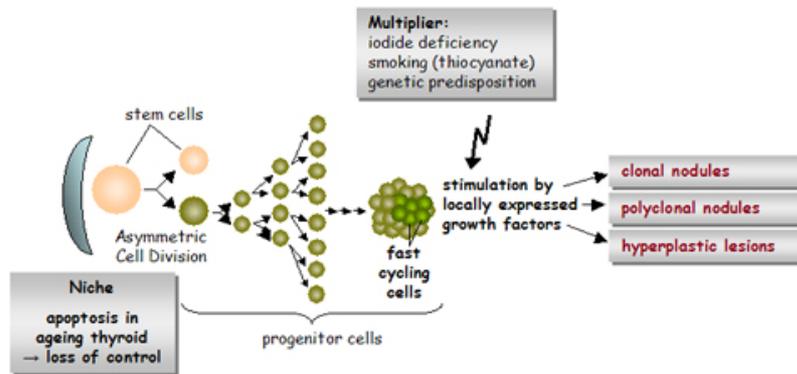


Figure 1. **Thyroid nodules and nodular goitre as a stem cell disease.**

Apoptosis, operative in ageing thyroids, may limit growth control by niches. In turn, a essentially quiescent stem cell may proliferate to give birth to a daughter stem cell and a progenitor cell (asymmetric cell division). Under the influence of locally expressed growth factors, one of these cells (or different cells) with a higher than average growth rate may grow out to form nodules or hyperplastic lesions, whereas unstimulated cells differentiate into normal thyroid cells as shown in vitro (27). Progenitor cells that do not achieve full differentiation, may be the origin of a non-functioning nodule or adenoma.

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The past, present and future status of iodine nutrition in Latin America.

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I. Background

Iodine deficiency is recognized as the most common cause of preventable brain damage and mental retardation. It also causes goiter, damaged reproduction, induces neurological and mental conditions, and other disorders, all together termed Iodine Deficiency Disorders (IDD) (1). Iodine deficiency is a permanent natural phenomenon widely distributed around the world and the association of severe iodine deficiency with endemic cretinism was recognized as a geographical-epidemiological fact long ago (2,3). Pioneering studies in the Region (4,5), and elsewhere (6,7), pointed the lack of iodine during pregnancy as a major cause of irreversible brain damage in the fetus, and this, rather than goitre, emerged as the gravest consequence of iodine deficiency. At the beginning of the 1990's it was estimated that at least 1500 million people, about one third of the world's population, were at risk of iodine deficiency, 655 million were affected by goiter, 11 million were overt cretins, and another 43 million had some degree of mental retardation (8).

The elimination of IDD as a public health problem by the year 2000 was advocated at the UN World Summit for Children in 1990, This unprecedented Declaration was endorsed by 71 Heads of States attending the meeting and representatives from other 88 governments. The goal of the elimination of IDD by the year 2000 was adopted by the World Health Assembly in 1991, and reaffirmed by the UN General Assembly in May 2002, and by the International Conference on Nutrition in 1992. In 1993, WHO and UNICEF recommended universal salt iodization (USI) as the main strategy to achieve elimination of IDD (9). As the goal was not achieved, the UN System at the UN General Assembly Special Session for Children (UNGASS) in May 2002 set the new target for elimination of IDD the year 2005.

Although enormous progress in the sustained elimination of IDD has been made by a number of countries, according to the latest report of the WHO to the 60th World Health Assembly (May 2007), about 31% (1900.9 million) of the world's population still have insufficient iodine intakes, and, as a result, twenty-two million children each year are at risk to fail to reach their full intellectual potential. The most affected Regions are South-East Asia and Europe, while the American Region has achieved the most significant progress (10).

Recognizing the importance of IDD elimination the World Health Assembly in 2005 adopted the Resolution WHA58.24 committing to reporting on the global IDD situation every 3 years.

II. The past and present situation of IDD in Latin America

1. Past situation

The American countries have a rich history of iodine deficiency. Pre-Colombian statues in the Andean regions and in Mexico show that endemic goiter existed there long before Columbus arrived. In the early 20th century, iodine deficiency was recognized as a public health problem in most of the Latin America countries, The severity of iodine deficiency followed geologic patterns similar to those elsewhere in the world. The worst endemias were in isolated mountain communities. The Andean Regions and Central Mexico were the most affected, but many other parts of the hemisphere were also severely involved, and virtually no country in mainland Latin America was free of iodine deficiency (11-13).

Modern surveys for goiter within individual counties began in the 1930s. Almost all had at least some regions where the goiter prevalence was more than 50% and several countries such as Bolivia, Brazil, Ecuador, Peru, Mexico and Guatemala, had iodine deficiency in most of their territory.

In the 1950s to 1970s, most countries passed legislation on iodized salt, establishing arbitrarily a wide range of iodization levels (11,12), likely because of poor information on the daily physiologic needs of iodine (Table 1). Unfortunately, after some initial success in most of the countries, later some of them relapsed, e.g.. Guatemala, Colombia and Mexico, mainly because several common problems emerged. First, laws were not enforced and did not fix responsibility for absorbing the cost of salt iodization. No Latin America country addressed these issues satisfactorily. Secondly, monitoring was either absent or inadequate. Thus, after initial enthusiasm on the part of the government and the salt industry for regular checks on iodine levels in salt, interest waned, monitoring lapsed, and the iodine content of randomly selected salt samples either was absent or greatly diminished. Thirdly, the importance of iodine deficiency and its correction was not adequately communicated to the concerned sectors, such as different branches of the government, the health establishment, industry, and most important, the consumers. Hence, about thirty years later, as shown in Table 1, only a few countries were nearing iodine sufficiency, and goiter prevalence had no significantly changed (13). In 1999, the WHO reported that despite significant regional progress, iodine deficiency remained a public health problem in 19 countries in the region (14). This general failure in Latin America provides a valuable lesson related to iodine prophylaxis elsewhere in the world.

2. Present situation

Most Latin American countries have reassessed their iodine status over the last 15 years and have implemented programs for the control of IDD (Table 2). Since 1985 great progresses have been made in the fight against iodine deficiency, particularly by aggressive push for iodized salt use. The achievements to date have been remarkable and indicate that the American Region will be among the first regions to attain the goal of the sustained elimination of iodine deficiency. The virtual elimination of IDD has been declared in six countries by external evaluations, Peru in 1996, Colombia in 1998, Ecuador and Venezuela in 1999, Panama in 2002, and Cuba in 2004. Bolivia was also declared to be free of IDD in 1996, but because of lack of sustainability relapsed three years later.

It must be noticed, however, that despite the great progress made by governments and agencies in the past 15 years, problems remain, such as a low level of governmental support and lack of effective monitoring of salt iodization in some countries that prevent from an effective and sustained elimination of IDD in the whole region. Some countries have regressed in the past five years, others never achieved iodine sufficiency, and still other countries have been incompletely assessed, and the risk of iodine excess has risen in more than one (Table 3).

The present article summarizes some recent information collected principally by ICCDD in the Region (15), the countries' reports to the Regional Meeting Optimal Iodine Nutrition in the Americas (Lima, Peru, 2004) (16), and the experience with the ThyroMobil project, which visited 13 countries in the Region in 1998-2000 (17).

a. Iodized salt: supply, consumption and quality

As shown in Table 4 currently all the countries in the region have reinforced their activities to reach the goal of universal salt iodization (USI) for human consumption. The legislation concerning the level of iodization of the salt has been corrected during the last decade in seven countries (Brazil, Chile, Ecuador, Mexico, Panama, Paraguay, and Venezuela) where used to be very low or very high, and in Uruguay where the iodization of the salt was required in only half of the country.

With the exception of three countries (Haiti, Dominican Republic and Guatemala), in all the others the production/importation volume of iodized salt is practically covering the human potential demand, estimated on the bases of an average annual consumption of 4-5 kilos of salt per person.

Monitoring of iodized salt is being carried out in practically all the countries (Table 2). Although in the majority of countries more than 80% of the salt at retail contains more than 15ppm of iodine, the recommended figure of more than 90% has not been met or sustained in many. It is of particular concern the situation of Guatemala and Dominican Republic, as well the lack of information in Haiti. At least 90% of households use adequately iodized salt.

b. Urinary iodine and iodine nutrition

Urinary iodine excretion analysis is recognized as the most important indicator of the impact of intervention and of the iodine nutrition. However, regular monitoring is carried out in only a few countries (Table 2), in some the only data available is the one collected with the ThyroMobil campaign.

As shown in Table 4, urinary iodine appears normal in most of the countries. The median urinary iodine is above 100µg/L (i.e., iodine sufficient) in 16 out of the 20 (Table 2). There are 3 countries with a median <100mU/L. It must be noticed that in 11 countries the median value is above 200µg/L, and that in 5 of them (Brazil, Colombia, Honduras, Paraguay and Uruguay) their medians are above 300µg/L, signaling the risk of iodine excess. Recently Camargo et al (18) reported on a higher prevalence (17.5%) of chronic autoimmune thyroiditis in the urban area of São Paulo, Brazil, that was considered to be linked to the relatively higher iodine intake (1998-2004) by the Brazilian population (UIE median: 305 µg I/L).

c. Goiter prevalence

Less and less emphasis is placed on assessing the prevalence of goiter because the palpation method is unreliable and the ultrasound method is not available in all the countries. Recently, the ThyroMobil Project in Latin America examined schoolchildren in 13 countries and the results showed that prevalence was still above 5% in the majority of countries (Table 5). This is probably due to the fact that the goiter disappearance following iodine prophylaxis is a long lasting process.

III. Contributing factors to the sustained elimination of IDD in Latin America

There have been important global and regional accounts that have significantly contributed to strengthen the development and progress of the national IDD control programs in the Region. Some of these are listed below.

1. PAHO/WHO Technical Group on Research in Endemic Goiter

In the early 60's the Panamerican Health Organizations/World Health Organization gave high priority to the study of the iodine deficiency problem and founded the Technical Group on Research in Endemic Goiter, convening various prestigious Latin American scientists to promote and carry out epidemiologic as well as research studies for the prevention and treatment of IDD. The group, headed by John B Stanbury, a well recognized international authority in this field, hold five regional meetings, the first one in Venezuela (1963) and the last one in Peru (1983).

2. Foundation of the International Council for the Control of Iodine Deficiency Disorders (ICCIDD)

No doubt that the foundation of the ICCIDD in March 1986 has been the most important and decisive push for the countries to abandon their many decades neglected and ineffective attitude in front to the iodine deficiency problems. Moreover, the important role played by the ICCIDD in a series of Resolutions passed by the World Health Assembly and for the adoption of the goal of virtual elimination of IDD at the UN Children´s Summit in 1990, strengthen the governments commitments against IDD.

3. Andean Sub-Regional Program for the Control of IDD

This Program was founded by UNICEF with the collaboration of PAHO/WHO and ICCIDD in 1991, and lasted for about ten years. The program included originally Bolivia, Colombia, Ecuador, Peru and Venezuela, and later on Paraguay as well. The objective of the program was to assist the country members to reach the goal of the World Summit for Children. Its main asset was to promote the exchanges of methodology and experiences among countries in IEC, social marketing, salt iodization technology, epidemiology, and monitoring. It proved effective in strengthening the IDD control programs of individual countries.

4. Regional research

Many studies carried out in the Region underscore the importance and deleterious effects of iodine deficiency on human development and the urgent need for its elimination. The following paragraphs briefly describe some of these.

a. *Maternal-fetal studies*

Pioneering studies in the Region during pregnancy (4, 5) pointed to iodine deficiency as a high risk situation for the pregnant woman and the fetus, and as the major cause of impaired mental and neurofunctional development. Concern about this finding, confirmed by others around the world (6, 7), led the World Summit for Children to declare the elimination of IDD as a target for the year 2000. These studies also demonstrated that this damage can be prevented by the appropriated supplementation of iodine during the critical periods of life.

b. *Use of iodized oil for correcting and preventing IDD*

Research studies on iodised oil for correcting and preventing iodine deficiency paved the way for rapid control of the problem in Peru (19) and its widespread use around the world, while awaiting the more slowly paced implementation of USI. This method has proved to be effective and long lasting, one single injection protects from iodine deficiency for three to five years without complications, it is of easy application and low cost. Its administration to pregnant women was safe and effective to protect the fetus from the consequences of iodine deficiency. Its use has been recommended by WHO in those areas at high risk of IDD while the iodized salt is not available (20,21).

c. *ThyroMobil Project in Latin America*

This is the first survey assessing iodine nutrition in a whole continent by using the same standardized methods for the estimation of the three key variables recommended by WHO/UNICEF/ICCIDD (22), i.e. the iodine content of salt in the community, the median urinary iodine and the prevalence of goiter in school children. The ThyroMobil model used in this study confirmed its specific performances in providing, on a strictly independent basis, standardized data, communication and social mobilization, partnership with the private sector and mobilization of national experts and authorities. The results show remarkable success in the elimination of iodine deficiency by iodized salt programs in Latin America. (17)

Thirteen Latin American countries (Mexico, El Salvador, Guatemala, Honduras, Nicaragua, Argentina, Bolivia, Brazil, Chile, Ecuador, Paraguay, Peru and Venezuela) were selected for the I project on the basis of mutual agreement between the health authorities in each country and the Regional Coordinator of ICCIDD. A total of 16,288 school children were examined for thyroid volume estimated by ultrasound and spot urine samples were randomly collected in about half of them (8,208). Salt samples were also obtained in local markets or at local homes.

This study disclosed a wide variation in the levels of salt iodization reflecting the different laws, which established a markedly lower or markedly higher content of iodine in salt than the recommended range of 20 to 40 ppm. The health authorities in five countries (Brazil, Chile, Ecuador, Paraguay and Mexico), following the report of the study have changed the level of salt iodization.

An important role was played by the ThyroMobil system in terms of social mobilization and awareness creation. The launching and development of the project in each country included press conferences, lectures and distributions of education materials, aimed to increase awareness of IDD, as well as to reinforce commitments towards its sustained elimination among national authorities, academicians, salt industry, and the population in general.

d. *International reference values for thyroid volume by ultrasound*

A multinational project which objectives was to develop international reference values for thyroid gland volume by ultrasound in school-age children from areas of long-standing iodine sufficiency. The study was carried out in 6 sites selected from the Americas, Europe and Western Pacific: Manama, Bahrain; Tokyo, Japan; Lima, Peru; Zurich, Switzerland; Boston, USA; and Cape Town, South Africa. These criteria can now be used to define goiter in the context of iodine deficiency disorders (IDD) surveillance (23).

5. Salt industry

The salt industry is absolutely recognized as one of the important partners for reaching the goal of the sustained elimination of iodine deficiency. As a matter of fact, its accelerated growing has been a key issue in the success of the IDD control programs in the region. The expansion of its market has been favored by the social marketing and the IEC campaigns carried out by the national IDD programs.

6. Support to laboratories processing iodine

Since urinary iodine is the most important indicator of iodine nutrition, it is important the quality assurance of laboratories processing it. The Andean Sub- Regional Program initiated a trial in 1998 to provide support for quality assurance to the laboratories processing iodine in urinary and in salt in the country's members. Most recently, the participation of ICCIDD in the International Resource Laboratories for Iodine (IRLI) Network has maintained and reinforced the support to improve the quality and efficiency of the laboratories in all the Latin American Region. Two laboratories in the region have been selected to integrate the IRLI Network, one in Peru – Endocrinology and Metabolism Unit, High Altitude Research Institute, Cayetano Heredia Peruvian University (6) and the other one in Guatemala – Food Safety and Fortification Area, INCAP.

7. Regional meetings

A number of scientific meetings have been organized in the Region and important decision has been recommended.

a. *Meetings of the PAHO/WHO Technical Group on Research in Endemic Goiter*

A series of five meetings were hold by the PAHO/WHO Technical Group on Research in Endemic Goiter which took place in Caracas, Venezuela (1963), Cuernavaca, Mexico (1965), Puebla, Mexico (1968), Gauruja, Brazil (1973), and Lima, Peru (1983). These meetings were aimed to review new knowledge on the pathophysiology of the IDD, research studies on prevention and correction of iodine deficiency, regional epidemiology, and other important related subjects, as well as to formulate a series of recommendations on definitions, research, and approaches to prophylaxis.

b. *UNICEF-PAHO/WHO-ICCIDD Meeting, Quito-Ecuador 2000*

A landmark meeting in Quito, Ecuador, in April 1994, attended by high-ranking officials from UNICEF, PAHO/WHO, ICCIDD, and government, issued a declaration, signed by

representatives from 23 countries in the region, stating their commitment to universal salt iodization in the Region by the year 1995 as the mid-decade goal, to be followed by the final goal of eliminating iodine deficiency as a public health problem by the year 2000.

c. *Salt 2000. The Latin American and the Caribbean Regional Meeting, Bogota, Colombia 2000*

In 2000 a salt regional meeting in Bogota, Colombia, reached a number of important decisions for the salt industry: (i) encourage salt producers to produce and distribute top quality iodized salt at a reasonable price; (ii) pursue permanent political will for support of IDD programs; (iii) maintain regular monitoring of quality of salt production and its effects in human nutrition; (iiii) develop social mobilization programs to encourage consumption of iodized salt; (iiiii) create a trust fund for implementing regional communication programs o iodized salt consumption; (iiiiii) include instruction on iodine deficiency and iodized salt use in the educational system.

d. *Regional Meeting Optimal Iodine Nutrition in the Americas, Lima-Peru 2004*

This meeting took place in Lima, Peru in May 2004, sponsored by PAHO/WHO, UNICEF, ICCIDD and the Iodine Network. The meeting was convened to review the current status of iodine nutrition and iodized salt in each of the Latin American and the Caribbean countries, to identify obstacles to sustainable optimal iodine nutrition, and to develop a strategy to overcome them.

The following key issues in achieving and sustaining optimal iodine nutrition were discussed: (i). organization of national efforts for sustained IDD eliminations, (ii). national coalitions to promote and sustain optimal iodine nutrition, (iii). assuring adequately iodized salt, (iiii). effective systems for monitoring iodine in people and in salt, (iiiii). role of the education and communication, (iiiiii). regional cooperation-harmonization of salt iodization levels and regulations, laboratory networks, information sharing, (iiiiiii). current and future role of agencies and NGOs. The meeting was attended by twenty countries (Argentina, Bolivia, Brazil, Chile, Colombia, Costa Rica, Cuba, Dominican Republic, Ecuador, El Salvador, Guatemala, Haiti, Honduras, Mexico, Nicaragua, Panama, Paraguay, Peru, Uruguay, Venezuela) were represented by their Ministers of Health or senior leadership in health and nutrition at the Regional Meeting. Governmental officials responsible for iodine nutrition, and representatives of the salt industry were also participants., as well as representatives of eight international agencies and organizations (PAHO/WHO, UNICEF, ICCIDD, Kiwanis International, Micronutrients Initiative, Salt Institute, Network for Sustained Elimination of Iodine Deficiency).

IV. **Conclusions and recommendations**

- 1) Globally the countries of The Americas have made significant progress towards the elimination of IDD; however, problems remain that threaten the effective and sustained elimination of IDD in the whole region. A few countries are still deficient, others have been incompletely assessed, and the risk of iodine excess has risen in some.
- 2) Much of the America´s severe iodine deficiency of the past has been corrected. At least 80% of salt at retail is adequately iodized, and only 3 countries (Guatemala, Dominic Republic, Haiti) have currently median urinary iodine in the deficient range.
- 3) The achievements so far reflect effective collaboration among may partners, both national –governments (especially Ministries of Health, Education, and Commerce), the salt industry, the health sector, consumers, and advocacy groups – and international – ICCIDD, UNICEF, PAHO/WHO, Kiwanis, bilateral donors, private foundations, and others. This collaboration offers useful models for tackling other health issues.
- 4) The great challenge now is sustaining the progress. The failures after previous success in the past decades in Latin America emphasize the perils of relaxed vigilance. The following elements are essential to sustain progress toward optimum iodine nutrition:
 - √ Maintain high level political commitment to and priority on the prevention and correction of nutritional deficiencies, such as infant brain damage due to inadequate intakes of iodine, while at the same time preventing the excess intake of essential nutrients. It includes a permanent funding from regular budget, mobilization of social demand, securing adequate resources, ownership and empowerment of salt producers, and an enabling legal environment linked to a system of transparent and effective enforcement.
 - √ Implementation of a national watch committee. Each country must take long-range responsibility for its own program to achieve permanent optimal iodine nutrition. A National Coalition is the critical tool to ensure that all stake holders continue their contribution to the progress in iodine nutrition, each according to agreed-upon and responsibilities.

- √ Monitoring of iodine in people and in salt is still non-existent, fragile, or inadequate in many countries. Monitoring of iodine in salt and in people is essential to assess and maintain optimum iodine nutrition status. It must be sustained and systematic, and results must be communicated to the appropriate levels of decision to make necessary corrections. Laboratories with adequate quality assurance systems in place are required to guarantee the validity of monitoring results.
- √ Ensuring adequately iodized salt. Provided with adequate assistance, all salt producers – small and large – can iodize salt effectively. Some countries need watching for iodine excess, as a result of high iodine in salt.
- √ Communication, including advocacy and education, needs to be planned comprehensively as an integral part of the overall national effort. To bridge the generation gap it is important to infiltrate and pervade national education systems permanently.
- √ Report every three years on the status of national programs and on the efforts being made to ensure progress and sustainability at the highest national level as well as to PAHO/WHO, in accordance with the WHA Resolution WH58.24.

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VI. Tables

Table 1. Laws on salt iodization in Latin America and their impact on prevalence of goiter

Country	Legislation		Iodine requirement ppm	Prevalence of goiter*	
	Approved year	Implemented year		1950-1960 %	1980-1990 %
<i>North America</i>					
Mexico	1963	1963	10	5-46	5-50
<i>The Caribbean</i>					
Cuba	1999	1999	18-23	NA	30
Dominican Rep.	1994	1994	30-100	NA	NA
Haiti	NA	NA	NA	NA	NA
<i>Central America</i>					
Costa Rica	1941	1972	33-50	19	4
El Salvador	1961	1972	30-100	30	25
Guatemala	1954	1959	30-100	38	21
Honduras	1960	1971	50-100	22	9
Nicaragua	1968	1978	30-100	27	20
Panamá	1955	1969	66-100	17	13
<i>South America</i>					
Argentina	1953	1962	30	34-83	10-42
Bolivia	1968	1977	40-80	68	61
Brazil	1953	1977	10	27	29-35
Chile	1959	1979	100	25	10
Colômbia	1955	1960	50-100	53	14
Ecuador	1968	1973	50-100	34	37
Paraguay	1958	1966	60-80	50	49
Peru	1940	1971	30-40	28	36
Uruguay	1961	1963	30-40	7-38	9
Venezuela	1966	1968	20-30	33	33

* By palpation NA = not available. Modified from Pretell et al (ref. 13)

Table 2. National IDD Control Programms

Country	Responsability		Monitoring		IEC
	MOH	Other	Iodized Salt	Urinary Iodine	
Mexico	Yes		Yes	No	Yes
Cuba	Yes		Yes	Yes	Yes
Dominican Republic	Yes		No	No	No
Haiti	No		No	No	No
Costa Rica	Yes		Yes	No	No
El Salvador	Yes		Yes	No	Yes
Guatemala	Yes		Yes	No	Yes
Honduras	Yes		Yes	No	Yes
Nicaragua	Yes		Yes	Yes	Yes
Panama	Yes		Yes	Yes	Yes
Argentina	No	FASEN *	No	No	No
Bolivia	Yes		Yes	Yes	Yes
Brazil	Yes		Yes	No	Yes
Chile	No	INTA **	Yes	Yes	No
Colombia	Yes		Yes	Yes	Yes
Ecuador	Yes		Yes	Yes	Yes
Paraguay	Yes		Yes	Yes	Yes
Peru	Yes		Yes	Yes	Yes
Uruguay	Yes		Yes	Yes	Yes
Venezuela	Yes		Yes	Yes	Yes

* Federación Argentina de Sociedades de Endocrinología

** Instituto de Nutrición y Tecnología de Alimentos, Universidad de Chile

Modified from Pretell EA (ref. 13)

Table 3. Iodine nutrition status in Latin America and the Caribbean (2005)

Deficient	Mild	Likely Deficient	Sufficient	Likely Sufficient	Risk of Excess*	Unknown
Haiti	Guatemala	Guyana	Bolivia	Argentina	Brazil	Barbados
	Dominican Rep.		Chile	Belize	Colombia	
			Cuba	Costa Rica	Honduras	
			Ecuador	El Salvador	Paraguay	
			Nicaragua	Mexico	Uruguay	
			Panama	Surinan		
			Peru			
			Venezuela			

Modified from Pretell EA (ref.15)

Table 4. Data on iodized salt and urinary iodine monitoring.

Country	Actual Legislation Iodization level ppm	Coverage of population demand * %	Iodine content retailed market ≥ 15 ppm %	Urinary iodine Median μ/L
Mexico	20-40	100	91	176
Cuba	15-25	100	83	214
Dominican Republic	30-100	--	7	--
Haiti	--	--	--	<100
Costa Rica	23-46	91	91	214
El Salvador	30-100	100	67	180
Guatemala	20-60	--	77	72
Honduras	50-100	100	99	356
Nicaragua	33-60	100	97	235
Panama	20-60	100	93	209
Argentina	33	--	73	46-314
Bolivia	40-80	90	90	192
Brazil	20-60	100	88	360
Chile	20-60	100	97	230
Colombia	50-100	100	73	409
Ecuador	30-50	100	97	254
Paraguay	40-60	100	95	373
Peru	30-40	100	90	180
Uruguay	30-50	100	89	310
Venezuela	40-70	100	57	185

* 4-5 kg./pc/year

Modified from Pretell EA (ref.13)

Table 5. Prevalence of goiter in relation to age and body surface area (BSA) in schoolchildren (6-12 y-old)

Country	By age		By BSA	
	n	%	n	%
Mexico	2118	20.4	2041	29.5
El Salvador	572	13.3	541	29.0
Guatemala	532	4.5	478	15.6
Honduras	544	21.0	520	39.2
Nicaragua	531	25.0	501	37.5
Argentina	1146	15.7	1083	12.7
Bolivia	1239	3.1	1178	6.4
Brazil	1563	4.5	1452	8.1
Chile	936	12.7	894	8.5
Ecuador	1195	3.3	1027	18.1
Paraguay	902	17.1	878	22.4
Peru	2072	6.5	1928	18.3
Venezuela	1257	6.8	1219	10.5

Data collected during the ThyroMobil project (1998-2000)

Modified from Pretell et al (ref. 17)

Iodine Deficiency Disorders (IDD) in the Asia Pacific Region

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INTRODUCTION

According to the WHO over one billion people living within the Asia Pacific region have urinary iodine excretion concentrations less than the minimum level of 100 µg per litre and are at risk of developing one or more of the adverse effects of IDD (1). The vast geographical expanse of this region, incorporating approximately 40 countries, from tiny Pacific islands to some of the most populous nations on earth, such as India, Indonesia and China, poses a challenge in identifying and addressing the problems of IDD in this part of the world. Poverty and social disadvantage characterise many of these underdeveloped countries. IDD is most commonly, but not always, associated with lack of social and economic development, isolation and mountainous terrain. UNICEF has estimated there are more than 20 million babies born annually within the Asia Pacific region that are not protected from iodine deficiency. The majority of these babies are born in India, Bangladesh and western China

Within the Asia Pacific region, IDD has been recognised as a serious public health problem in India, Bangladesh, Indonesia, Myanmar (Burma), Cambodia, Thailand, China (western provinces including Tibet), Mongolia, North Korea (DPRK) Laos, Malaysia, Papua New Guinea, East Timor and the Philippines. This is not an exclusive list. In some of these countries serious iodine deficiency is limited to remotely located pockets of the population that are difficult to reach with iodised salt on a sustainable basis. Despite all the difficulties, there has been considerable improvement in household iodised salt coverage in most of these countries over the past decade, giving hope to the realisation of the goal of sustainable IDD control within the next decade (Figure 1).

In the South Pacific there have been sporadic reports of goitre being endemic in parts of Vanuatu, New Caledonia, Fiji, Samoa and some of the other smaller islands such as Tuvalu. Because many of the smaller countries in the Asia Pacific region are island states, with large expanses of coastline and presumed easy access to seafood, it has erroneously been assumed that iodine deficiency is not a significant endemic problem in the Pacific. As a consequence, little or no data on IDD has been collected in many of the poorer, smaller nations and a disregard for the crucial role of iodine nutrition has characterised the position of some of the more affluent nations such as Australia and New Zealand (2).

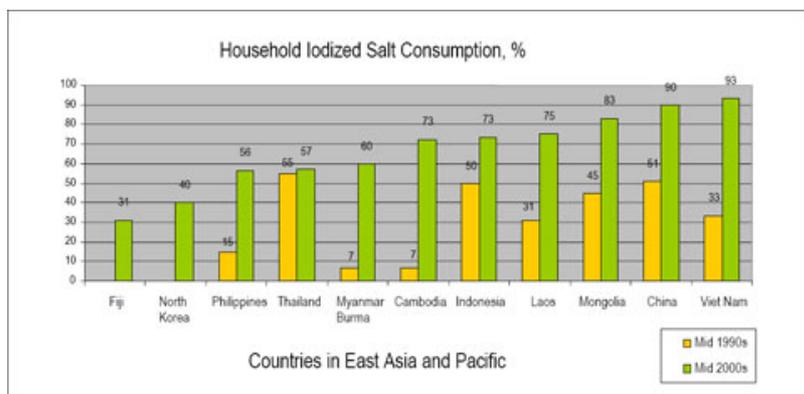


Figure 1: Household Iodised Salt Consumption (USI) in East Asia and Pacific Region (data courtesy of UNICEF 2007)

Examples of successful IDD elimination programs

China, Vietnam, Thailand and Indonesia are good examples of countries that have invested enormous effort and resources in IDD elimination programs and are now reaping the social and health benefits of these investments (3). For these reasons we will look at the recent history and achievements of two of these countries, China and Vietnam, with which we have had first-hand experience in combating IDD. Both China and Vietnam have met the overall target of achieving iodised salt coverage in excess of 90% of all households.(Figure 1)

Peoples Republic of China

Goitre has been recognised as a problem in China for thousands of years. Although the role of iodine was unknown until the 19th century, seaweed and burnt sea-sponge were advocated for the treatment of goitre in China over 4000 years ago. However, it was not until the 1960s that endemic goitre was acknowledged as a nationwide, public health issue. The connection between iodine deficiency and brain damage, that is far more common and subtler in its manifestations than classical cretinism, was first recognised by Chinese scientists (Drs Ma Tai and Lu TZ) three to four decades ago. They called this condition "subclinical cretinism" and drew attention to the fact that severe iodine deficiency could affect whole communities, adversely impacting on the intellectual, physical, economic and social development of the people (4). Over the next two decades numerous surveys conducted in many regions of China revealed that goitre was widespread throughout the country and cretinism and subclinical cretinism were common in severely iodine deficient areas. Before any iodised salt prophylaxis programs were commenced endemic goitre was estimated to be present in 20% to 30% of the population in the majority of provinces, thus affecting millions and millions of people. It is a matter of speculation as to how many tens of millions of the population suffered from some form of brain damage due to iodine deficiency. There are stories of places being labelled "idiot villages" because overt cretinism was common and most of the population suffered from goitre and mental disability (4). The recognition of defective cognitive performance, coupled with impaired child development, elevated the concern about iodine deficiency from an endocrine problem of thyroid dysfunction resulting in goitre to that of a reduction in human resources and constrained economic development (5). A recent meta-analysis undertaken on observational studies in China confirmed an average IQ loss of approximately 12 points in children born in moderate to severe iodine deficient areas and that this loss can be prevented by appropriate iodised salt intervention programs (6). The findings of reduced cognitive performance and lower intelligence in many segments of the population in China, and not the high prevalence of endemic goitre, has provided the impetus for the serious national commitment and effort to eliminate IDD from the country. The story of how this has been achieved is quite remarkable and an inspirational triumph in public health achievements in modern times (4,5).

At the historic UN Summit for Children held in 1990, China was a signatory to the declaration of the goal of virtual elimination of IDD by the year 2000. With the assistance of international agencies and bilateral aid donors from Australia and Canada, the State Council hosted a high-level advocacy meeting in 1993 involving governors of all provinces, and representatives of government, the salt industry, health care institutions, together with civic leaders and international experts to develop a commitment and plan for the elimination of IDD in China. The State Council approved regulations for mandatory iodisation of all edible salt (Universal Salt Iodisation or USI) and appointed a multi-sectoral leadership and management group to oversee the effort and to provide support for training of personnel and education of the population. A national monitoring program was put in place to support the implementation of this initiative. When the first national survey was performed in 1995 the population coverage of iodised salt was approximately 30% and rose to over 90% by year 2000 to achieve the target for USI. Iodine concentrations in edible salt have been adjusted to prevent excessive intake (4).

IDD in Tibet

While the national targets of household coverage of iodised salt and a goitre rate of less than 5% in schoolchildren have been achieved, there remain some serious problems in several provinces in the remote western region of China. In particular, Tibet continues to suffer from serious iodine deficiency. Goitre rates have been as high as 50%, cretin rates in some villages up to 13% and the average IQ of children being only 85 (7). In 1999 with support from WHO, AusAID, and UNICEF, we undertook a feasibility study for the development of a whole of Tibet IDD elimination program. The project comprised support for the development of an iodised salt industry, development of health education and communication materials, training, capacity building, management support and implementation of an interim iodised oil supplementation program for infants and all women of childbearing age. AusAID contributed over \$2 million and WHO provided umbrella support. This program has been successful in reaching over 90% of the target population with the iodised oil supplements. It is estimated that over 170,000 newborn babies have had their brains protected from iodine deficiency through this program (8). We are in the process of transferring all responsibilities to the Tibet government and the Tibet Department of Health who are committed to providing iodised salt to all of their citizens.

Vietnam

The first IDD control programs in Vietnam were established in the early 1970s. The initial focus was on the mountainous provinces in North Vietnam where goitre rates were as high as

55 %. A nationwide survey was undertaken in 1993 and the average goitre rate in children was found to be 22 % and the median urinary iodine excretion was only 32 ug/l. The government of Vietnam responded by establishing a National IDD Control Committee and developed a nationwide network of salt iodisation plants. Australian development aid, through AusAID and Westmead Hospital, provided technical and other assistance in establishing this program. In 1999 the government issued a decree relating to the production and supply of iodised salt for human consumption. Currently, the rate of coverage for adequately iodised salt in Vietnam has risen to over 90%. The Vietnamese government has a policy for subsidising iodised salt for some 12 million ethnic minorities in mountainous areas of the country. There is a very well organised and efficient national IDD committee that oversees monitoring of the IDD elimination program. Provincial IDD committees undertake monitoring surveys three times a year and there is a national survey every second year. There is a central laboratory in the Hospital for Endocrinology in Hanoi that oversees all laboratories monitoring for urinary and salt iodine levels. Information Education and Communication (IEC) activities are well developed. Recently the government strengthened legislation to eliminate non-iodised salt from the marketplace. Vietnam has achieved the USI goal of over 90 % coverage of the population and reduction of goitre rates to less than 10 % (Figure 1)

What are the lessons we have learned in these developing countries?

- Framing the message of iodine deficiency around brain damage, loss of IQ and impaired mental and physical development is far more powerful and persuasive than advocating iodine prophylaxis programs for endemic goitre
- Advocacy at government level is essential to influence those that have the power to make decisions. IDD is a "whole of government" issue affecting social and economic development and not simply a public health problem. Obtaining and maintaining high-level political commitment to eliminating IDD is essential.
- Acceptance at a national level that USI (mandatory fortification of all edible salt with iodine) is the most appropriate vehicle for normalising iodine nutrition within a community and that acceptance of USI must be followed by implementation of legislation and regulations underpinning this initiative.
- Multi-sectoral involvement is a prerequisite for success. The salt industry is as an important player as the health sector in achieving USI and must accept responsibility to ensure supply of iodised salt.
- Education of the public is necessary to create demand for iodised salt and sustainability of optimal iodine intake.
- A vital component of successful outcome is the implementation of a program for regular monitoring of the population iodine intake and the quality assurance of iodised salt at production and retail level with mandatory public reporting of the data.
- Where iodised salt cannot be provided to all citizens, and in particular children and women of childbearing age, an iodised oil supplementation program is an effective means of protecting the brains of the newborn and the growth and development of infants and children and should be employed as an interim strategy.

Re-emergence of Iodine Deficiency in Australia and New Zealand

Mild iodine deficiency has re-emerged in Australia and New Zealand over the past decade and poses a significant health risk to future generations of these countries (9,10). Median urinary iodine excretion (UIE) levels in Australia have decreased from over 200 ug/l in 1990 to less than 100 ug/l by 1999 (9). Iodine deficiency has resurfaced after more than half a century of iodine sufficiency because of changes in work practices in the dairy industry and because consumers do not purchase iodised salt and the food industry does not use iodised salt in food preparation. The dramatic decline in iodine intake in the Australian population has been attributed to the major decrease in iodine concentrations in dairy milk (2,11). Since the early 1960s the major source of iodine in the Australian diet has been from iodine contamination of milk by iodophores used as sanitising agents in the dairy industry. These chemicals have been replaced by chlorine-based disinfectants. Because Australia has not had an ongoing iodine nutrition monitoring program (except for the island state of Tasmania), the problem of iodine deficiency has only come to light through the efforts of researchers interested in IDD (2). Health authorities have shown little or no interest in addressing the problem because mild iodine deficiency has not been seen as a threat to human health.

Sales of iodised table salt represent less than 20 % of the edible salt market sales in Australia. There is no legislation mandating the use of iodised salt in the food industry and there is little awareness among the public of the problems of IDD. The situation in New Zealand is very similar to that in Australia. Both countries share a common food standards authority (Food Standards of Australia and New Zealand FSANZ) so any changes to legislation regarding mandatory salt iodisation will need to be agreed between them before we can expect change.

A National Iodine Nutrition Study has recently been completed in Australia to obtain a snapshot of urinary iodine levels and thyroid size in 8 to 10 year old schoolchildren. The median urinary iodine level is under 100 ug/l, and there is increased size of thyroid glands by ultrasound measurement, consistent with mild iodine deficiency (11). Similar results have been obtained in studies of New Zealand children.

Conclusions

The goal of achieving USI, and virtual elimination of IDD, within the Asia Pacific region by end of 2010 remains a formidable challenge, and is most likely not achievable. Unless IDD elimination efforts can be reinvigorated in a number of countries, where there has been little or no progress in recent years, the goal is not attainable in the foreseeable future. The commitment and achievements in countries such as China, and more recently Vietnam, provide inspiration to others that sustainable IDD elimination can be realised throughout the Asia Pacific region.

More information is needed from properly constructed surveys within the smaller island states of the Pacific to define the extent and severity of iodine deficiency in these countries. Government intervention banning the importation of all non-iodised edible salt would provide an immediate solution to the problem. Within the more affluent countries such as Australia and New Zealand education of the public is needed to create a demand for iodised salt and to lobby governments for implementation of mandatory salt iodisation. In the interim there is a good case to be made for all pregnant and breastfeeding women in these countries to be taking daily iodine supplements to ensure optimal iodine nutrition during critical times of human development.

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How do we optimize iodine intake to minimize the occurrence of thyroid disorders in Europe ?

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Both insufficient and excessive iodine intake may lead to disease in an individual, and the epidemiology of thyroid disorders depends on the iodine intake level of the population (1). Historically, the occurrence of thyroid disease in Europe has been dominated by iodine deficiency with some geographical variation. Severe iodine deficiency with endemic cretinism and goitre in the major part of the population was primarily found in the Alps and in other mountainous regions, but milder forms of iodine deficiency were present in regions of nearly every European country (2). As reviewed previously by Delange (3), iodine deficiency has been eradicated in some European countries for many years, but other countries have lagged behind, especially in prevention of mild to moderate iodine deficiency. Fortunately, and due to keen efforts by dedicated, hard-working people, the situation has improved in recent years. The number of European countries affected by iodine deficiency is steadily decreasing (4). However, continued focus on the necessity to monitor and adjust the iodine intake of European populations is necessary.

Challenges in the field of iodine intake

Thyroid disease may occasionally cause permanent impairment of health and undiagnosed and untreated thyroid function abnormalities in pregnant women may permanently harm fetal brain development leading to mental deficits(5). Even small aberrations in thyroid function at the population level may change the occurrence of overweight (6,7) and blood pressure abnormalities (8,9). Thus, thyroid disorders should be prevented if possible. Without going into the complicated discussion on interaction between genes and environment, the three factors most important for the occurrence of thyroid abnormalities are: 1) The level of iodine intake 2) The genetic background 3) Tobacco smoking. How should iodine intake be modified to prevent thyroid disorders? The primary concern is always to avoid iodine deficiency as this may most seriously affect the health of a population. However, the level of iodine intake should optimally be increased in a way that will not cause unnecessary disease from excessive iodine intake.

Optimal iodine intake to prevent brain damage

It is a balance to discuss optimization of iodine intake and avoidance of excessive iodine intake without bringing iodization programs into discredit, which may lead to reoccurrence of iodine deficiency, and thereby worsen the situation. As illustrated in fig. 1, the risks from iodine deficiency are much higher than those from a higher than necessary iodine intake. The most severe complication from low iodine intake is developmental brain damage, which is well documented in severe iodine deficiency (10).

Fig 1

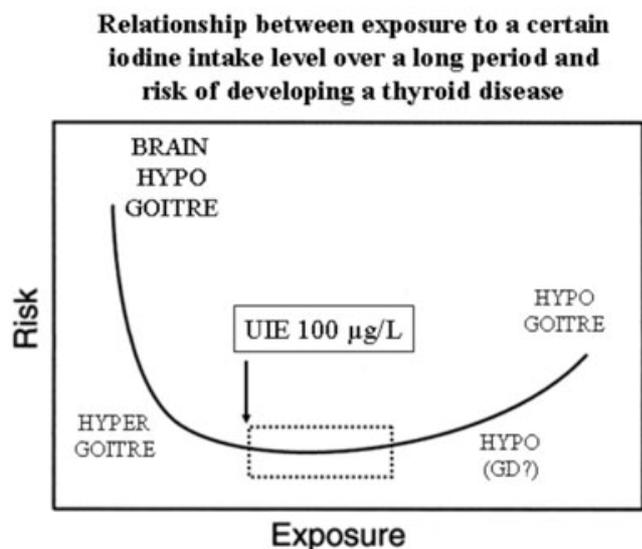


Fig. 1.

Theoretical curve showing the U-formed relationship between exposure to a certain level of iodine intake and the risk of disease. The stippled box illustrates the optimal level of iodine intake, with the lower level in homogenous population groups being a median urinary iodine concentration of 100 µg/L (45).

The damage is caused by an iodine deficiency induced decrease in thyroid hormone production in the pregnant woman and/or the fetus/neonate, that may also lead to stillbirth and other pregnancy complications. The iodine intake level in a population at which this may occur may depend on individual genetic factors as well as on deficiency of other nutrients (11) and on intake of goitrogens that may hamper iodine utilization of the maternal and fetal thyroid, and iodine transport into the mother's milk (12). Moreover, in a population there is a certain spread in iodine intake between individuals with some people having intake considerably below average, and the iodine needs of every single pregnant woman should be covered. In a group of men living in a moderately iodine deficient area, we found that thyroid function at the individual level varied in parallel with the iodine intake of the person when average urinary iodine excretion over one year was below 50 µg per day (13).

In pregnancy iodine needs are higher than normal (5), but the extra needs may to some degree be covered by thyroidal iodine stores if the woman lives in an area with sufficient iodine intake. Corresponding to this, signs of impaired thyroid function in pregnant women have been observed in small studies when iodine intake was only mildly to moderately deficient (14,15). Thus, to cover the increased needs of iodine during pregnancy the population should be iodine sufficient with a median urinary iodine concentration above 100 µg/L. As an additional measure in countries with a low normal iodine intake, iodine should be part of the vitamin + mineral supplements taken by many European women during pregnancy (16).

Table 1 lists a number of abnormalities that may be less common after an increase in iodine intake of a population. The evidence for such a beneficial effect according to the authors' opinion ('confidence') is indicated, ranging from possibly true to established. The outcome in a given population would depend on the levels of iodine intake before and after. As indicated, the evidence for less disease when iodine deficiency is eradicated is in general good.

Some disorders may become more common after an increase in iodine intake as discussed below, although the evidence is less firm (table 1), and the overall burden of disease will be lower (fig. 1).

Less common		More common	
A.	Brain damage caused by maternal/fetal/neonatal iodine deficiency (***)	A.	Brain damage caused by maternal hypothyroidism (*)
B.	Goitre caused by iodine deficiency (***)	B.	Goitre caused by autoimmunity (**)
C.	Hyperthyroidism caused by thyroid autonomy (***)	C.	Hyperthyroidism caused by Graves' disease (*)
D.	Hypothyroidism caused by iodine deficiency (***)	D.	Hypothyroidism caused by autoimmune thyroiditis (**)
E.	Follicular and anaplastic thyroid cancer (**)	E.	Papillary thyroid cancer (**)
F.	Thyroid cancer after radioactive iodine fall-out (**)		

Table 1

Abnormalities that theoretically may be less common or more after an increase in population iodine intake. The actual change in risk would depend on the level at which the change in iodine intake occurs. A rough estimate of confidence is given (possibly true (*), probably true (**), established (***)).

Optimal iodine intake to prevent development of goitre and thyroid autonomy

Iodine intake below the recommended level leads to a dose dependent increase in the incidence and prevalence of goitre and thyroid autonomy in a population. In severe iodine deficiency the highest prevalence of goitre is seen in young adults (17), but in areas of mild to moderate iodine deficiency goitre and autonomous thyroid nodules develops with age (18). The DanThyr study which is the monitoring of the Danish iodine program (19) showed that even relatively small differences in population iodine intake of around 30 µg per day were associated with considerable differences in the risk of goitre (18) and hyperthyroidism caused by thyroid autonomy (20). Moreover, a moderate increase in iodine intake of 50-60 µg per day has led to a clear reduction in thyroid size in the Danish population after a few years (21). The DanThyr study has confirmed that the lower limit of sufficient iodine intake of a population set by the WHO (median urinary iodine concentration 100 µg/L) corresponds to the limit below which the risk of goitre and thyroid nodules may increase in a European population.

The cause for development of the multifocal thyroid abnormalities characteristically found in many elderly people when iodine intake is below recommended has not been fully elucidated (22). One candidate is H₂O₂ generated in the thyroid as part of the thyroid hormone synthesis process (23). As recently reviewed (24) thyroid cells generate large amounts of H₂O₂ and this process is up-regulated when iodine intake is low as part of thyroidal iodine-auto-regulation, similar to many other processes involved in iodine utilization. Genetics and tobacco smoking are two other factors important for development of simple goitre (25). As both may act via partial impairment of thyroid iodine utilization, a sufficient iodine intake in a population is a most effective measure to prevent goitre and hyperthyroidism caused by thyroid autonomy. The importance of abnormalities caused by mild to moderate iodine deficiency at the population level is considerable. In a population study of 68 year old people living in Randers, Denmark (with mild to moderate iodine deficiency) before the Danish iodization program, 8 % of the women had undergone thyroid surgery for non-toxic goitre, 4 % had a clinically significant goitre and one out of 30 had undiagnosed overt hyperthyroidism caused by thyroid autonomy (26). The economic consequences of mild to moderate iodine deficiency are considerable (27).

Risks from an increase in population iodine intake

The major concern when iodine intake has been abruptly increased from insufficient to high has been a transient surge of hyperthyroidism, as reviewed by Stanbury et al (28). Iodization programs in Europe as elsewhere should be carefully planned and executed. However, as illustrated in fig. 1, a higher iodine intake level may also be associated with a more permanent increase in the occurrence of some thyroid abnormalities.

The three abnormalities which will be discussed are primary hypothyroidism, Graves' disease and diffuse goitre. In a comparative study of elderly people living in Iceland (with high iodine intake) and Jutland, Denmark (with moderate iodine deficiency) the prevalence of subclinical hypothyroidism was much higher in Iceland than in Denmark (26). Similar findings have been reported when comparing people with low and high iodine intake in Hungary (29), people with sufficient and high iodine intake in China (30,31) and people with different degrees of excess iodine intake in Japan (32). In the DanThyr register of overt hypo- and hyperthyroidism (19) we found a considerably higher incidence rate of overt hypothyroidism in an area with

mild (Copenhagen, Sealand) compared to an area with moderate (Aalborg, Jutland) iodine deficiency (20). The difference was caused by 50 % more cases of spontaneous autoimmune hypothyroidism in the area with the highest iodine intake level (33). After increasing the iodine intake of the Danish population with around 60 µg/day to a low normal level, a small increase in the incidence of overt hypothyroidism has been observed (34).

The exact dose/response curve for this association between higher iodine intake and more hypothyroidism is at present unknown. There are large differences between the occurrence of hypothyroidism between populations, part of which may be genetically determined. In Scotland, hypothyroidism is as common in young women in fertile age as it is in 80 year old women in Denmark (35). It is beyond the scope of this article to discuss in detail the possible mechanism involved in the association between higher iodine intake and more hypothyroidism. Iodine has a series of inhibitory effects on thyroidal processes involved in hormone synthesis and secretion. Some people seem to be less able to escape from these inhibitory effects (36) which may be more prevalent if the thyroid is affected by autoimmunity.

In our comparative study of overt hyperthyroidism in Iceland and Jutland, Denmark (37) the life-time risk of Graves' disease was not much different between the two populations, but on average the disease developed 15-20 years earlier in the population in Iceland. This may suggest a genetic predisposition with a higher risk of overt disease if the iodine intake is higher. Such a mechanism is supported by studies showing risk of reoccurrence of hyperthyroidism with higher iodine intake in people who are in remission after a previous episode of Graves' disease (38,39).

A higher frequency of diffuse goitre with excessive iodine intake has been observed in school-age children both in the USA (40) and in China (30).

Ways to achieve and maintain optimal iodine intake in European populations

Iodine intake depends on the dietary habits and the iodine content of the individual food items. Fig. 2 illustrates the relative contribution of different sources to iodine intake in the DanThyr population study before the Danish iodization program (41).

Fig 2

Iodine from various sources in the Danthyr cohort

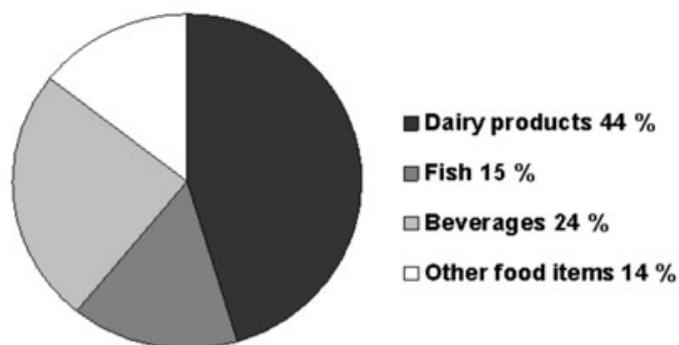


Fig. 2. The main sources of iodine intake in Denmark before the Danish iodization program estimated from food frequency questionnaire. Without individual supplements. N = 4649 adults. Median urinary iodine concentration 45-70 µg/L. Data from the DanThyr study (41).

Iodine in dairy products contributed nearly half of the average iodine intake. The lactating mammary gland concentrates iodide from blood by way of an iodine transporter identical to thyroidal NIS, and iodine content of dairy milk depends on the contents of iodine and of NIS inhibitors in cow feed (42). Because dairy products are so important for iodine intake in many populations iodine feeding of dairy cows should be monitored and regulated.

In the DanThyr cohort the second most important source of iodine was beverages (fig. 2). The iodine content of fresh water varies depending on the aquifer (43), and regional differences in iodine intake may be caused by different sources of water (44). It is relatively easy to identify areas with high iodine content of water. In such areas individual intake of iodine supplements should probably be avoided.

In many European areas with low iodine content of natural food, an iodization program has to be running. This is normally done by salt iodization. Because both low and high iodine

intake may lead to more disease than seen with optimal iodine intake, attempts should be made to create programs that keep the spread of iodine intake between individuals relatively narrow. This is illustrated in figure 3A.

Fig 3

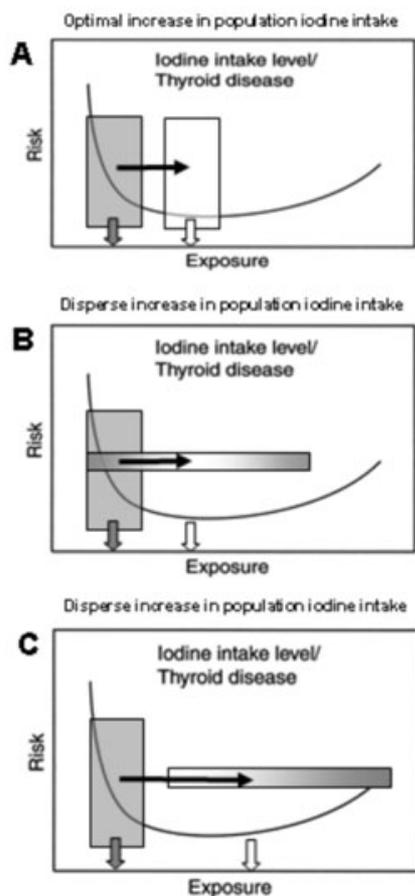


Fig. 3. Illustration of the principle in an optimal increase in iodine intake of an iodine deficient population, A:

The boxes represent the distribution of iodine intake in the population before and after a good program, with nearly unaltered distribution. B illustrates a situation with the same average increase in iodine intake, but a much larger dispersion in individual iodine intake because iodine has been added in larger amounts to a more narrow set of foods. C: the same dispersion if iodine deficiency should be entirely avoided in the population; the average increase in iodine intake has to be considerably higher than in A and B, and many more people will be exposed to excessive iodine.

Iodization of all salt used by households and the food industry will distribute iodine quite even in the population and increase the iodine intake without marked changes in the dispersion of individual iodine intake. However, such iodization is often difficult to achieve because of political and commercial concerns. In Denmark, household salt and salt used for commercial bread production is mandatorily iodized (19). Bread is a stable dietary component in Denmark, and this type of iodization gives nearly as good a distribution of iodine as universal salt iodization.

If, on the other hand, iodide is added to only a minor part of food the amount of iodide added has to be considerably higher to give the same increase in medium iodine intake of the population. After such a program the dispersion of iodine intake between individuals may be much higher depending on dietary habits (fig. 3B). For the same average increase in iodine intake some people will still be iodine deficient and others will have an excessive intake (compare fig. 3A and 3B). To avoid iodine deficiency in part of the population after such a program, it will be necessary to increase the iodization level further, and more people will have excessive iodine intake (see fig. 3C).

Conclusion

Eradication of iodine deficiency has always the highest priority. The situation in Europe has improved in recent years.

Optimal prevention of thyroid disease by modification of iodine intake in the population is achieved by keeping iodine intake in individuals within a relatively narrow interval around the recommended level. To run an optimal iodization program it is necessary to have information on dietary habits in the population, and on iodine contents of different food items. Iodine used for enrichment of food should be well distributed in different food items, e. g. by universal or nearly universal iodization of salt. Optimal methods may differ between European countries depending on dietary habits.

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Iodine Nutrition in North America

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INTRODUCTION

Iodine is critical for the synthesis of thyroid hormone, and deficiency may result in goiter, impaired cognition, developmental abnormalities, and cretinism. Iodine deficiency is the most common cause of preventable mental retardation worldwide and poses an important public health issue in many areas. In this review of iodine nutrition in North America, we will focus on the state of iodine nutrition in the U.S., as there have been no recent assessments of iodine nutrition in Canada and only limited data from Mexico. Dedicated efforts to eliminate iodine deficiency and varying consumption of iodine-containing foods have influenced the status of the North America iodine supply dramatically over the last century.

The status of North American dietary iodine deficiency before the 1920s

Endemic iodine deficiency disorders occur commonly in areas where iodine in the soil is depleted by erosion and other natural processes. In North America, iodine deficiency was prevalent in the Great Lakes, Appalachian, and Northwestern U.S. regions and in most of Canada up until the 1920s. Goiter was present in 26-70% of children living in this "goiter belt", and mental retardation and hypothyroidism were common (1). In 1917 midwesterner David Marine performed studies in schoolgirls that demonstrated that goiter could be eradicated by iodine supplementation (2). Based primarily on his work, voluntary salt iodization was initiated in the U.S. in 1924, resulting in the elimination of the goiter belt. Salt iodization was also begun in Canada in the 1920s. A survey in 1950 demonstrated that the goiter prevalence across 8 states of Mexico ranged from 5 to 46 percent (3). Mandatory iodized salt was introduced in some regions of Mexico in 1963, but salt iodization programs were not consistently maintained.

What are the recommendations for iodine intake?

The U.S. Institute of Medicine has established Recommended Daily Allowances (RDA) for iodine in U.S. and Canadian infants, children and adolescents, pregnant women, lactating women, and other adults range from 110 – 290 mcg/L. For infants less than one year of age, a recommended iodine daily intake has not been established, and an Adequate Intake (AI) level is instead utilized (Table 1).

In the population subset of women of childbearing age, iodine nutrition is particularly important, given the importance of iodine in the development of the central nervous system. The American Thyroid Association has recently recommended that all U.S. and Canadian women receive 150 µg iodine supplements above their dietary intake during pregnancy and lactation, and that the iodine content in all prenatal vitamins should be standardized at 150 µg (4).

Type of Recommendation	Population	µg iodine per day
Adequate Intake	Infants 0 – 6 months	110
	Infants 7 – 12 months	130
Recommended Dietary Allowance	Children 1 – 8 years	90
	Children 9 – 13 years	120
	Adolescents and adults > 13 years	150
	Pregnant women	220
	Lactating women	290
Tolerable Upper Limit	Infants 0 – 12 months	Unknown
	Children 1 – 3 years	200
	Children 4 – 8 years	300
	Children 9 – 13 years	600
	Adolescents 14 – 18 years	900
	Adults 19 – 50 years	1,100

Table 1. Institute of Medicine Recommendations for Dietary Iodine

[Adapted from Food and Nutrition Board, Institute of Medicine 2001 Dietary reference intakes.

National Academy Press, Washington, D.C.]

What is the current status of iodine nutrition in North America?

The median urinary iodine concentration in the first U.S. National Health and Nutrition Examination Survey (NHANES I, 1971-1974) was 320 µg/L, which is consistent with adequate to excessive dietary iodine intake (5). However, the median urinary iodine decreased by more than 50 percent to 145 µg/L, by NHANES III (1988-1994). It is reassuring that data from the most recent NHANES survey (2001-2002) found that the median U.S. urinary iodine concentration has stabilized at 168 µg/L (6) (Figure 1A). In Mexico, there have been no surveys examining iodine nutrition across the population. However, several small studies, mostly in schoolchildren, in the last decade have suggested likely iodine sufficiency. The median urinary iodine level in a 2006 study of 100 schoolchildren was adequate at 125 µg/L, with only 6% of these children having median urinary iodine levels < 50 µg/L (7). In contrast to the U.S., where both iodized and non-iodized salt are available, all table salt in Canada is iodized; both the U.S. and Canada fortify table salt with 100 ppm potassium iodide, which corresponds to approximately 77 µg iodine per gram of salt. Although no recent studies in Canada have examined measures of iodine status across the general population, it is considered likely to be an iodine sufficient region (8).

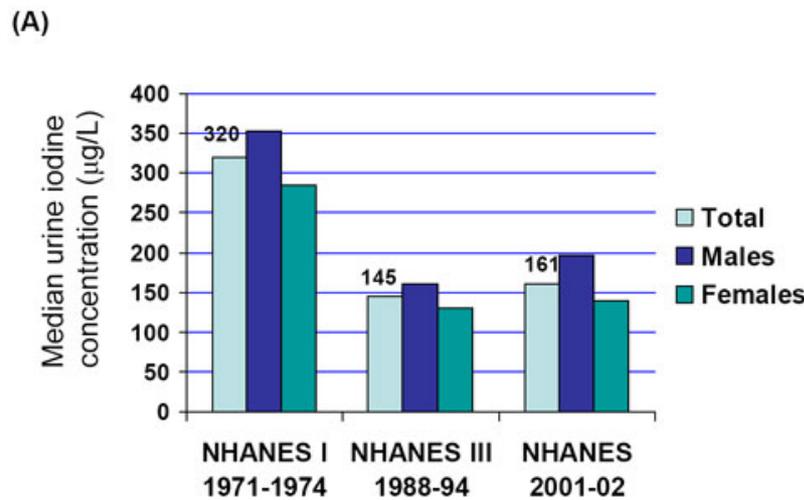


Figure 1. (A) Median U.S. urinary iodine concentrations in males and females, 1971-2002 (B)

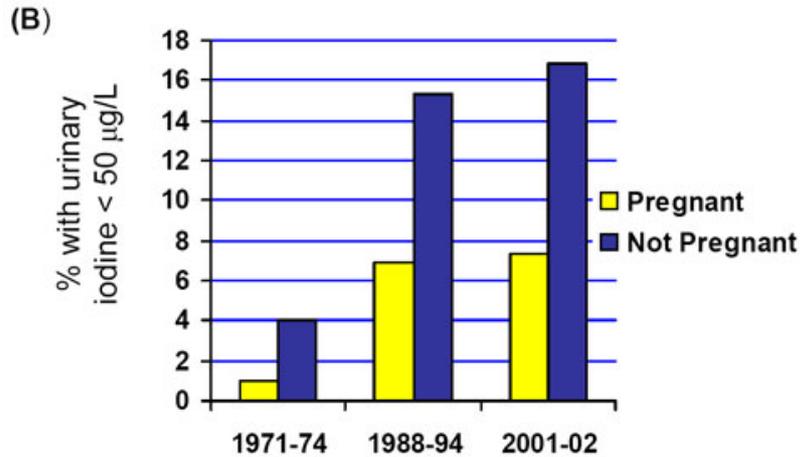
Median U.S. urinary iodine concentrations in pregnant and non-pregnant women of child-bearing age (15- 44 years old), 1971-2002.

[Adapted from Hollowell et al, JCEM 1998; 83:3401-8 & Caldwell et al, Thyroid 2005;15:692-9]

Women of childbearing age

Women of childbearing age (15-44 years) and their infants are especially susceptible to iodine deficiency. This is because thyroid hormone, requiring adequate maternal iodine intake, is

critical for neural development in utero. Between NHANES I and III, the most pronounced decline in U.S. median urinary iodine values occurred in women of childbearing age (Figure 1B). The median urinary iodine value in pregnant women ($n = 208$) from NHANES I was $327 \mu\text{g/L}$, with 1% of the women sampled having urinary iodine levels $< 50 \mu\text{g/L}$. The median urinary iodine level among pregnant women from NHANES III ($n = 348$) was $141 \mu\text{g/L}$, with 6.9% having urinary iodine levels $< 50 \mu\text{g/L}$ (5). The most recent NHANES survey (2000-2001) demonstrated that urinary iodine values in pregnant women appear to have stabilized since NHANES III. The median urinary iodine value was $173 \mu\text{g/L}$ for the 126 pregnant women sampled, with 7.3% having urinary iodine levels $< 50 \mu\text{g/L}$ (6). We assessed spot urine specimens from 100 healthy pregnant Boston-area women in 2004 and found a median urinary iodine of $149 \mu\text{g/L}$ (range, 13 – $1200 \mu\text{g/L}$), but 9% were $< 50 \mu\text{g/L}$ (9).



[Adapted from Hollowell et al, JCEM 1998; 83:3401-8 & Caldwell et al, Thyroid 2005;15:692-9]

Children

Several studies have examined urinary iodine in samples of school-aged U.S. children. NHANES I reported that in children ages 6-11, the median urinary iodine was $421 \mu\text{g/L}$, while by the time of NHANES III, this value had decreased to $237 \mu\text{g/L}$ (5). Since then, urinary iodine values in U.S. children, as for other groups, appear to have stabilized. The median urinary iodine in a 1996 sample of 302 Atlanta children was $282 \mu\text{g/L}$ (10). In a 2002 sample of 565 Boston-area children, median urinary iodine was $289 \mu\text{g/L}$ (11). Finally, in the most recent NHANES survey (2001-2002; $n = 374$) median urinary iodine was $249 \mu\text{g/L}$ (6). Interestingly, children's urinary iodine values are consistently higher than those of U.S. adults and boys' urinary iodine values are consistently higher than those for girls.

Breastfed infants

Infants who are breastfed are reliant on maternal iodine intake for adequate iodine nutrition (12). During lactation, iodine is avidly concentrated in the breast, due to increased expression of the sodium/iodide symporter in lactating mammary tissue (13). However, current data regarding iodine sufficiency among lactating U.S. women are very limited. The median breast milk iodide level in a 1984 sample of 37 U.S. women was $146 \mu\text{g/L}$ (14). In 2005, Kirk et al reported a substantially lower median breast milk iodide value of $33.5 \mu\text{g/L}$ in a sample of 23 U.S. women recruited via the internet (15). Finally, we reported a median urinary breast milk iodine concentration of $155 \mu\text{g/L}$ in a sample of 57 lactating Boston-area women (16). Forty-seven percent of women sampled may have been providing breast milk with insufficient iodine to meet infants' requirements in this study (16).

What are common sources of iodine in the U.S. diet?

It has been difficult to identify sources of dietary iodine in the U.S. There are a wide range of potential sources, and varying dietary practices likely contribute to wide fluctuations in individual iodine intake. Food iodine levels are further affected by regional variations in topsoil content and irrigation practices. The U.S. Food and Drug Administration does not require iodine content to be listed on food packaging.

Salt

In 1990, the World Health Assembly adopted universal salt iodization as the route to eliminate iodine deficiency disorders. Though iodized salt was responsible for eliminating the goiter belt in the U.S. beginning in the 1920s, it has never been mandated in the U.S. 70% of salt sold for household use in the U.S. is fortified with 100 ppm potassium iodide (i.e. $400 \mu\text{g}$ iodine

is present in one teaspoon of iodized table salt) (17), but household table salt accounts for only about 15% of daily salt intake in the U.S. Among the possible reasons for the decrease in U.S. iodine consumption between the early 1970s and the 1990s are recommendations for reduced salt intake for blood pressure control, and increasing use of non-iodized salt in processed foods (18). Finally, the salt used in manufacturing many processed foods may not be iodized and warrants further investigation. In Canada, all salt is required to be fortified with 77 ppm potassium iodide, while Mexico has mandated fortification of all salt with 20 ppm potassium iodide. Whether this is consistently so in Mexico is unclear.

Milk

Between 1965 and 1980, U.S. milk iodine content increased by 300-500%, primarily because of changes in cattle feeds (19). Then in 1986, the allowable amount of organic iodine ethylenediamine dihydroiodide (EDDI) in cattle feed was limited to 10mg per cow daily, resulting in decreases in the iodine content of U.S. cows' milk, which accounts for another likely reason for the decrease in U.S. dietary iodine intake between the 1970s and 1990s. In addition, iodophor disinfectant pre- and post-milking teat dips and udder washes, which are widely used in the U.S., contain up to 1% available iodine, and are absorbed through the skin and subsequently incorporated into cows' milk (20, 21) and may also directly contaminate milk during the milking process. The iodine content of 18 varieties of cows' milk from Boston-area supermarkets was recently measured (22). The average iodine content of milk in this sample was 110 µg per cup (464 µg/L), with iodine content being slightly higher in the winter than in the summer. Another recent study examined 39 samples of cow's milk from around the U.S. and found that the average iodide content was 89.2 µg/L (15).

Commercially-baked breads

Commercially-baked breads have been another major source of iodine in the U.S. diet. Iodate bread conditioners, added to bread to maintain freshness, were widely used starting in the 1940s. London et al reported in 1965 that bread was a source of large quantities of dietary iodine with iodine content as high as 150 µg per slice (23). This was considered to be a cause of decreasing radioactive iodine uptakes in the U.S. during the 1960s (24, 25). The use of iodate bread conditioners has decreased in recent decades, another probable contributing factor to the reduction in U.S. dietary iodine levels between the 1970s and the early 1990s. In 2002 we measured iodine content of 18 different breads from Boston-area supermarkets (26). Three varieties of bread contained >300 µg iodine per slice (313.5 to 587.4 µg), while the average iodine content in the other 17 brands was 10 µg iodine/slice. We found that the labeling of bread packages did not accurately predict the content of iodine.

Infant formula

We recently measured the iodine content of 17 varieties of infant formula sold in the Boston area, which ranged from 84-224 µg/L, similar to concentrations found in breast milk (16).

Other sources

Other important sources of dietary iodine in North America include eggs, meat, and poultry. The iodine in eggs is found primarily in the yolk, and a large egg contains about 29 µg of iodine (26). The amount of iodine in meat and poultry is highly variable, depending largely on the amount of iodine supplementation of animals' feed. Seafood can also be a large source of dietary iodine and may contain 2–10 times as much iodine as meats (27). However, iodine content varies widely depending on the type of seafood and location (28). In general, saltwater seafood contains more iodine than freshwater seafood. Edible seaweeds may contain up to 2500 µg iodine per gram (29), but are not a major component of the North American diet. Erythrosine dye (FDC Red #3) is sometimes described as a major contributor to U.S. dietary iodine intake, but this is untrue. First, this colorant is no longer widely used in U.S. foods. Second, the iodine contained in erythrosine is not readily bioavailable, as only about 1% of iodine in ingested erythrosine is actually absorbed (30).

Multivitamins

Multivitamins, in both adult and infant formulations, may be an important source of iodine in the U.S. Only 51% of the adult multivitamin formulations on the U.S. market contain iodine (generally 150 µg iodine daily) (31). 45% of the types of U.S. children's multivitamin formulations contain iodine, and importantly, none of the infant liquid multivitamin formulations marketed in the U.S. contain iodine (31). Based on concerns about adequate iodine intake in the perinatal period, the National Academy of Sciences recently recommended that consideration be given to adding iodine to all prenatal vitamins (32). However, only 44 of 69 (64%) prenatal multivitamins marketed in the U.S. currently contain any iodine; of those, only 15% contain more than 150 µg, and most contain less than the 220 µg daily recommended for pregnancy or the 290 µg daily recommended during lactation (31).

Medications

Although they do not contribute to North American dietary sufficiency overall, in some individuals, medications can be an important source of ingested daily iodine (Table 2). Amiodarone is an antiarrhythmic agent frequently used in the U.S. (33), Canada, and Mexico, and contains 75 mg iodine per 200mg tablet. Iodinated intravenous radiographic contrast agents contain

up to 380 mg of iodine per mL. Some topical antiseptic contain iodine, but systemic absorption of these agents is generally not clinically significant except in patients with severe burns (34), those treated with betadine irrigation for severe wound infections (35), and in preterm infants (36). Use of iodine-containing vaginal douches has declined over the last 15 years, but remains a common practice among some groups of U.S. women (37); frequent use has been shown to increase serum and urine iodine concentrations (38). In the past, there were several iodine-containing anti-asthmatic medications and expectorants on the U.S. market, however these are no longer available. Finally, some dietary supplements may contain large amounts of iodine; these are not regulated by the U.S. Food and Drug Administration and the prevalence of such supplement use is unknown.

Medication	Iodine Content
Amiodarone	75 mg/200mg tablet
Iodoquinol	134 mg/tablet
Povidone-iodine vaginal douche	10 mg/ml
Povidone-iodine topical antiseptic	10 mg/ml
Iodinated intravenous contrast dye	150 – 320 mg/ml
SSKI	25 mg/drop
Lugol's solution	5 mg/drop

Table 2. Iodine-Containing Medications Currently Marketed in the U.S.

What are the assessments used to ensure iodine adequacy in North America?

The U.S. Total Diet Study in the 1980s was a market basket study that assessed iodine consumption in a wide variety of common foods. The estimated individual daily iodine intake was 150 – 550 µg/day. The NHANES studies have measured trends by urinary iodine concentrations across the U.S. population since the early 1970s. Larger and systematic studies examining iodine nutrition in different population subgroups, and the assessment of the iodine content in different foods and other sources, are warranted to ensure iodine sufficiency in the future. There have been no recent systematic assessments of the status of iodine nutrition in Canada or Mexico.

CONCLUSIONS

Iodine deficiency was prevalent in the Great Lakes, Appalachian, and Northwestern U.S. regions and in most of Canada until the 1920s, when efforts to eliminate endemic goiter with the use of iodized salt were introduced. However, the most significant routes of iodine exposure are difficult to determine, and further studies are needed to assess the role of other foods and other sources with large iodine loads. Although assessments of U.S. iodine nutritional status in the last 40 years have shown a decreasing trend of urinary iodine values, the U.S. population remains generally iodine sufficient. Despite this, achieving stable adequate iodine levels in pregnant women and those of childbearing age may remain an area worthy of public health concern. We feel there needs to be increased awareness of the importance of adequate iodine nutrition especially in this particularly susceptible population, and that iodine-containing multivitamins should be recommended for pregnant and non-pregnant women of childbearing age. There have been no recent systematic assessments of iodine intake in Canada and Mexico, though these countries are likely iodine sufficient. Studies are needed to determine the status of iodine nutrition in these areas and in population subgroups across the U.S.

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The TSH receptor – a new crystal structure

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In 2006 our laboratory was able to determine the high resolution (2.55 Å) crystal structure of the TSH receptor (TSHR) leucine rich domain (LRD) in complex with the thyroid stimulating monoclonal autoantibody M22 (1). As a consequence, the structure of the TSHR LRD is now established. Also the molecular interactions between the receptor and M22 are known in detail. These results have important implications for our understanding of glycoprotein hormone interactions with their receptors. Furthermore, there are important implications for the rational design of new molecules which can interact with the TSHR as agonists or antagonists. In addition, the structure provides key insights into the nature of the autoimmune response to the TSH receptor. A brief review of these aspects of the TSHR and how it interacts with M22 and TSH is presented.

TSH receptor structure

The overall structure of the TSHR determined by comparative modelling (2,3) prior to crystal structure analysis is shown in Figure 1. It consists of three major domains: the LRD, cleavage domain (CD) and transmembrane domain (TMD). The concave surface formed by the LRD (with some contribution from the CD) is principally responsible for ligand binding.

Obtaining highly purified preparations of the TSHR suitable for crystallisation and analysis by X-ray diffraction has been a major challenge. This was due in part at least to the susceptibility of native and recombinant TSHR preparations to degradation and denaturation. Furthermore this instability is seen in preparations of the isolated LRD.

Early studies (4,5) with preparations of native TSHR extracellular domain (ECD) showed that when the ECD bound patient serum TSHR autoantibodies a stable complex was formed.

This complex consisted of one molecule of ECD and one molecule of antibody and it could be purified by various chromatographic procedures and isoelectric focussing. Once the thyroid stimulating human monoclonal autoantibody M22 became available (6,7) it was possible to use a similar approach to these earlier studies to obtain preparations of the TSHR-M22 complex suitable for structural analysis. In particular, a TSHR260 construct (coding for amino acids 1-260 with a 6 His tag at the C-terminus) was expressed in insect cells with M22 Fab included in the culture medium.

As TSHR260 was secreted into the culture medium, it was bound by M22 and a stable complex formed. The complex could then be purified by various chromatographic procedures to give a preparation about 95% pure containing equal proportions of M22 Fab and TSHR260 (1). After deglycosylation, crystals suitable for analysis by X-ray diffraction were obtained and the structure solved at 2.55 Å resolution (1).

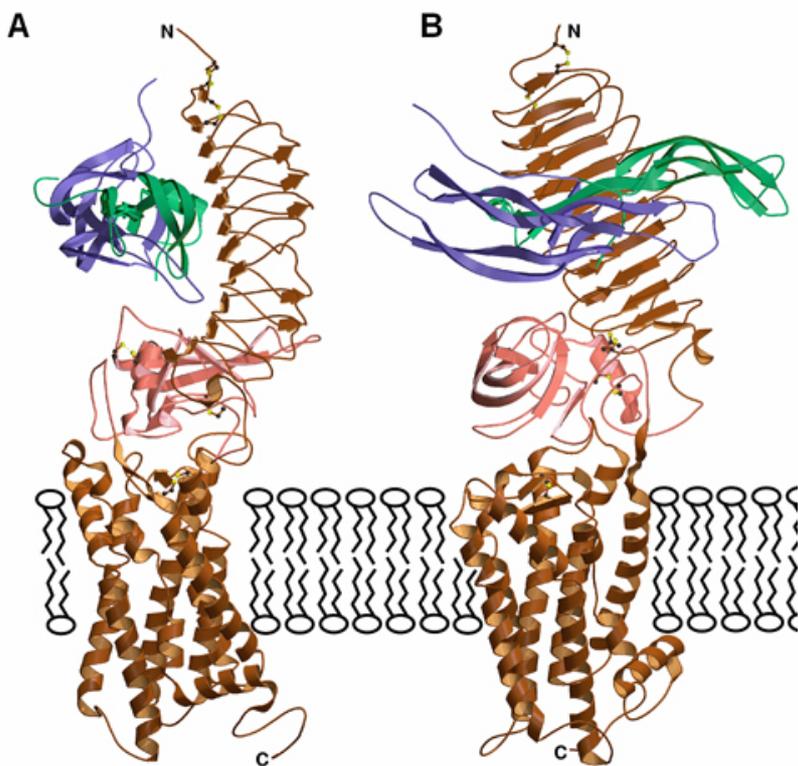


Figure 1

Structural model of the TSHR-TSH complex (2,3). (A) and (B) are the same models with (B) turned by 90° relative to (A). The α -chain of hTSH is in green and the β -chain is in blue, the TSHR transmembrane domain (TMD) and leucine-rich domain (LRD) are in brown and the TSHR cleavage domain (CD) is in pink.

TSHR LRD structure

The structure of the TSHR LRD is shown in Figure 2 and the receptor has the shape of a slightly curved helical tube (Figure 3) constructed from leucine-rich repeat motifs. It has opposed concave and convex surfaces with a 10-stranded β sheet located on the concave surface. The inner surface of the tube is lined with hydrophobic residues. The TSHR N-terminal cysteine 31 and cysteine 41 are linked via a disulphide bond. All five glycosylation sites are located on the convex surface of the LRD (Figures 2 and 3), away from the regions involved in ligand binding.

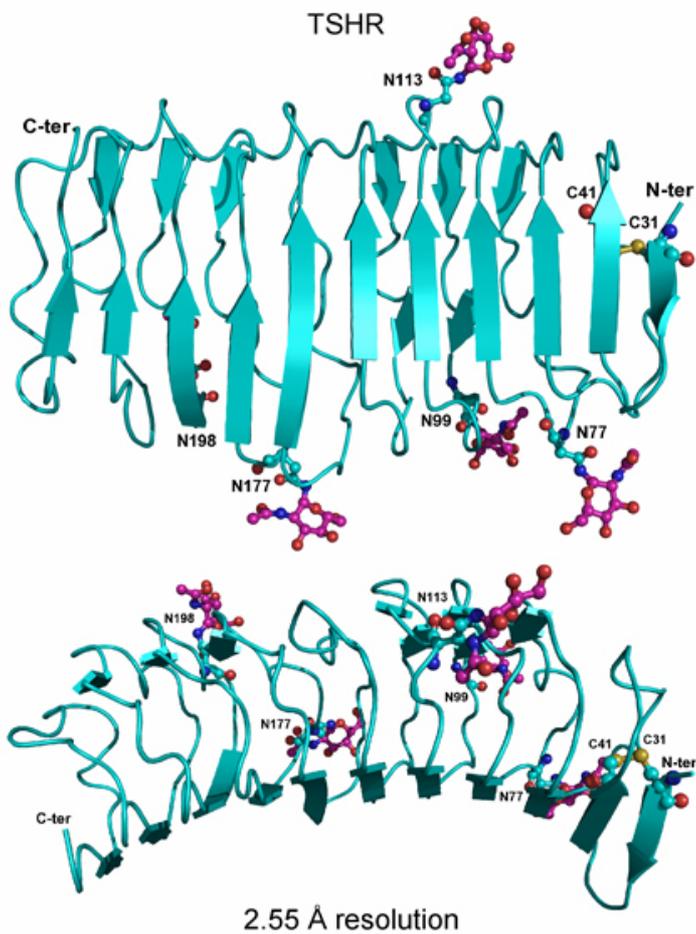


Figure 2

The crystal structure of the TSHR at 2.55 Å resolution. The N-linked carbohydrate residues are shown as ball and stick (carbon atoms in pink, oxygen atoms in red and nitrogen atoms in blue) and carbohydrate-bound asparagines are labelled. The carbohydrate-bound asparagines and the disulphide bonded cysteines at the N-terminus (N-ter) are shown as ball and stick (oxygen atoms in red, nitrogen atoms in blue, sulphur atoms in yellow and carbon atoms in cyan and blue for TSHR and FSHR respectively). The Figure was generated using PyMOL. C-terminus = C-ter.

The TSHR-M22 complex

M22 Fab clasps the concave surface of the TSHR LRD at 90° to the axis of the LRD tube and a large part of the LRD surface is involved in interactions with M22 (Figure 3). These extend from the LRD N-terminus to C-terminus and a total of 2500 Å² of solvent accessible surface area is buried in the interface (1). This area is larger than typically observed for antibody-antigen interactions (8). There are a large number of hydrogen bonds and salt bridges in the interface together with non-hydrogen bonding polar interactions, hydrophobic contacts and van der Waals interactions. The heavy chain (HC) of M22 Fab has more amino acids in interaction with the LRD than has the light chain (LC). Both chains form hydrogen bonds and salt bridges with the TSHR, 14 in the case of the HC and 8 in the case of the LC. Most of the residues in M22 which interact with the TSHR are in the hypervariable regions, particularly H2, H3 and L2.

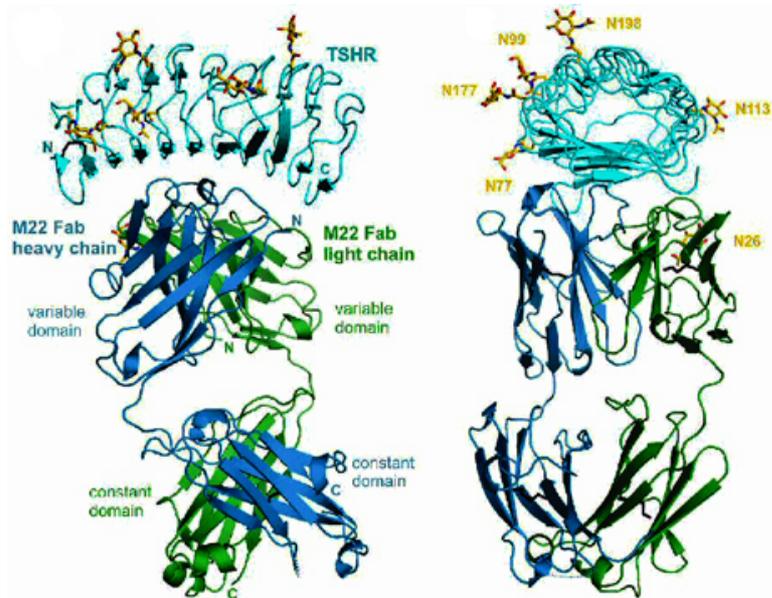


Figure 3

The TSHR-M22 Fab complex structure shown in differently aligned views related by a 90° rotation about the vertical axis. TSHR LRD is in cyan, M22 Fab LC is in green, M22 HC is in blue, the N-linked carbohydrates are in yellow and carbohydrate-bound asparagines are labelled. The amino-(N) and carboxyl-(C) termini are indicated. Disulphide bonds are in black.

Comparison of receptor bound and free M22

As the crystal structure of M22 Fab is known at 1.65 Å resolution (7), when the TSHR-M22 structure was solved, it was possible to compare receptor bound and free M22 structures in detail. This comparison showed that the majority of M22 variable region residues involved in interactions with the TSHR had almost identical positions in the receptor bound and free structures. The highest deviation of an atom from M22 backbone residues was 1.1 Å. In addition, only six M22 amino acids in the complex showed a deviation of their side chains of greater than 2 Å compared to unbound M22. Consequently, there is essentially no movement of the atoms of M22 when receptor binding occurs. As a result, the free energy loss which would happen as a consequence of a conformational change in M22 does not occur and this is consistent with the high affinity of the M22-TSHR interaction (6,7). The TSHR LRD itself is a relatively rigid structure and a major change in LRD conformation on M22 binding is unlikely. However binding of M22 to the LRD must induce changes in the receptor which cause signal induction but to date the nature of these changes is not known.

The TSHR-TSH and FSHR-FSH complexes

Once the structure of the TSHR LRD was known, it was possible to use the structure, together with the structure of the FSHR LRD (9) to prepare a new comparative model of the TSHR LRD bound to TSH. This involved (a) building a model of the TSHR-TSH complex based on the crystal structure of the FSHR-FSH complex (2.9 Å resolution), (b) replacing the FSHR structure in the model with the TSHR crystal structure (2.55 Å resolution) and (c) minimisation of the interface in order to optimise interactions between TSH and the TSHR. This model of the TSHR-TSH complex is shown in Figure 4 together with the FSHR-FSH structure for comparison.

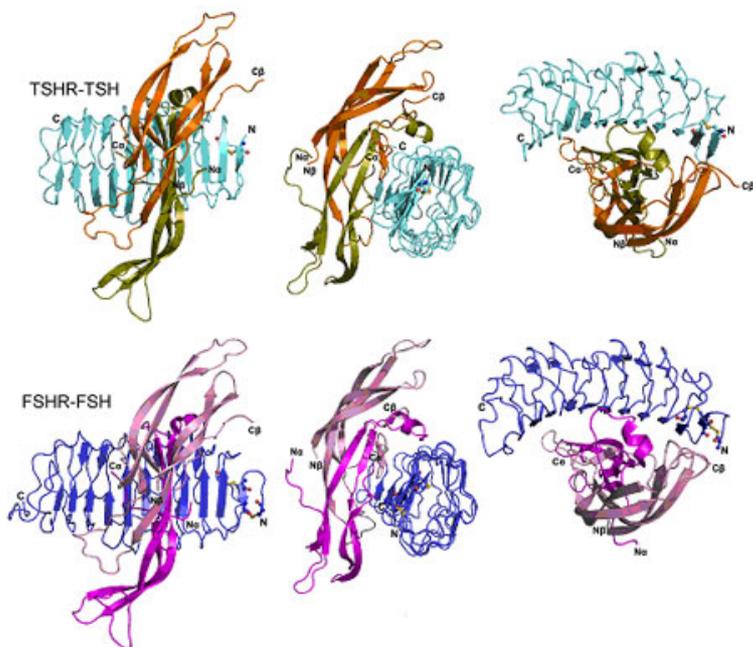


Figure 4

Binding arrangements in the TSHR-TSH complex (TSHR shown in cyan, α -TSH shown in deep olive and β -TSH shown in orange) and the FSHR-FSH complex (FSHR shown in blue, α -FSH shown in magenta and β -FSH shown in pink). The disulphide bonded cysteines at the receptors' N-termini are shown as ball and stick (oxygen atoms in red, nitrogen atoms in blue, sulphur atoms in yellow and carbon atoms in cyan and blue for TSHR and FSHR respectively). Three different perspectives of each complex are shown.

Comparison of TSHR interactions with hormone and with autoantibody

When the structures of the TSHR LRD-M22 complex and the FSHR LRD-FSH complex are superimposed, the positions of M22 Fab and FSH relative to the concave surface of their respective receptors are remarkably similar (1). As TSH and FSH position themselves on their respective receptors in essentially identical ways (Figure 4) this means that M22 positions itself on the TSHR in a very similar way to TSH. Consequently, two ligands with very different origins and structures show remarkably similar TSH receptor binding features. This is an example of evolutionary convergence and the nature of the evolutionary forces which have resulted in the production of an autoantibody which mimics the actions of the native hormone TSH so well are intriguing.

Comparison of hormone-receptor and autoantibody-receptor interactions

As described above, M22 positions itself on the TSHR LRD in a very similar way to TSH and in a very similar way to FSH on the FSHR LRD. Also, the solvent accessible surface areas buried in the interfaces between the TSHR and M22 and between the FSHR and FSH are similarly large (2500 \AA^2 and 2600 \AA^2 respectively) (1,9). In addition, the overall structures of both complexes are remarkably similar with the rmsd on C_{α} core atoms of the two receptor LRDs being 1.1 \AA (1). M22 and FSH interact with residues contributed by all 10 β -strands on the concave surface of the TSHR or FSHR respectively. However, there are 14 hydrogen bonds in the TSHR-M22 complex but only 6 hydrogen bonds in the FSHR-FSH complex. In addition, M22 interacts with residues in the C terminal part of the TSHR LRD but the equivalent residues in the C terminal part of the FSHR LRD are not involved in FSH binding (1,9). Also, the conformational change in FSH which occurs on binding to the FSHR (9) is not seen in M22 on binding to the TSHR.

Blocking and stimulating TSHR autoantibodies

Although the details of how a thyroid stimulating autoantibody interacts with the TSHR LRD are now known at the atomic level, there is not as yet similar data relating to a TSHR autoantibody which blocks TSH action. The crystal structure of a mouse MAb (RSR-B2) which blocks the stimulating activity of TSH and thyroid stimulating autoantibodies is known (10). Also extensive experiments on the interactions of RSR-B2 with mutated TSHR preparations have been able to establish which amino acids on the LRD concave surface are important for RSR-B2 binding (11). These studies indicate that TSHR residues important for the blocking activity of RSR-B2 are in the N-terminal part of the LRD whereas about half of the TSHR

residues important for the stimulating activity of M22 are C-terminal of all the residues important for blocking activity of RSR-B2 ie RSR-B2 interacts with residues in the N-terminal part of the TSHR LRD whereas M22 interacts with amino acids in both the N- and C-terminal parts. Although the TSHR residues important for the activities of the stimulating antibody (M22) and the blocking antibody (RSR-B2) differ, these two antibodies compete effectively with each other for binding to the TSHR and there is evidence for a considerable overlap of their respective binding sites on the TSHR (6,7,10,11). Consequently differences in the regions of the receptor recognised by the two MAbs might be responsible for their different activities.

Mechanism of TSHR activation by TSH and TSHR autoantibodies

The structural information currently available and comparative models derived from it have provided little insight as yet into how ligand binding activates the TSHR. Ligand induced dimerisation has been proposed to be an important step in activation of GPCRs in general (12) and the TSHR in particular (13,14). Analysis of the TSHR LRD-M22 complex however indicates that this does not form dimers (see above) and consequently receptor-receptor interactions involving the first 260 amino acids of the TSHR are unlikely to be important in any dimer formation. In X-ray diffraction analysis of the FSHR-FSH complex, two FSHR-FSH complexes were observed in the asymmetric unit and dimerisation of the FSHR-FSH complex was suggested (9). However this apparent difference between the two complexes may reflect differences in crystal packing.

Implications of recent structural studies

The structures of the regions of the TSHR and of the FSHR which are responsible in the main part for binding activating ligands are now known at 2.55 Å and 2.9 Å resolution respectively. Also, the details of how these receptors interact with their ligands are known at the atomic level. Careful comparison of the TSHR and FSHR structures and how they interact with their respective activating ligands should provide key insights into how the distinct specificity and functions of the two receptor-hormone systems have evolved. In terms of how ligand binding activates the TSHR (and FSHR), structural information on the entire ECD at least and its position relative to the TMD in the 3-domain structure of the receptor (unbound and bound to a ligand) is likely to be needed before this process can be better understood. There is good reason to believe that determining these structures is an attainable goal using a similar approach to that employed to solve the structure of the TSHR LRD in complex with M22. Knowledge of the TSHR-M22 interactions at the atomic level provides a basis for the rational design of small molecules which inhibit the binding of autoantibodies to the receptor. Such compounds are likely to have a higher degree of specificity for the TSHR than the low molecular weight inhibitors of TSHR TMD function which have been described (15,16). This is because the sequence similarities between the glycoprotein hormone TMDs is high (about 70%) whereas that between their LRDs is relatively low (about 40%). Inhibitors of the autoantibody-receptor interaction designed using the TSHR-M22 structure are likely to provide a new generation of drugs which will control thyroid function by targeting the actions of the autoantibodies responsible for Graves' disease with a high degree of specificity.

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Conflict of interest statement

RSR Ltd is a developer of medical diagnostics including kits for measuring thyroid autoantibodies.

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Microchimerism and thyroid disease - 2007

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Introduction

Defining microchimerism

The term microchimerism was coined by Liegeois (1) in 1977. The name recalls the Lyon-goat-snake merged mythological figure (Figure 1). Microchimerism is defined by the presence of cells in an individual tissue that derives from another genetically distinct individual. This situation can result from a natural process such as pregnancy or between twins or from the mother (natural microchimerism) or after an artificial intervention such as a tissue transplant or blood transfusion (artificial microchimerism). It has also been suggested that sexual intercourse may involve male cell trafficking and theoretically microchimerism could also arise from an older sibling transferred via the maternal circulation to the foetus of a later pregnancy. Furthermore, long-term persistence of foetal cells in the mother (foetal microchimerism) or maternal cells in her offspring (maternal microchimerism) indicates the prolonged potential for a role in immune modulation and/or disease pathology (Table 1).



Figure 1

Chimera. Etruscan bronze sculpture from Arezzo, Italy, 5th–4th century BC. Archaeological Museum, Florence.

Table 1: Types of microchimerism

Type	Commentaries
Natural	The microchimeric cells come as a result of a current biological situation such as pregnancy, miscarriage or twinning.
Artificial	The source of microchimeric cells is due to a medical intervention as organ transplantation (such as bone marrow, kidney and heart) or blood transfusion.
Bad	It is assumed that the microchimeric cells elicit a graft vs. host like immune reaction and therefore start or maintain a disease. Examples may be the autoimmune diseases as Hashimoto's thyroiditis or Graves' disease.
Good	Microchimeric cells may participate in tissue repair processes.
Maternal	Persistence of maternal cells in offspring.
Foetal	Persistence of foetal cells in the mother.

The consequences of microchimerism

The role of these alien cells in the health of the recipient is of great interest and has not been fully elucidated. However experimental data support important hypotheses concerning their biological significance. It can be assumed that microchimerism may have adverse, neutral, or beneficial effects, depending on a variety of factors (2):

- The bad microchimerism hypothesis was initially proposed by Nelson (3) who suggested that the presence, in women, of foetal cells following pregnancy leads to a graft-versus-host-like response in parous women. As a consequence the maternal immune response to these 'foreign' cells may contribute to autoimmune disease pathogenesis.
- There is potential for a good microchimerism, where persistent foetal cells, instead of inducing an immune reaction, are tolerated and have a positive effect as a resource of progenitor cells that may have the capacity to participate in maternal tissue repair processes.
- In addition, there may be neutral microchimerism, where foetal cells are innocent bystanders. It remains possible that foetal cells act as innocent local observers in a process triggered by other mechanisms and play no role in biology at all.

These three options may each occur under different circumstances and maternal or foetal factors must determine the different type of responses. A variety of such circumstances have been identified:

- It is well known that artificial microchimerism can result in chronic graft-vs-host disease, a disorder not dissimilar to autoimmune disease.
- The contribution of natural microchimerism to the origin or exacerbation of autoimmune diseases has been widely documented (4-9) although not yet universally accepted (10). The mechanism of this association has been based on the degree of HLA disparity between host and foreign cells which may determine the strength of any potential graft v host relationship.
- Controversy exists around the role of microchimeric cells in the pathogenesis of diseases of non-immune origin. Part of this controversy is the consideration that the presence of these foreign cells in tissues may be a consequence rather than the cause of disease. Since there may be a variety of foreign cell types persisting within the host tissue, including microchimeric stem cells, and there is potential for a variety of roles for such cells in disease including host tissue repair (11). Hence, factors that may influence foetal cell involvement include foetal or placental cell activities besides histocompatibility (4).
- The number of foetal cells that are transferred into the maternal circulation is considerably greater than the number of maternal cells transferred into the foetal circulation and cell number may be an important factor in any disease initiation process.

Detecting microchimerism

The commonest approach to detecting foreign cells has been the assay of male-specific gene markers in females such as the SRY gene. This can be studied by Y chromosome-specific Polymerase Cell Reaction (PCR) amplification (12,13) or by Fluorescence In-situ Hybridisation (FISH) with labelling of X and Y-chromosomes (14).

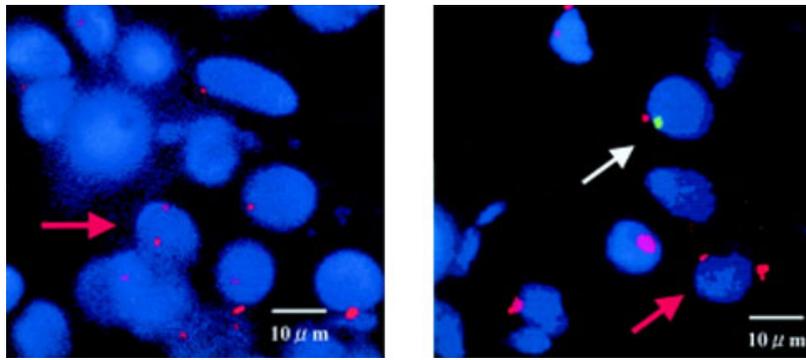


Figure 2

Fluorescence in situ hybridisation to detect the Y chromosome-containing cells in the biopsy specimens of the labial salivary glands.

The Y chromosome probe was labelled with fluorescein (green signal), and the X chromosome probe was labelled with rhodamine red (red signal).

(A) A female patient without Sjögren's Syndrome (SS). The cell nuclei contain two X chromosomes (red signals). (B) A female patient with SS who tested positive for a Y chromosome-specific sequence by PCR. White arrow - nucleus containing one Y chromosome (green signal); red arrow, nucleus containing two X chromosomes (red signals).

From Kuroki et al (55).

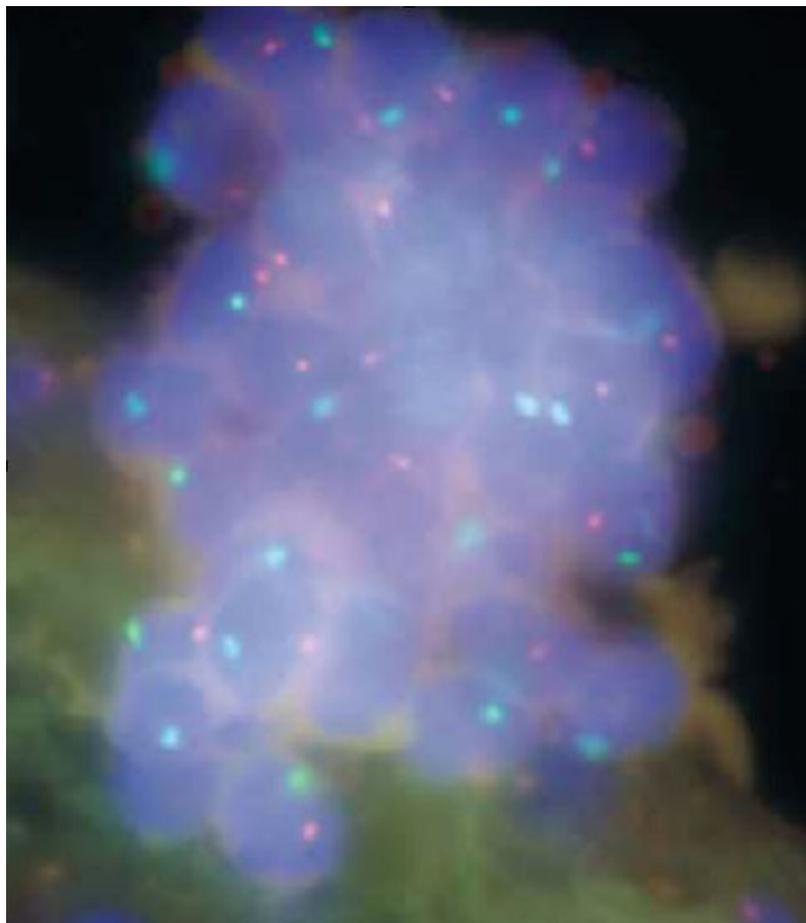


Figure 3

Interphase fluorescence in situ hybridization (FISH) of thyroid tissue showing a group of microchimeric cells identified by the presence of X and Y chromosomes (orange and green, respectively). The X or Y chromosome may not be observed in each nucleus, as they may not be in the same plane of focus (magnification X400). The FISH assay employed Cy3-labeled X (orange) and fluorescein isothiocyanate conjugated-labeled Y (green) chromosome probes. Nuclei were counterstained with 4',6-diamidino-2-phenylindole (blue). From Khosrotehrani K et al (40).

More recently, to identify the foetal cell type, immunological isolation, such as cell sorting with antibody-coated magnetic beads and flow cytometry, followed by detection of male DNA has also been used (6). Single cell RT-PCR after laser captures dissection is also a potential identifier. However, the most straightforward approach is to use fluorescent labelled male mice such that at least half the foetuses will be labelled and their cells easily identified histologically (15).

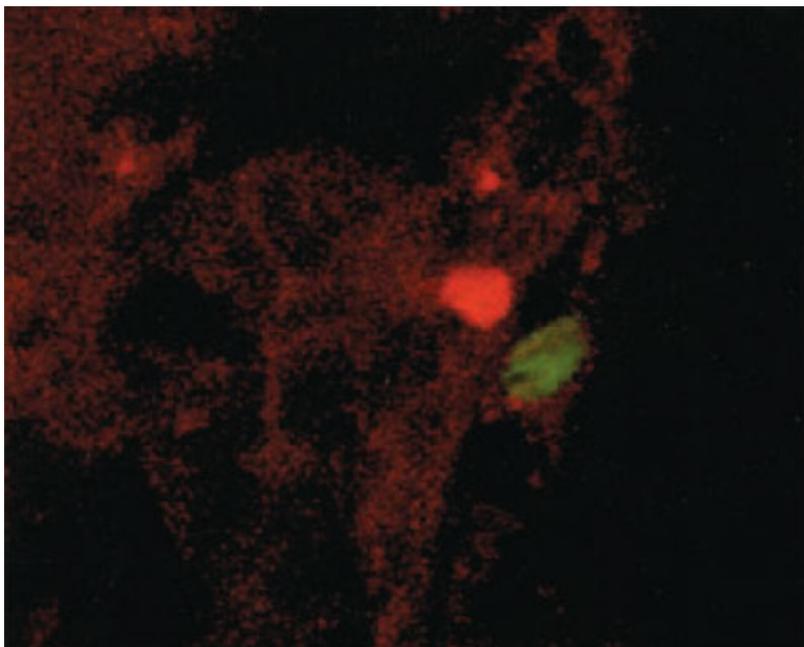


Figure 4

Intrathyroidal foetal cell expressing GFP seen in a murine model of autoimmune thyroiditis during pregnancy induced by mating with a male GFP-transgenic mouse. From Ando and Davies (8).

2.0. Pregnancy

Immunity in pregnancy

For a successful pregnancy outcome the maternal immune system must not reject the foetus. Trophoblast cells serve as physical barrier, expressing several immune modulating molecules as well as secreting a variety of cytokines. Thus placental immune suppression, which includes reduced activity of T regulatory cells (16), helps establish foetal microchimerism.

Table 2: Immune changes in pregnancy

Level of change	Changes	Consequences
Local control	Trophoblast cells, express several immune modulating molecules (Fas-L, HLA-G and indoleamine 2,3-dioxygenase) as well as secreting a variety of cytokines.	Fas-L induce apoptosis on foetal antigen-reactive maternal lymphocytes. HLA-G inhibits both NK cell function and maturation of dendritic cells. Indoleamine 2,3-dioxygenase catalyses triptophane in lymphocytes which is critical in the maintenance of allogenic pregnancy.
Systemic control	T Cell	Decreased CD4+, increased CD8+ T cells and increasing activity of T regulatory cells. The immune response is turned from Th1 (cellular) to Th2 (humoral), with an increase in Th2 cytokine production.
	B Cell	Despite the shift to Th2, the relative B cell production and activity is downregulated leading to a reduction in antibody production.
	Hormonal environment	Increase in plasma levels of oestrogen, progesterone and corticosteroids. Oestrogen produces negative regulation of B cell activity. Progesterone generates variation in cytokine profiles. Corticosteroids induce immune cell apoptosis and immune-suppression.
Postpartum	Recovery of pre-pregnancy immune function.	Increased titres of serum antibodies, reversed ratio CD4+/CD8+ T cells, and change in cytokine profiles favour Th1 responses.

In normal pregnancy there are important changes in maternal immune responses. A physiological immuno-suppression occurs to create an immune-privileged state protecting the foetus from rejection. Both arms of the adaptative immune responses (cell-mediated and humoral) are attenuated as is natural immunity. This placental immune suppression is thought to help establish fetal microchimerism to different degrees in different women.

Therefore, once foetal cells migrate and take up residence in maternal tissues, they may survive during the pregnancy and this immune suppression may remain some months after delivery (17), allowing foetal cells to establish themselves and to survive the postpartum period (6). Furthermore, a degree of long term tolerance for such cells may develop in some individuals, since fetal cells may survive for many years. Similarly, maternal cells may pass into the foetus whose immune system is immature and rapidly develops tolerance for such cells. The presence of microchimeric female cells in cord blood samples of male infants was described more than ten years ago by Hall et al (18). Since then, the presence of maternal cells has been repeatedly detected in adult healthy subjects (19,20) and in neonatal ill infants (21). Experimental data also showed the presence of maternal cells in bone marrow cavities of developing bones in a murine model (22). Investigations have estimated that maternal-to-foetus transfer should be as frequent as foetus-to-maternal trafficking, because maternal DNA has been detected in 40-100% of cord blood samples when PCR techniques were used (23,24).

Nelson's group demonstrated the presence of long-lasting maternal microchimeric cells in lymphoid (25%) and myeloid (14%) compartments of peripheral blood in healthy adult women (25). They speculated that the circulating level of maternal T lymphocytes in the foetus was present at levels with the potential for immunological effects. At the same time they raised the question such as whether an autoimmune disease may result from a breakdown in tolerance to maternal microchimerism. Maternal cells had been previously identified in neonatal lupus syndrome, an autoimmune disease that develops in uterus, and that finding indicated that maternal cells could migrate to and expand in the child's diseased tissue (21). A recent observation of maternal circulating cells in the foetus during pregnancy suggests an association of maternal HLA DQ*0301 with microchimerism (26). The presence of maternal microchimerism has been detected in a new-born thyroid autopsied at day 2 with multiple congenital abnormalities, but so far has not been reported in thyroid disease (19) but it remains possible that maternal cells may be involved in chronic inflammatory responses leading to tissue damage.

Pregnancy and microchimerism

Pregnancy is the cause of foetal microchimerism.

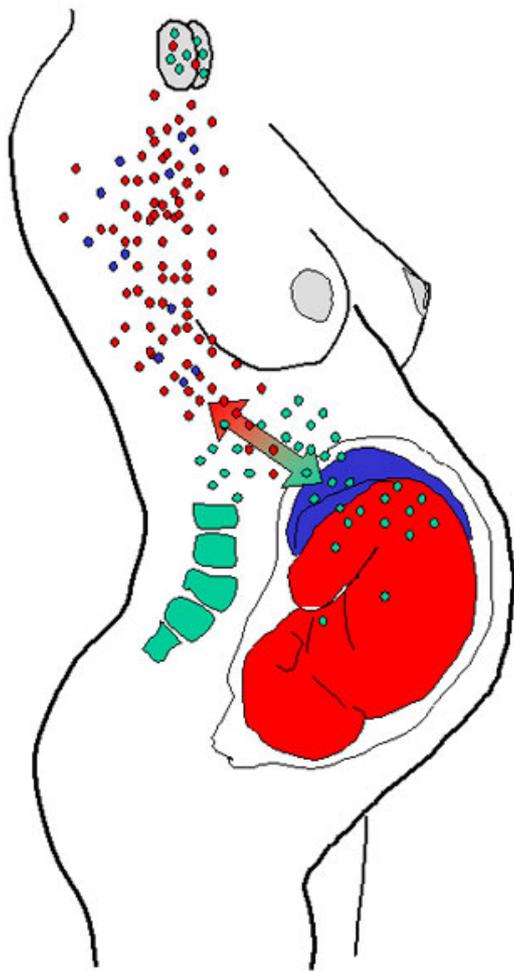


Figure 5

Cell traffic during pregnancy is bi-directional for maternal, foetal and placental cells.

Microchimeric cells enter the circulation and persist in the host tissues and are tolerated for many years.

Pregnancy microchimerism results from imperfections in the physical barrier of the placental trophoblastic tissue that separates the maternal circulation from the foetal circulation. In humans, such cell transfer has been detected as early as 4-5 weeks post conception (27). After the 34th week of gestation, circulating male cells have been detected in 100% of women bearing male foetuses (28,29). However, the concentration of these cells is generally very low, around 1-6 foetal cells/mL of maternal venous blood (30) or 1:500,000 male:female cells in male bearing pregnant women (31). Although their concentration decreases with time, such foetal cells can persist after delivery for more than 25 years (32). Studies have detected male cells of presumed foetal origin in 30% to 50% of healthy women many years after a prior male pregnancy (33).

Microchimeric cell types in pregnancy

The origin of microchimeric cells in pregnant women can be foetal or placental cells. It is assumed that foetal cells that enter the maternal circulation are predominantly of haematopoietic origin, such as nucleated red blood cells, lymphocytes, dendritic cells or haematopoietic stem cells (4,32,34-37). However trophoblasts and mesenchymal stem cells also circulate within maternal blood (38,39). Microchimeric foetal cells have been found in almost all maternal tissues at variable frequencies (40). One report found 14-60% of microchimeric cells expressing cytokeratin in three thyroid tissues which suggested that foetal microchimeric cells may differentiate in maternal host tissue (40). It has also been suggested, albeit not proven, that fetal stem cells may persist postpartum and have multilineage capacity. Therefore it is also plausible that foetal microchimeric cells may differentiate even into cells with endodermal lineage such as thyroid cells. In that sense it has been reported that foetal cells exist with leukocyte, or hepatocyte markers, in a variety of maternal tissue specimens (40). Immune cells are a significant proportion of these microchimeric cells and may arise directly from foetal or placental origin or may have been derived from pluripotent microchimeric stem cells. Differentiation of such foetal stem cells may be in response to pre-existing tissue injury such as in autoimmunity and infection (6). Furthermore, the microchimeric T cells are immunologically competent as shown by proliferation to maternal antigen (41). Therefore it has been hypothesised that such cells may modulate autoimmune diseases in the postpartum by playing a role in antigen

presentation and immunoregulation (8,16).

Autoimmune diseases and pregnancy

Most autoimmune diseases, and especially autoimmune thyroid diseases, are much more frequent in the middle-aged female population and are well known to be modulated by pregnancy (6,16,42). Autoimmune diseases also have an especially high prevalence in the postpartum period (43). Whereas pregnancy leads to an amelioration of autoimmunity, the postpartum period is associated with an exacerbation of autoimmune disease. For example, Graves' disease and postpartum thyroiditis can affect 8-10% of women and up to 40% of TPO-Ab positive women in the post partum (16). It has been presumed that the postpartum exacerbation of the autoimmune diseases is more likely due to loss of placental immune suppression rather than maternal exposure to persisting specific allogeneic foetal antigens but there is no evidence to dismiss a microchimeric cause (6). Up to 60% of reproductive Graves' patients reported the development of Graves' disease within one-year of delivery (44).

Microchimerism and pregnancy related abnormalities

Some pregnancy abnormalities such as pre-term labour, pre-eclampsia and aneuploidy have been related to increased numbers of circulating foetal cells in women (9). A significant association between foetal loss and microchimerism has also been observed (45). Polymorphic eruptions of pregnancy are pruritic, non-follicular erythematous papules in the skin where male foetal cells have been detected (31). In each of these situations there is a reported association but no mechanistic relationship has been described.

3.0. Autoimmune Disease and Microchimerism

Autoimmune disease and maternal microchimerism

Many scientists had linked cells transferred from foetus to mothers to the development of some autoimmune diseases, but the evidence linking microchimeric maternal cells to the development of these diseases is less clear. However new discoveries have shed light on the physiological activity of maternal microchimeric cells in offspring. Nelson et al have recently shown that cells which passed from mother to child during pregnancy can differentiate into pancreatic islet beta cells in type 1 diabetes patients (46). The authors speculated that these cells may play a role in aiding the production of insulin. They found that 51% of the blood samples from 94 patients with autoimmune type 1 diabetes had maternal microchimeric cells, as did 33% of 54 unaffected siblings and 17% of 24 healthy controls. The differences between groups were significant. The researchers also found maternal cells in pancreatic islet tissue of four male cadavers. They concluded that maternal cells help to regenerate damaged tissue. In this paper, in contrast with their previous opinion (25), Nelson et al. hypothesised that maternal microchimeric cells do not appear to trigger the autoimmune response that contributes to the development of type 1 diabetes. However this interesting finding, while confirming previous results, does not rule out other possible capabilities for maternal microchimeric cells. It seems, therefore, premature to conclude that microchimeric maternal cells were not causing an autoimmune response as well as facilitating repair of the pancreas tissue. Other authors have found maternal cells in inflammatory lesions of scleroderma patients which express similar antigens to those of the autologous cells in the same lesion, supporting the contribution of maternal microchimeric cells in the origin of autoimmune diseases in their children (9).

Autoimmune disease and foetal microchimerism

The presence of microchimeric male cells in blood samples of women with previous male pregnancies ranges from 25 to 31% (8). This frequency increases in those women who harbour any autoimmune disease, ranging from 45 to 60%. The existence of foetal microchimerism in relation to human autoimmune disease was first confirmed in systemic sclerosis patients (47). Studies demonstrated the presence of male DNA in skin lesions of women with systemic sclerosis (48). However this association it is not as clear in other autoimmune diseases with female preponderance such as primary biliary cirrhosis, Sjögren's syndrome or erythematosus systemic lupus (49). In addition some reports have shown that foetal cell microchimerism is a relatively common occurrence in women with both autoimmune and non-autoimmune diseases (2). Therefore, such data are contradictory in relation to the health consequences of persistent foetal cells in maternal tissues.

Autoimmune thyroid disease and microchimerism

The thyroid is the most common target for autoimmunity in humans. The high female preponderance and the high prevalence in women after childbearing suggest that pregnancy related factors have a strong influence on thyroid autoimmune disorders such as Graves' disease and Hashimoto's thyroiditis. Autoimmune thyroid disease may initiate or exacerbate in the postpartum period, whereas during pregnancy the activity of these disorders is reduced in relation to placental immune suppression.

The influence of pregnancy in autoimmune thyroid disease was investigated in our laboratory in a murine model of experimental autoimmune thyroiditis, showing that thyroglobulin

immunisation leads to foetal loss in specific allogeneic pregnancies (42). In later studies we focused on the investigation of the relationship between foetal microchimerism and autoimmune thyroiditis. The results demonstrated a significantly higher accumulation of foetal cells in the maternal thyroids from thyroglobulin immunised animals (46%) when compared with control non-immunised pregnant mice (20%) (15). The microchimeric cells included cells of foetal T cell and dendritic cell lineage. These results suggested that the inflamed thyroid gland was capable of accumulating foetal immune cells that may have a regulatory role on maternal autoimmune thyroiditis. We also found that the presence of foetal cells in the maternal thyroids decline rapidly after delivery, indicating the recovery of the maternal immune system in the postpartum period. However, in allogeneic pregnancies this was followed by an increase in the intrathyroidal infiltrate, as a consequence of the conclusion of the pregnancy related immune-privileged status (15). In contrast to the mouse model, many women show persistence of fetal cells for many years and this is reflected in the thyroid gland of those women with autoimmune thyroid disease. The reasons for the persistence of apparently foreign cells in the human thyroid are not completely understood. Currently the only known factor that determines the persistence of microchimeric cells in the host is the Human Leukocyte Antigen compatibility between mother and foetus (50). In addition the immunogenetic susceptibility markers, HLA DQA1*0501-DQB1*0201 and DQB1*0301, that are more frequent in patients with thyroid autoimmunity are also more common in patients of mother-child pairs with microchimerism (51).

In theory, the presence of immune foetal cells within the maternal thyroid gland may elicit a response once the immune-privileged pregnancy status is finished in the postpartum period (8). Therefore, it has been hypothesised that immune foetal cells interact with maternal immune cells during the postpartum to initiate or exaggerate autoimmune postpartum thyroid disease in a host versus graft reaction.

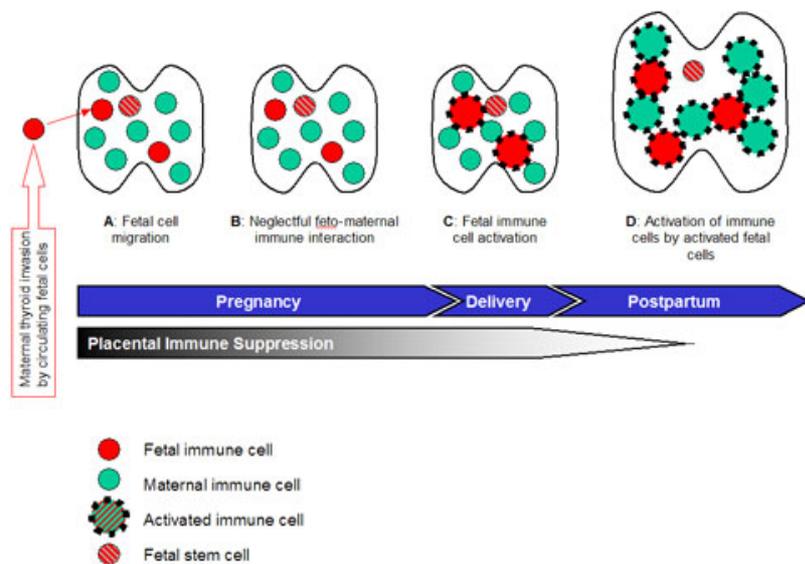


Figure 6

Hypothetical mechanism by which foetal cells may modulate autoimmune thyroid disease in the postpartum period.

- Foetal microchimerism is established during pregnancy by placental immune suppression. The mechanism that attracts foetal immune cells (or other cells as foetal stem cells) to migrate into the maternal thyroid gland has not been fully elucidated but is exaggerated by inflammation. Cytokines, chemokines, and adherent factors may be involved.
- Due to placental immune suppression, immunological interaction between maternal and foetal immune cells is minimal and/or neglectful during pregnancy.
- After delivery, partially sustained immunosuppressive effects facilitate the survival of foetal cells, but eventual loss of placental immune suppression results in activation intrathyroidal foetal immune cells.
- Activated foetal immune cells initiate a graft vs. host reaction against maternal

antigens by secreting immunomodulatory cytokines and/or expressing immunomodulatory molecules, which activates intrathyroidal maternal autoreactive T cells and eventually initiates and/or exacerbates postpartum autoimmune thyroid disease. Modified from Ando and Davies (8).

However, the presence of activated immune foetal cells in the maternal tissues may only trigger the development of autoimmune disorders in susceptible women (6). The mechanism that attracts foetal immune cells to migrate into the maternal thyroid gland has not been fully studied but it is clearly related to the local inflammatory response (15). Cytokines, chemokines, and adherent factors such as integrins may be involved (42). It is also plausible that a local intrathyroidal or distant insult may initiate immune responses and foetal cell accumulation (6). Clinical studies have shown that the accumulation of intrathyroidal foetal cells is common in autoimmune thyroid disease and that intra-thyroidal foetal cells are more abundant in thyroids that are affected by autoimmune diseases than in those that are not affected (6,52).

Table 3: Summary of studies of foetal microchimeric male cells in the thyroid gland

Disease	(n) Studied population	% Where male cells were found
Graves' disease	43	24 (56%)
Hashimoto's thyroiditis	80	39 (49%)
Thyroid adenoma	26	7 (27%)
Multinodular Goitre	56	7 (13%)
Control	39	4 (10%)

This table summarises the population of females with different thyroid diseases in whom the presence of microchimeric male cells has been studied. The information was collected and combined from five very different studies: Ando et al (12), Klintschar et al (13) Srivasta et al (14), Renne et al (52) and Klintschar et al (53). For the correct interpretation of this information it is important to consider that the design and techniques of these studies were diverse. Ando et al and Klintschar et al used a PCR approach while Srivasta et al and Renne et al used FISH technique. It, therefore, provides only a rough guide to the situation.

FISH: Fluorescence In-situ Hybridisation. PCR: Polymerase Cell Reaction.

The presence of male foetal cells has been identified in both Hashimoto's thyroiditis (13,14) and in Graves' disease (12). The specific frequency of foetal microchimeric cells in postpartum thyroiditis has never been clinically studied. Nonetheless, these results support the presence of active microchimeric cells within the thyroid and that microchimerism is associated with thyroid disease.

The presence of microchimeric cells in non-autoimmune thyroid disease varies extensively, albeit the criteria for choosing these samples varies between studies. Some authors have studied glands from necropsy specimens without known thyroid disease and found no microchimeric cells (14). Other investigations studied females with a history of a past male pregnancy and patients with past history of polycystic ovary syndrome who were never pregnant (12). Circulating male cells were detected by PCR in 28% of the subjects from the first group, whereas no male cells were found in the blood samples of the latter. Recently Klintschar et al (53) did not detect any male cells in 17 women with healthy thyroid glands from autopsies that were preserved in paraffin blocks. The interpretation of these results is complex since paraffin samples may underestimate the yield of the PCR (12). At the same time the problem of contamination is always present because pathologists usually do not clean paraffin baths after the preparation of the samples.

Hashimoto's thyroiditis

Four studies have found male cells in samples of Hashimoto's thyroiditis, assuming they were microchimeric cells related to a previous pregnancy (13,14,52,53). The presence of these cells in the studied females ranged between 38 to 83%. This discrepancy probably reflects the low number of samples examined, the differences in the study designs and the variation in the evaluation techniques (FISH or PCR). The studies also showed a wide discrepancy in the quantification of the presence of male cells per subject. The two laboratories which utilised FISH showed results that range from 0 to 35 male cells per slide. Analysis by quantitative real time PCR showed that thyroid of Hashimoto's thyroiditis patients contained male cells that ranged from 0 to 4,900 per 100,000 female cells. The median of male cells detected in those women with positive results was 97 male cells/100,000 female cells. The differences between the Hashimoto's thyroiditis patients and control groups in individual studies are constant. In the first study published by Klintschar et al (13), sequences were derived from the Y-chromosome in 8 out of 17 thyroids of women diagnosed with Hashimoto thyroiditis. In contrast these cells were found only in 1 of 25 nodular goitres that were used as a control group.

This highly significant difference between both groups, despite similar characteristics in age and number and gender of children, was strong evidence of an etiological role of

microchimerism in the pathogenesis of Hashimoto's disease (13). When compared both autoimmune entities (Graves' disease and Hashimoto's thyroiditis), the frequency of microchimerism was highest in patients with Hashimoto's thyroiditis (52). Similarly, when compared, microchimeric cells were found in more subjects with Hashimoto's thyroiditis (83%) than in any other thyroid diseases (14).

Graves' disease

The main study of the association of Graves' disease with microchimerism was performed in a sample of 27 glands of affected subjects by ELISA-PCR technique (12). Twenty thyroids of Graves' disease patients had been preserved in paraffin block while seven had been frozen. In the first group only 4 (20%) samples were positive for SRY gene, while 6 (86%) of the frozen samples showed the Y chromosome gene. In addition intrathyroidal microchimerism was more common and profound in samples from patients with autoimmune Graves' disease than in benign adenoma (12). Surprisingly the study also showed that many women with male cells in their thyroids declared no past history of male pregnancies. However this did not necessarily exclude the possibility of undetected first trimester pregnancies, because it has been demonstrated that foetal microchimerism can be established in the first month of pregnancy (12,27). Some authors have argued that as they only detected male cells in mothers with at least one son, pregnancy appeared to be (in absence of transplants or transfusions) a condition sine qua non for microchimerism (53). However we believe that the sources of natural microchimerism are much more complex and involve other factors such as cell trafficking from maternal origin or intercourse. At the same time, the techniques for detecting microchimeric cells are limited, and the majority of the studies have looked only for male cells. This procedure is unable to detect microchimeric cells from daughters, which presumably are the source of similar amounts of microchimeric cells as sons.

Microchimerism and non-autoimmune diseases

It is also conceivable that microchimerism contributes to non autoimmune thyroid disease (52). The presence of microchimeric cells in nodular disease might result from anatomical changes or associated with local expression of growth factors re-lated to goitrous growth and repair processes (14). The relationship between foetal cell microchimerism and non-autoimmune diseases, including infectious disease and cancer, has led to speculation that foetal cells may provide a rejuvenating source of progenitor cells. These cells may potentially participate in maternal tissue repair processes. It has been hypothesised that microchimeric cells have the ability to migrate from the circulation, home to diverse tissues, and differentiate or fuse with host cells (2). In fact male cells of presumably foetal origin with follicular morphology have been observed in a thyroid of a woman affected with a multinodular goitre (14). Foetal cells have also been identified in thyroid adenomas and adenomatous goitres although a local inflammatory infiltrate may have been responsible. The presence of microchimeric cells ranges from 45 to 57% of cases of nodular thyroid disease although it is unclear if this was related to an associated perinodular thyroiditis (13,14,52,53). The highest presence was detected in a FISH study where up to 156 male cells were found in a sample from a multinodular goitre. This was fourfold higher than that found in a Hashimoto's sample. Klintschar et al (13) found that only one of the 25 (4%) thyroids of women with multinodular goitre was positive for male gene PCR. Ando et al (12) did not show SRY gene in the adenomas which were preserved in paraffin but their SRY PCR came out with positive results in 25% of the frozen adenoma samples. Renne et al (52) showed similar results (22%) for microchimeric cells in nodular thyroid disease. The role of these cells in adenoma formation is unknown.

Points of controversy

Although some data conflict with the hypothesis of a direct relationship between microchimerism and autoimmune disease it remains a consistent observation with many examples. The absence of foetal cell microchimerism in some female preponderant autoimmune disorders such as primary biliary cirrhosis or Sjögren's syndrome may simply imply a different immunopathologic mechanism at work in these disorders. Furthermore, the presence of microchimerism in non-autoimmune diseases including infectious hepatitis and even thyroid adenomas may simply be a reflection of an inflammatory response in susceptible individuals attracting foetal cell accumulation (2,14). An Australian study of more than one thousand women of varying parity concluded that pregnancy was not a risk factor for autoimmune disease (54). But that does not exclude the fact that that pregnancy modulates autoimmune disease when it exists. Nevertheless, it remains to be shown that the presence of foetal cells directly influences the natural history of autoimmune disease.

4.0. Conclusions

There is a large body of evidence that indicates the thyroid gland as an important organ where foetal microchimeric cells settle and persist and this is more common in autoimmune than in non-autoimmune thyroid diseases. The biological consequences of foetal immune cells within the maternal thyroid gland, which may become activated in the postpartum period, as maternal immune suppression is lost, remains an attractive explanation for the postpartum exacerbation of the autoimmune thyroid diseases. Understanding the types of foetal cells transferred to mothers, the location of these cells within diverse maternal organs and their function may provide incite into their role in immunopathology and tissue repair and allow us to take advantage of new therapeutic procedures.

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EMERGING NEW FEATURES OF PATIENTS WITH THYROGLOBULIN MUTATIONS, INCLUDING INCREASED INCIDENCE OF THYROID CANCER

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Abstract

Until recently, mutations of the thyroglobulin (Tg) gene were believed rare. However, an increasing number of Tg mutations have been identified, as well as many new features of patients carrying such mutations elucidated. Notably, in adult patients who were born before initiation of neonatal screening, the presence of an enlarged thyroid gland partially compensated impaired thyroid hormone synthesis. Consequently, their thyroid functions were normal or subclinically hypothyroid. In addition, many patients developed cancer in the enlarging thyroid gland. In this article, we summarize recent advances in the clinical and pathogenic features of patients with Tg mutations.

Introduction

Congenital hypothyroidism, which affects 1 in 3,000-4,000 live births, is divided into nongoitrous and goitrous forms. The nongoitrous form (85% of congenital hypothyroidism) is caused by defective thyroid gland development and the goitrous form (the remaining 15%) results from abnormal hormone synthesis due to mutations in genes encoding the sodium iodide symporter, thyroglobulin (Tg), thyroperoxidase, thyroid oxidase 2, and pendrin (1).

Tg, the most abundant protein in the thyroid, functions as a matrix for thyroid hormone synthesis. Immediately after translation, Tg polypeptide in the endoplasmic reticulum (ER) forms high molecular weight aggregates which are mediated by nearly 60 interchain disulfide bonds as well as molecular chaperones, such as GRP78 (BiP) and GRP94 (2,3). During transport through the Golgi apparatus, Tg polypeptides mature through monomeric form to dimers with a molecular weight of 660kDa, which are ultimately secreted into the follicular lumen. Specific tyrosyl residues are iodinated and coupled to form thyroxine (T4) and triiodothyronine (T3) by thyroperoxidase.

Clinical Characteristics of Tg Mutations

Clinical characteristics of patients with Tg mutations are diverse, ranging from congenitally hypothyroid patients detected in fetal life to adult goitrous cases with normal thyroid functions. Since a recent review of Tg mutations (4) which described 35 different inactivating mutations (20 missense mutations, 8 splice site mutations, and 2 single nucleotide deletions), two new missense mutations G59S and S2113L have been reported (5), which together comprise a total of 37 different mutations. A typical case is a 33 year old female who was first identified as having a Tg mutation by genetic analysis (6). She was born to parents of first cousins and two of her five siblings had a voluminous goiter. A splice site mutation IVS3-3C>G caused short stature (132cm in height) and marginal mental retardation (IQ 86). Her serum T4 level was remarkably reduced, whereas the level of T3 was in the upper normal or slightly higher than normal range. Her serum TSH was increased (57 m U/ml). Her goiter was huge and increased in size gradually, necessitating multiple operations. The level of serum Tg was low compared to the enlarged thyroid gland and radioactive iodine uptake was increased. Tg mutations in patients with congenital goiter and hypothyroidism with similar characteristics were found in a Brazilian kindred with three different genotypes R1511X/R1511X, R277X/IVS34-1G>C, and R277X/R1511X (7,8), in Brazilian and Argentinean families with R277X/R277X (9,10), and in two siblings with IVS30+1G>T (11) and IVS5+1G>A (12). Goiter was detected even in fetal life with a 22 year old mother in successive pregnancies with the mutation G362fsX382/R2223H in infants (13) and in a dichorionic twin pregnancy with mutations R1511X/G59S and R1511X/S2113L in each infant (5). Fetal hypothyroidism was confirmed by low T4 and high TSH concentrations in umbilical veins. In the former cases, an intraamniotic injection of levothyroxine was performed with successful reduction of goiter size, whereas without intraamniotic injection of levothyroxine in the latter cases respiratory obstruction required tracheal intubation.

On the other hand, mild adult cases, characterized by enlarging goiter with almost normal thyroid function, were reported in patients with congenital euthyroid goiter and the variant type of adenomatous goiter due to two missense mutations C1245R and C1977S (14,15). In a large cohort of 52 patients from 41 families carrying 26 different mutations (18 missense

mutations, 4 splice site mutations, 3 nonsense mutations, and 1 single nucleotide deletion), 36 patients were born before 1979 when mass screening of congenital goiter was initiated in Japan (16). Their thyroid functions were deemed normal or subclinical hypothyroid, with TSH levels being less than 10 mU/ml in all of the patients, except for one patient with the mutation C1245R/Q2638X. Among 13 patients who were born after 1979, 12 patients were positive at mass screening when 3 patients without medical records were excluded. Only 7 out of these 12 patients were maintained on levothyroxine therapy, with the other 5 patients being either transiently treated or not treated at all. Their thyroid function tests showed subclinical hypothyroidism at the age of five years. TSH suppression therapy was usually not effective in reducing goiter size in adult patients, whereas early treatment during the neonatal period seems to work with respect of prevention of goiter enlargement (17).

The distinction between severe and mild forms of Tg mutations seems dependent on dietary iodide supply or the type of mutation. Since a sufficient amount of iodide is contained in Japanese foods, many patients in Japan with Tg mutations present normally or with subclinical hypothyroid. The severe cases usually involve nonsense, splice site, or frame shift mutations, whereas most of the mild cases are associated with missense mutations, the latter presumably causing smaller structural changes leading to preserved homogeneity (14).

Prevalence of Tg mutations

Analysis of the Kumamoto Prefecture in Japan showed that the incidence of Tg mutations is one in 67,000 individuals (16), which gives the estimate that occurrence of heterozygous carriers is one in 130. This figure is compatible with one in 66,000 occurrences of total iodide organification defects, the majority of which are caused by mutations in the thyroperoxidase gene (18).

Founder effects

Some of the mutations are clustered in particular areas. For example, the mutation R277X was reported only in Brazil and Argentina, while the mutations C1058R, C1245R, and C1977S were found to occur in Japan. Single nucleotide polymorphism (SNP) and microsatellite analysis in patients with the mutation R277X showed that two affected individuals do not share a common Tg allele, suggesting that the mutation R277X is a mutational hot spot, not inherited from a common ancestor (10). However, the haplotypes of eight patients with the mutation C1058R and seven patients with the mutation C1977S were identical (16). When calculated from allelic frequencies of the SNPs in normal controls, the occurrence of the mutations C1058R and C1977S in the Japanese population would be one in 810 million and one in 37 billion, respectively, confirming that the homozygous occurrence of the mutations C1058R and C1977S were the result of a founder effect. The SNP analysis of 12 patients with the mutation C1245R suggested that C1245R is an independently recurrent mutation, or, taking into account that nine patients share a common allele, an alternative explanation is that C1245R is an old mutation with new SNPs being subsequently created.

Defective intracellular transport of Tg

Abnormal three-dimensional structure due to Tg mutations results in defective intracellular transport of Tg. The microscopic observation of tissues with Tg mutations was scant colloid in the follicular lumen, which was dilated to various degrees. Immunohistochemistry revealed that Tg immunoreactivity is in the follicular cells but not in the follicular lumen.

Electronmicroscopy showed that numerous dilated ER occupied the cytoplasm of the follicular cells, suggesting that Tg is retained in the ER, thereby contributing to endoplasmic reticulum storage disease (ERSD). In fact, the overall tissue content of Tg was remarkably reduced. Abnormal Tg formed disulfide-linked high molecular weight aggregates, which are only transiently formed immediately after translation in physiological conditions. Carbohydrate analysis by endoglycosidase H treatment showed that the carbohydrate residues on the mutant Tg proteins are of a simple ER-type, contrary to the complex Golgi-type found in the case of normal Tg. This defective intracellular transport of Tg was confirmed in cultured cells expressing Tg with mutations, C1245R, C1977S (14, 15), and G2356R (20). The ERSD seems a universal mechanism for Tg mutations in humans, as well as in *cog/cog* mice (21) and *rdw* rats (22, 23), both of which are caused by the mutations L2263P and G2300R, respectively.

The retention of abnormal Tg in the ER also induces upregulation of molecular chaperones, such as calreticulin, GRP78 (BiP), GRP94, PDI, ERp72, ERp29 (19, 24, 25), which either assist the proper folding of proteins or escort proteins through intracellular compartments to their final destinations. The mechanism which results from excessive accumulation of proteins is called the unfolded protein response (UPR), which consists of two pathways, namely transcriptional induction of genes that enhance the ER-folding capacity (molecular chaperones) and general translational attenuation aimed at reduction of the protein load on the ER (26). The transcriptional pathway is activated by ATF6, a basic leucine zipper transcription factor. The activation of ATF6 was observed in the human tissues with the mutations C1245R and C1977S, as well as in *rdw* rats (25).

Increased activation of T4 to T3 by D2

Normal to high serum T3 concentrations with disproportionately low serum T4 levels were frequently found in patients with Tg mutations (6, 17, 20, 27). This tends to be a universal mechanism to avoid tissue hypothyroidism in the context of limited supply of T4. Type 2 iodothyronine deiodinase (D2) activates conversion from T4 to T3 and is postulated to play an

important role in providing serum T3. Thyroidal D2 activities in patients with the mutations C1245R/G3456R, C1245R/C1245R, and C1977S/C1977S were higher than wild-type thyroid gland and positively correlated with FT3/FT4 ratios (20).

Thyroid cancer development in long-standing goiter with Tg mutations (Figure 1).

Whether hyperplasia of the thyroid in patients with dysmorphonogenesis may proceed to malignant change has long been discussed (28, 29). Morphological findings of dysmorphonogenetic goiter are diverse, ranging from nodular hyperplasia to cystic degeneration, focal fibrosis, and calcification. Because of atypical cellular findings often found in hyperplastic nodular regions, it had been sometimes difficult to differentiate malignant transformation from benign hyperplasia. By applying strict criteria for malignancy with definite vascular invasion, local aggressive growth, and metastasis, six cases were confirmed as thyroid cancer associated with dysmorphonogenetic goiter (30). Subsequently, in a meta-analysis of 109 patients with dysmorphonogenesis undergoing surgery, 19 patients (17%) had thyroid follicular cancer (31). After genetic analysis was instituted, sporadic cases of thyroid cancers were reported in patients with mutations in thyroperoxidase (32) and pendrin (33). In terms of Tg mutations in a cohort of 11 patients who underwent surgery, seven developed thyroid cancers consisting of six patients with papillary cancer and one patient with follicular cancer (17). Two patients showed a single locus of cancer and the other five had multifocal thyroid cancers. The incidence of 63.6% (7/11) is higher than the 27.4% incidence of microcarcinoma detected in thyroid benign diseases (34) or 28% incidence of histopathologically confirmed thyroid cancer in thyroid nodular lesions (35). There was another case report of metastatic follicular thyroid cancer arising in a patient with the Tg mutation IVS5+1G>A (12).

Somatic activating mutations of the BRAF gene are responsible for some of the thyroid cancers developed in long-standing goiters with Tg mutations (17). Of the five patients, two heterozygous mutations V600E and K601E were found in cancerous, but not neighboring non-cancerous tissue from two patients. One of the mutations V600E is the most common type of mutation in papillary cancers (36). The other mutation K601E which was detected in follicular thyroid cancer is a rare mutation.

The factors which play a role in the development of goiter leading to malignant transformation would be multiple. Obviously, prolonged stimulation of TSH is generally accepted in the development of goiter. However, in some cases of Tg mutations, other unknown mechanisms should be postulated since TSH suppression therapy is not usually effective in adult cases whose serum TSH were not elevated (17).

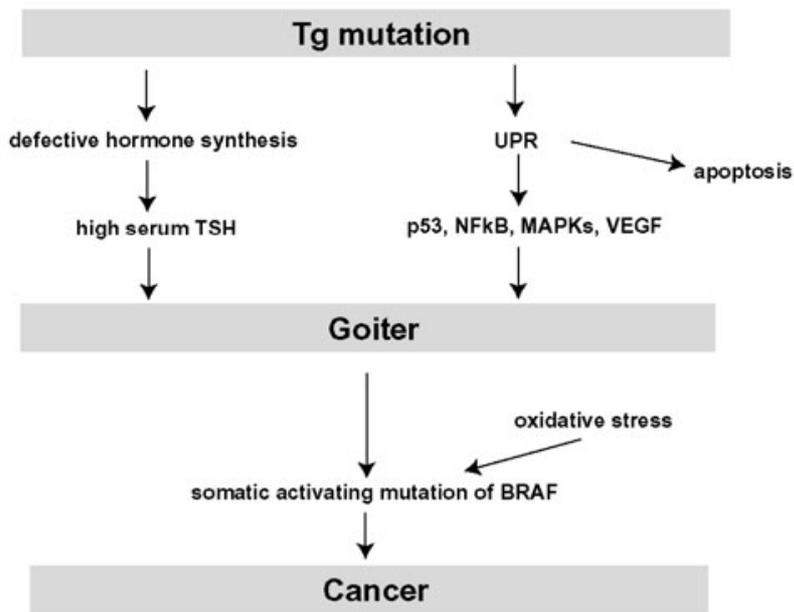
Intrinsic to UPR, many mechanisms have been reported to stimulate cellular proliferation. Overload of misfolded proteins in the ER induces adaptation responses consisting of upregulation of protein folding capacities by molecular chaperones (37) and repression of global protein synthesis by the eIF2 α (eukaryotic initiation factor-2 α) kinase PERK as a means to decrease the protein overload in the ER (38). If these adaptive mechanisms are not sufficient to alleviate ER stress, an apoptotic program is activated. However, these defensive mechanisms also activate cell survival and may even paradoxically stimulate cell proliferation to reduce accumulation of misfolded proteins in the ER. In fact, thyroid tissues from patients with Tg mutations display heterogeneous appearance, hypercellularity with numerous small follicles in some areas and cystic degeneration in others. The tumor suppressor protein p53 is a transcription factor which regulates the expression of many genes, whose functions are related to cell cycle arrest, DNA repair, or apoptosis (39). ER stress induced p53 cytoplasmic localization and accelerated degradation, leading to prevention of p53-dependent apoptosis (40, 41). During impaired protein folding assembly in the ER, phosphorylation of eIF2 α by PERK activated NF- κ B (42), which has a crucial role in cancer development and progression (43). Upon disruption of calcium homeostasis in the ER, PERK is required to activate p38 MAPK and induced the expression of immediate-early genes, including c-myc, c-jun, egr-1, and fra-1 (44). Under hypoxic stress when PERK is activated, formation of functional microvessels was promoted by translation of many angiogenic genes, including VEGF, in PERK-dependent manner (45).

In the thyroid, a strikingly high mutation rate was shown most likely to be due to oxidative DNA modifications (46). Oxidative stress by free radicals and reactive oxygen species generated in the course of thyroid hormone synthesis might cause DNA damage and somatic mutations. Enhanced multiplication of thyroid epithelial cells in patients with Tg mutations, in conjunction with high mutagenesis rates via oxidative stress, would lead to somatic mutations in certain oncogenes.

Conclusion

We described diverse clinical manifestations of Tg mutations, some of which involve enlarging goiter in iodide sufficient areas. The pathogenesis of Tg mutations is a defective intracellular transport of Tg stimulating an unfolded protein response. Thyroid cancers develop frequently in enlarging goiter. Multifocal occurrence of cancer warrants total thyroidectomy to avoid future recurrence of cancer in the remaining tissue. Elevated TSH or the pathways through unfolded protein response (UPR) would lead to thyrocyte proliferation and, partly in conjunction with oxidative stress present in thyrocytes, ultimately create activating somatic mutations of BRAF.

Figure 1. Postulated mechanisms of development of cancer through goiter in patients with Tg mutations.



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CONTRASTING ROLES FOR THYROID HORMONE IN THE DEVELOPING AND ADULT SKELETON

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INTRODUCTION

The role of the hypothalamic-pituitary-thyroid axis in the regulation of skeletal development and adult bone mass is a rapidly emerging and controversial field. Recent studies have greatly advanced our understanding and clearly demonstrated the importance of the field to osteoporosis, a major healthcare priority that affects 50% of women and 20% of men over 50 and costs €31 billion per annum (1). Skeletal development and adult bone turnover are regulated by numerous systemic and paracrine factors that act within the complex multi-cellular bone microenvironment (2-4). Disruption of these signalling pathways affects growth, acquisition of peak bone mass and skeletal remodelling and may lead to osteoporosis and fragility fracture.

During skeletal development long bones are formed by endochondral ossification, whereas the craniofacial skeleton is formed by intramembranous ossification. In endochondral ossification mesenchyme-derived chondrocytes form a cartilage model and undergo hypertrophic differentiation followed by apoptosis with subsequent calcification of the surrounding collagen X-rich matrix scaffold. Accompanying vascular invasion mediates the entry of osteoblasts and initiation of bone formation. An organised programme of chondrocyte maturation, proliferation and differentiation continues within the growth plate until adolescence, resulting in linear growth and the acquisition of normal peak bone mass. In intramembranous ossification mesenchymal cells differentiate directly into osteoblasts and bone formation subsequently follows. By contrast, the mechanical strength and three-dimensional architecture of adult bone is maintained by skeletal remodelling, a cyclical process that requires precise coupling of osteoclastic bone resorption followed by osteoblastic bone formation (5).

Thyroid hormone is an essential regulator of both skeletal development and adult bone turnover. The actions of thyroid hormone (T3) are mediated by the thyroid hormone nuclear receptors (TRs). TR α 1, α 2 and β 1 are expressed in chondrocytes and bone forming osteoblasts (6), but it remains unclear whether T3 also acts directly in bone resorbing osteoclasts or whether it regulates osteoclastogenesis indirectly via actions in osteoblasts (7,8).

THYROID HORMONE ACTION IS ANABOLIC IN THE DEVELOPING SKELETON

Thyroid hormone deficiency during development

The developing skeleton is exquisitely sensitive to the actions of T3 (6). Childhood hypothyroidism results in growth arrest, epiphyseal dysgenesis and delayed bone age. Thyroid hormone replacement leads to a period of rapid "catch up" growth but a deficit in final height that is proportional to the duration and severity of the preceding hypothyroidism (9,10).

The skeletal phenotype of developmental hypothyroidism has been studied in detail in thyroid hormone manipulated rats (11), congenitally hypothyroid mice (Pax8^{-/-}) (12), mice lacking all TR α isoforms (TR α ^{0/0}) (13) and mice harbouring 2 different dominant negative mutations of TR α 1, (TR α 1^{+R384C}) (14) and (TR α 1^{+PV}) (15) (Table 1). Although TR α ^{0/0}, TR α 1^{+/}

R384C and TR α 1^{+PV} mice are systemically euthyroid, T3 target gene analysis in chondrocytes and osteoblasts has demonstrated that the skeleton is hypothyroid (16-19). These animal models consistently demonstrate growth retardation, delayed endochondral ossification, reduced cortical bone thickness and reduced bone mineral deposition (17-19). Growth retardation results from impaired recruitment of chondrocyte progenitor cells and delayed hypertrophic differentiation of proliferating chondrocytes. TR α 1^{+R384C} and TR α 1^{+PV} mice with dominant negative mutations of TR α have a more severe phenotype than TR α ^{0/0} mice and also exhibit delayed intramembranous ossification (18,19), suggesting a deleterious effect of either the mutant TR α 1 or unliganded apoTR α 1 in the developing skeleton.

Thyroid hormone excess during development

Childhood thyrotoxicosis results in accelerated growth, advanced bone age and paradoxical short stature due to premature fusion of the epiphyseal growth plates. Moreover, severe thyrotoxicosis in early childhood may also lead to premature closure of the skull sutures and craniosynostosis (20).

The skeletal phenotype of thyrotoxicosis during growth has also been characterised in thyroid hormone manipulated rats (11), mice from two different genetic backgrounds that lack all TR β isoforms (TR β ^{-/-}) (21,22) and mice harbouring a dominant negative mutation of TR β (TR β ^{PV/PV}) (23) (Table 1). Both the TR β ^{-/-} strains and TR β ^{PV/PV} mice have central resistance to thyroid hormone (RTH) with elevated circulating thyroid hormone levels due to disruption of the hypothalamic-pituitary feedback loop, but T3 target gene studies revealed thyrotoxicosis in bone (17-19,24). Analysis of the skeletal phenotypes of these mice demonstrated accelerated early growth with advanced endochondral and intramembranous ossification accompanied by increased cortical bone thickness and increased bone mineral deposition but subsequent persisting short stature (16-19,24). The short stature results from early growth plate quiescence due to accelerated endochondral ossification associated with increased FGFR3 activation and premature hypertrophic chondrocyte differentiation.

Thus, demonstration of delayed ossification in euthyroid TR α mutant mice, together with the finding of advanced ossification in systemically hyperthyroid TR β mutants, indicates that actions of T3 are anabolic during skeletal development and mediated by TR α 1. Euthyroidism is essential for normal skeletal development, linear growth and the attainment of peak bone mass.

THYROID HORMONE ACTION IS CATABOLIC IN THE ADULT SKELETON

Adult thyroid hormone deficiency

Adult hypothyroidism results in reduced bone turnover due to reductions in both osteoclastic bone resorption and osteoblastic bone formation (25,26). Remarkably, adult TR α ^{0/0} and TR α 1^{+R384C} mice have an osteosclerotic phenotype characterised by markedly increased bone mass with robust and plate-like trabeculae. This phenotype occurs even though there is delayed ossification during growth and results from a remodelling defect accompanied by reduced osteoclast numbers and bone resorption surfaces. Furthermore, although skeletal mineralisation is normal in TR α ^{0/0} mice it is increased in the more severely affected TR α 1^{+R384C} mice that harbour a dominant negative TR α 1 mutant.

Adult thyrotoxicosis

In adults, thyrotoxicosis is an important cause of secondary osteoporosis. Uncontrolled hyperthyroidism increases bone turnover and accelerates bone loss by uncoupling bone resorption and bone formation (25). The duration of the bone remodelling cycle is reduced by 50% as the bone resorption and formation phases each occur more rapidly. Nevertheless, uncoupling of osteoclast and osteoblast activities is not balanced and favours a net increase in resorption that results in a 10% net loss of bone per remodelling cycle in thyrotoxicosis (25). Furthermore, in large population studies a prior history of thyroid hormone excess is associated with a life-long increased risk of fracture (27-29), whilst even a suppressed or low TSH from any cause is associated with a 4-fold increase in fracture risk in postmenopausal women (27-31).

Even though advanced ossification and increased mineral deposition occur in juvenile TR β ^{-/-} mice, adults display a phenotype of severe osteoporosis with markedly reduced numbers of gracile trabeculae, reduced cortical bone thickness and reduced bone mineralisation. This phenotype results from accelerated bone remodelling accompanied by increased osteoclast numbers and resorption surfaces (17,18).

Thus, in the adult skeleton, deletion or mutation of TR α results in reduced osteoclast numbers, impaired bone remodelling and osteosclerosis. By contrast, deletion or mutation of TR β causes osteoporosis, indicating that actions of T3 are catabolic in the adult skeleton.

THE CENTRAL AND PERIPHERAL ACTIONS OF THYROID HORMONE

Systemic thyroid status is determined by the hypothalamic-pituitary-thyroid-axis (HPT axis) (Figure 1). Thyroid hormones exert negative feedback on TRH and TSH resulting in the physiological reciprocal relationship between TSH and T4 and T3. The complex, opposing skeletal phenotypes observed in TR mutant mice (Table 1) arise because the hypothalamus and pituitary are TR β target tissues whereas TR α is markedly predominant in bone (24,32). Thus, disrupted TR α signalling in TR $\alpha^{0/0}$, TR $\alpha^{+/R384C}$ and TR $\alpha^{+/PV}$ mice does not influence regulation of the HPT axis, but causes hypothyroidism in bone even though circulating T3 and T4 levels are normal. By contrast, lack of TR β results in elevated T3 and T4 concentrations due to disrupted negative feedback regulation of TSH. These increased thyroid hormones excessively stimulate the wild-type TR α in bone resulting in skeletal thyrotoxicosis. Thus, the skeletal consequences of disruption of TR β are due to effects on the HPT-axis whereas disruption of TR α directly impairs TR α signalling in bone (33).

The Hypothalamic-Pituitary-Thyroid-Bone Axis

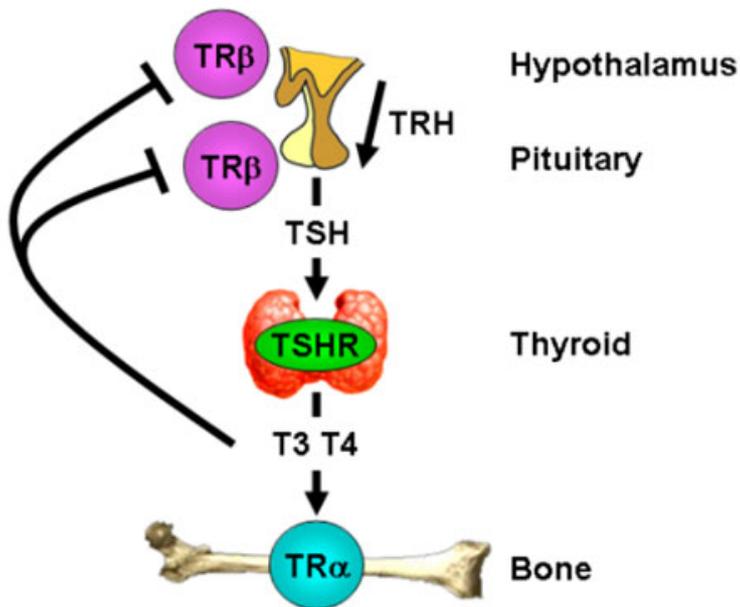


Figure 1

Schematic representation of the hypothalamic-pituitary-thyroid-bone axis. Thyroid releasing hormone (TRH) synthesised in the para-ventricular nucleus of the hypothalamus regulates thyroid stimulating hormone (TSH) synthesis and release from the anterior pituitary. Subsequently TSH, acting via the TSH receptor (TSHR) in thyroid follicular cells, stimulates thyroid hormone synthesis and secretion. In a classical negative feedback loop thyroid hormones, acting via TR β , inhibit TRH and TSH. T3 acts via TR α to regulate skeletal development and linear growth in juveniles and bone turnover and mineralisation in adults, thus dissociating the actions of TR α and TR β .

THYROID STATUS DURING DEVELOPMENT AND ADULT BONE STRUCTURE

The TR α^{R384C} mutant receptor has a 10-fold reduced affinity for T3 and acts as a dominant-negative inhibitor of wild type TR α 1 and TR β . Moreover, the resultant skeletal hypothyroidism in TR $\alpha^{+/R384C}$ mice is further exacerbated by a transient period of mild hypothyroidism during development between postnatal days 10 and 35 (Table 1). T3 treatment during this time period increases hormone levels sufficiently to allow the mutant TR α^{R384C} receptor to bind ligand and regulate target gene transcription normally, whereas the same T3 treatment results in a period of thyrotoxicosis in littermate controls (14,34). Remarkably, this transient T3 treatment of TR $\alpha^{+/R384C}$ mice during early post-natal skeletal development substantially ameliorates the subsequent adult phenotype by markedly reducing trabecular bone volume and mineralisation and increasing osteoclastic bone resorption. By contrast, transient thyrotoxicosis in control animals only results in a subsequent reduction of bone mineralisation in adults (17). The exaggerated response observed in TR $\alpha^{+/R384C}$ mice is similar to the period of accelerated catch-up growth seen in hypothyroid children following thyroid hormone replacement. These observations suggest that transient hypothyroidism or

hyperthyroidism during growth result in persistent skeletal abnormalities in adults and demonstrate that euthyroid status during development is essential for normal acquisition of peak bone mass and the establishment of normal adult skeletal structure and mineralisation.

DIRECT SKELETAL ACTIONS OF THYROID STIMULATING HORMONE

Despite these findings, a novel role for TSH as a direct negative regulator of skeletal remodeling has also been proposed (35) (Figure 2). In these studies TSH receptor (TSHR) expression was identified in osteoblasts and osteoclasts, increased bone turnover was observed in juvenile TSHR-null mice, and TSH inhibited osteoclastogenesis and osteoblast differentiation in vitro. In a recent study in ovariectomised aged rats, systemically administered TSH prevented bone loss and restored bone mass by inhibition of bone resorption and stimulation of bone formation (36). These intriguing findings raise the possibility that TSH deficiency may also contribute to the bone loss resulting from thyroid hormone excess in thyrotoxicosis. However, the patho-physiological importance of this possibility is not clear because the presence of TSHR stimulating antibodies in Graves' disease do not protect patients from bone loss, and osteoporosis occurs in the presence of markedly elevated levels of TSH in mice with mutation or deletion of TR β (17,18).

Actions of T3 and TSH in the skeleton

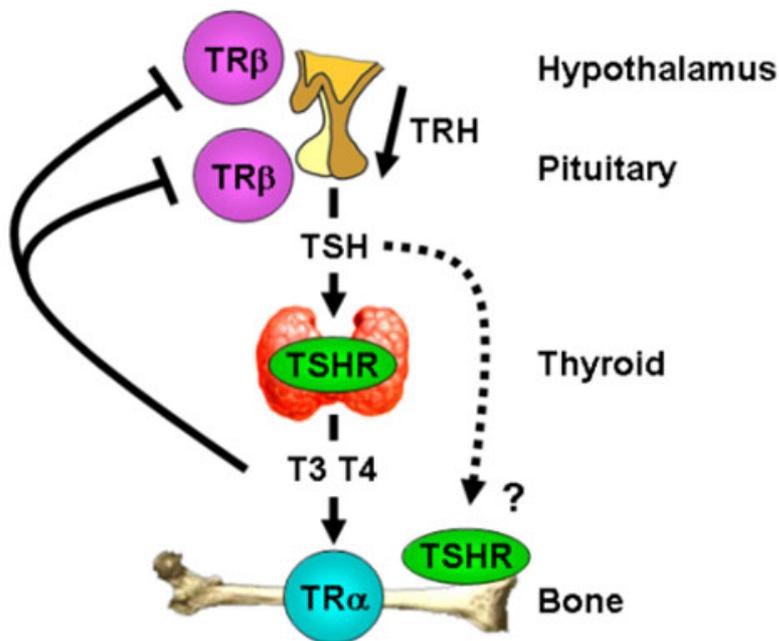


Figure 2

Normal T3/TR α 1 signaling is essential for bone development and maintenance of the adult skeleton. Recent studies have suggested that TSH may also be a direct negative regulator of bone remodelling acting via the TSHR expressed in osteoclasts and osteoblasts (35).

CURRENT CONTROVERSIES

The skeletal analyses of congenitally hypothyroid, TR knock-out and TR knock-in mice have resulted in important conceptual advances in the field and have identified a clear role for thyroid hormone and TR α 1 in developing and adult bone. At this stage, however, it is not clear to what extent the skeletal effects of thyroid hormones result from central, systemic and local actions of T3. Resolution of this important question will require cell-specific disruption of T3 action in bone using cre-lox gene targeting approaches. Furthermore, the relative contributions of thyroid hormones and TSH to skeletal development and adult bone maintenance will need to be addressed by genetic and pharmacological dissociation of their normal reciprocal relationship. A detailed understanding of these issues will provide a basis for the development of novel strategies for the prevention and treatment of osteoporosis.

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Abnormalities in Thyroid Function Parameters and Subclinical Thyroid Disease in the Elderly: A Brief Review

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Abnormal thyroid function tests consistent with either subclinical hypothyroidism or subclinical hyperthyroidism are not uncommon in the elderly. The question remains: do these changes represent authentic subclinical disease or are they simply a reflection of physiologic changes associated with aging? Interpretation of thyroid function in the elderly is further confounded by alterations secondary to chronic illnesses that may manifest as the nonthyroidal illness syndrome and by medication-induced changes in thyroid function parameters.

There are, however, predictable changes of thyroid function with aging¹. T4 secretion by the thyroid decreases with age, but since T4 degradation is also decreased with aging, the net result is an unchanged serum free T4. Both total and free T3 undergo age-related reductions likely due to decreased peripheral conversion of T4 to T3. It is hypothesized that the activity of 5'-monodeiodinase decreases with consequences of decreased outer ring deiodination of T4 similar to that seen in the nonthyroidal illness syndrome: (1) reduced T4 clearance, (2) reduced conversion of T4 to T3, and (3) increased T4 substrate available for conversion of T4 to rT3 and hence increased rT3 (Table 1).

Alterations in TSH associated with aging are complex. It is believed that increased TSH levels observed in the elderly are primarily identified in elderly women with antithyroid antibodies or in those with primary hypothyroidism, which is known to increase in prevalence with age². However, when those subjects with subclinical hypothyroidism are excluded, TSH levels are low or towards the lower end of the normal reference range reflecting the age-related decrease in TSH secretion by the pituitary. This may in part be due to reduced hypothalamic TRH secretion. However, pituitary thyrotrophs may also develop an increased sensitivity to thyroid-hormone induced negative feedback such that there is less TSH released for a given level of thyroid hormone sensed in the circulation or at the pituitary level. In addition to decreased TSH release, there are alterations in the circadian rhythm of TSH secretion with a 1-1.5 hour earlier shift in diurnal variation compared with young, healthy controls, and a blunted nocturnal peak in TSH in the elderly³.

Subclinical hyperthyroidism

Subclinical hyperthyroidism is defined as a low level of TSH in the setting of free T4 and T3 levels within the normal reference range. NHANES III found the prevalence of subclinical hyperthyroidism with a TSH < 0.1 mIU/L in the U.S. adult population to be 0.7% and to increase in those aged 80 or older⁴. The most common etiology of subclinical hyperthyroidism is multinodular goiter followed by Graves' disease and iatrogenic causes, namely thyroid hormone suppressive therapy (usually unintentional), in older individuals⁵. The natural history of subclinical hyperthyroidism in those aged 60 and older is the progression to overt hyperthyroidism in approximately 1 to 2% per year^{6,7}. Exposure to excess iodine (e.g., radiocontrast agent) may precipitate more overt thyroid abnormalities in individuals with subclinical thyroid disease.

Subclinical hyperthyroidism predominately exerts physiological effects on the bone and cardiovascular system⁸. Effects on the bone may consist of decreased bone density and increased bone turnover markers. In one study, there was no statistically significant difference in fracture rate between women over age 65 with normal TSH (0.9%) and those with TSH < 0.05 mIU/L (2.5%)⁹. However, more recently, a threefold increased risk for hip fracture and fourfold increased risk for vertebral fracture were reported in women 65 years or older with TSH < 0.1 mIU/L compared to normal TSH levels 0.5-5.5 mIU/L¹⁰. Cardiovascular effects include increased heart rate, increased cardiac contractility, increased left ventricular mass, delayed diastolic relaxation, and increased risk of atrial fibrillation¹¹. One prospective study of individuals 60 years or older found a threefold risk of the development of atrial fibrillation over 10 years in those with a low TSH (< 0.1 mIU/L) compared to those with a normal TSH¹², and another study found a greater than fivefold increased risk of atrial fibrillation, which was determined to be no different from that of overt hyperthyroidism¹³. However, in the recent prospective cohort study of individuals 65 years or older conducted by Cappola, et al, although there was an increased risk of atrial fibrillation associated with subclinical hyperthyroidism, there was no increased risk of stroke, coronary artery disease, cardiovascular death, or all-cause mortality¹⁴.

Recent guidelines following a consensus conference on subclinical thyroid disease¹⁵ stratified those with subclinical hyperthyroidism into two groups based on TSH level (Table 2). For those with TSH levels 0.1-0.45 mIU/L, the panel recommended against routine treatment for all patients, but stated that treatment should be considered in the elderly population for both

bone and cardiac adverse effects. However, treatment apart from beta-blockers for cardiac protection and bisphosphonates for bone density protection is not typically necessary. For the elderly with TSH lower than 0.1 mIU/L not ingesting levothyroxine, treatment is indicated for bone and cardiac protection on an individualized basis. Certainly, close monitoring for the development of atrial arrhythmia and for loss of bone mineral density are warranted in all elderly with subclinical hyperthyroidism.

Subclinical hypothyroidism

Subclinical hypothyroidism is defined as an elevated level of TSH in the setting of free T4 and T3 levels within the normal reference range. NHANES III found the prevalence of subclinical hypothyroidism with a TSH > 4.5 mIU/L in the U.S. adult population to be 4.3%, six times more prevalent than subclinical hyperthyroidism, and to increase precipitously in those age 70 or older⁴. The most common causes in individuals older than 55 years are autoimmune thyroiditis and prior therapy for previous thyrotoxicosis¹⁶; inadequately dosed thyroid hormone replacement is another cause. The natural history of subclinical hypothyroidism in this age group is the progression to overt hypothyroidism in approximately 8% per year. However in one study, 37% of patients were found to normalize their TSH levels without intervention over an average follow-up period of 31.7 months. In this same study, the presence of thyroid antibodies reduced the likelihood of spontaneous TSH normalization since 61.5% of those without antibodies compared to only 30% of those with positive antibodies were found to normalize their TSH levels¹⁷.

The possible physiologic effects of subclinical hypothyroidism include dyslipidemia and hence adverse cardiovascular risk, neurocognitive effects, and non-specific symptoms of overt hypothyroidism such as fatigue. A review of multiple studies found that thyroid hormone administration to individuals with subclinical hypothyroidism lowers total and LDL cholesterol but has no effect on HDL or triglycerides¹⁸. A recent randomized, double-blind, crossover study confirmed decreases in LDL and total cholesterol after 12 weeks of levothyroxine therapy as compared to placebo and found improvements in endothelial function, a known early marker of atherosclerosis¹⁹. However, the prospective cohort study of individuals 65 years or older by Cappola, et al found no increased risk of cardiovascular death or all-cause mortality in the subclinical hypothyroid group compared to the euthyroid group¹⁴. The effects on neurocognitive function are also inconclusive. Roberts, et al found that subclinical hypothyroidism was not associated with depression, anxiety, or cognition in a clinically meaningful way in individuals 65 years or older. In terms of hypothyroid symptoms, data suggest that there are no difference in the clinical signs and symptoms in elderly individuals with subclinical hypothyroidism compared to symptoms in those with either overt hypothyroidism or euthyroid individuals^{20,21}.

One prospective trial found that increasing levels of TSH were associated with a lower mortality rate in a cohort of octogenarians even after adjustment for baseline disability and health status²². Therefore, initiation of levothyroxine therapy in elderly individuals with subclinical hypothyroidism should be individualized to the specific patient. Current guidelines do not suggest routine levothyroxine therapy for those with normal free T4 and T3 but with slightly elevated TSH levels between 4.5-10 mIU/L¹⁵ (Table 3). Rather, asymptomatic individuals should be followed closely with repeat thyroid function testing every 6-12 months. In those patients with hypothyroid symptoms despite normal free T4 and T3, a several month trial of thyroid hormone therapy may be considered with careful monitoring.. In those elderly patients with TSH >10 mIU/L, treatment is usually considered. Although evidence suggests that starting levothyroxine at full replacement doses is likely safe even in elderly individuals without a history of cardiac disease²³, it is prudent to initiate lower starting dose of 25-50 mcg daily in this group and in those with pre-existing cardiac disease.

In conclusion, there are predictable changes in thyroid function parameters in the elderly including mild decreases in TSH and T3 levels, but free and total T4 levels are typically unchanged. As the current available data do not lead to definitive conclusions, therapy in the elderly with either subclinical hypothyroidism or hyperthyroidism should be individualized by the clinician to the specific patient until more definitive clinical studies are performed.

Table 1: Changes in thyroid function parameters in the healthy elderly

FT4 and Total T4	No change
FT3 and Total T3	Mild decrease*
rT3	Mild increase*
TBG	No change
TSH	Mild decrease*

(KEY: FT4: free T4; FT3: free T3; rT3: reverse T3; TBG: thyroid binding globulin; TSH: thyrotropin stimulating hormone. *Parameters may also remain within the normal range.)

Table 2: Treatment recommendations in the elderly with subclinical hyperthyroidism

	TSH 0.1-0.45 mIU/L	TSH <0.1 mIU/L
Endogenous subclinical hyperthyroidism	Consider treatment	Treatment indicated on individualized basis
Exogenous subclinical hyperthyroidism (i.e. on thyroid hormone suppression therapy for thyroid cancer)	Monitor closely for adverse cardiac and bone effects; review patient's goal TSH	Review patient's goal TSH; consider decreasing dose of thyroid hormone replacement

Table 3: Treatment recommendations in the elderly with subclinical hypothyroidism

TSH	Symptomatic?	Recommendations
TSH 4.5-10 mIU/L	no	Repeat TSH within a month and then every 6 months when asymptomatic. Consider levothyroxine therapy on an individual basis.
TSH 4.5-10 mIU/L	yes	Consider trial of thyroid hormone replacement
TSH >10 mIU/L	-----	In most circumstances administer thyroid hormone replacement

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Update on the genetics of autoimmune thyroid disease

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Introduction

Despite one third of patients with autoimmune thyroid disease (AITD) having an affected first degree relative, and a twin study showing that around three quarters of the susceptibility to AITDs can be attributed to heritable factors (1), progress in elucidating the genetic basis of these common disorders has been frustratingly slow. This article aims to review the current state of knowledge, examine the reasons why progress in genetics has been slow, and to consider future developments.

Current state of knowledge

Major Histocompatibility Complex: It has been known for more than 30 years that certain Human Leukocyte Antigen (*HLA*) alleles, encoded within the Major Histocompatibility Complex (*MHC*) on chromosome 6p21, are over-represented in AITD patients (2). The exact allele or haplotype combinations that are associated with AITD depend upon the nature of thyroid disease (i.e. Graves' disease [GD], Hashimoto's thyroiditis or post-partum thyroiditis) and the ethnic origin of the patient group. In white populations of European origin (hereafter "whites") the 'DR3' haplotype (*HLA DRB1*0301-DQB1*0201-DQA1*0501*) is typically found in about 50% of individuals with GD compared to about 25 to 30% of the background population. In patients with Hashimoto's thyroiditis, *HLA* associations have also been found with the *HLA DR4* and *DR5* haplotypes, however the effects are less consistent than those found in GD. This may be due to the smaller size of the study populations or the relatively inconsistent definition of Hashimoto's thyroiditis or autoimmune hypothyroidism (AH) between different studies. More recently, there have been attempts to fine-map the *HLA* disease associations in GD patient cohorts, using a sequence-based approach (3,4). These studies have confirmed that *HLA-DRB1* alleles encoding an arginine residue at position 74 are most strongly associated with GD in whites (3,4). However, despite these studies involving substantial numbers of patients, the linkage disequilibrium in this region is sufficiently strong that effects of polymorphisms in nearby genes still cannot be excluded as having a significant contribution to disease aetiology.

We make two observations from these studies: Firstly, with an odds ratio (OR) for association of the *DR3* haplotype with GD of around 2 in many studies, compared to autoimmune diseases such as type 1 diabetes or rheumatoid arthritis (OR for association at *HLA* between 3 and 4), there appears to be a lesser contribution of *HLA* to disease aetiology in AITD. This implies that *non-HLA* disease alleles have relatively stronger effects in AITDs than in other autoimmune conditions. Given the similar genetic contributions thought to be present in these common autoimmune conditions, this means it may be more straightforward to identify these *non-HLA* disease alleles through genetic means by studying AITD patients. Secondly, the *HLA* molecules serve to process and present peptide fragments as antigens to the T cell receptor, thus determining the antigenic specificity of an immune response. As the *HLA* associations are relatively heterogeneous in AITDs (different in distinct

populations, and only present in 50% of patients), this may mean that there is not a single T cell epitope (for instance, on the TSH receptor) that can be identified as being critical for disease onset or progression in AITDs.

Cytotoxic T-lymphocyte antigen-4: Alleles in the 3' region of the Cytotoxic T-lymphocyte antigen-4 (*CTLA4*) gene on chromosome 2q33 have been extensively associated with GD, and in fewer studies, with AH. CTLA4 is involved in the regulation of the costimulatory ("second") signal that permits T lymphocyte activation following HLA-peptide antigen encounter, and so was a good candidate gene for autoimmunity. Initial associations of *CTLA4* alleles with GD by Yanagawa and colleagues (5), were confirmed with a gamut of replication studies in many different GD populations (reviewed in 6). The true disease susceptibility allele at *CTLA4* remains to be defined but probably lies within a 6kb region including the 3'untranslated region (UTR) of the gene (7). The susceptibility haplotype at *CTLA4* is carried by about 50% of the healthy white population and its prevalence increases to 60% in subjects with GD, with an odds ratio for the most associated allele of about 1.5 (7). The mechanism by which these non-coding polymorphisms might modulate the immune response is still far from clear. One theory is that there is a circulating, soluble isoform of the CTLA4 protein that may be able to engage and occupy the CD28/CTLA4 receptors on antigen presenting cells (known as B7 molecules) and thereby modulate costimulatory signaling. Despite significant work in this area, this theory still lacks direct experimental evidence to support it (8). Another recent study has suggested a role for CTLA4 polymorphisms at an early stage of T cell differentiation and lineage commitment, with *CTLA4* genotypes being shown to correlate with the number of circulating CD4⁺, CD25⁺ T regulatory lymphocytes (9). Furthermore, it is notable that the allele associations at *CTLA4*, and at the adjacent inducible costimulator (*ICOS*) locus, appear to be subtly different in other autoimmune diseases (eg. systemic lupus erythematosus), than with AITDs (10). There may be an unsuspected level of complexity underlying the mechanistic effect of CTLA4 variant(s), with a possibility that different variants and hence aetiological mechanisms contribute to different autoimmune conditions.

Lymphoid tyrosine phosphatase: An additional locus that has recently come to light is *PTPN22*, which encodes the lymphoid tyrosine phosphatase (LYP). LYP is a negative regulator of T cell antigen receptor (CD3) signaling. A coding polymorphism, arginine to tryptophan at codon 620, activates the LYP molecule, paradoxically causing more potent inhibition of the T cell antigen receptor (CD3) signaling kinases, following engagement with MHC-antigen (11). The tryptophan allele is carried by about 7% of healthy subjects in northern European white populations, but is over-represented in GD subjects with a prevalence of about 13% (12,13). The odds ratio for the effect of this allele in GD is about 1.8, but because of its comparative rarity (12,13), it contributes slightly less to overall population Graves' disease susceptibility than CTLA4. Interestingly, the effects of the variant tryptophan 620 allele are very population specific, as the allele is only present at substantial frequency in white populations, being essentially absent in individuals of Asian and African descent (14). The mechanism by which the tryptophan 620 LYP variant predisposes to autoimmunity is unknown, but it is possible that it may mediate less efficient "weeding out" of T cells bearing potentially autoreactive T cell receptors in thymic development, leading to an autoimmune proclivity in later life.

Thyroid antigens: Disease specific loci for GD have started to be identified in recent years. After a period of negative investigations into the TSH receptor gene, alleles of SNP markers have now been shown to have unequivocal association with GD in 2 distinct patient cohorts (15,16). The associated polymorphism lies within the regulatory regions of the extracellular domain of the receptor, and fine mapping studies are in progress to more fully define the disease associated allele and hence the mechanism for the disease effect (15,16). In contrast, several studies have shown weaker evidence for association of GD with polymorphisms in the thyroglobulin (Tg) gene (17,18). On aggregate, these studies of the Tg gene have not shown convincing evidence for association with GD (although the effect may be different in AH) (19). Tg is a huge, 48 exon, gene and further work, to define and test the enormous diversity of haplotypes is currently awaited.

CD40 and CD25: The B lymphocyte surface immunoreceptor CD40 was identified as a candidate gene falling under a peak of linkage identified in GD families on chromosome 20 (20). A SNP marker, encoding a change in the context of the initiation of translation codon of CD40 has shown a weak association with GD in some studies (21,22) but not in others (23,24). Overall, a meta-analysis of the various studies confirms that *CD40* polymorphism does not have a major influence of GD susceptibility, but an effect with an odds ratio of 1.2 or less cannot entirely be excluded (25). The *CD25* gene encodes the α -chain of the interleukin-2 receptor, a key player in lymphocyte activation, and markers within this gene were found to be associated with T1D, with an odds ratio of about 1.3 (26). A replication case-control study using GD probands has confirmed a modest effect, with an odds ratio of 1.24 at the most associated marker (27). Although association at CD25 needs to be confirmed in a second AITD patient cohort before it is robust, it seems likely to be a further susceptibility locus making a small contribution towards Graves' disease pathogenesis.

Genome-wide genomics

Genome wide linkage: Genome-wide linkage scans have been completed in several patient cohorts with AITDs including Han Chinese, Japanese, the Old Order Amish and two studies using patients of mixed white origins (20, 28-32). The majority of these studies have used small "nuclear" families with 2 or more members each with AITD, most frequently a sib-pair. However, with one exception, families where one affected individual had GD and another AH have generally been included, with subanalysis of GD-only and AH-only families. Many chromosomal regions have been linked to AITD using this strategy including 1q36, 2q36, 5q11, 5q31-q33, 8q24, 11p15, 12q22, 13q32, 14p11, 17q21, 18p11, 19q13, 20q11 and Xq21 (20, 28-32). However, for relatively few of these loci has the evidence in favour of linkage (LOD

score) exceeded the 3.6 threshold for declaring definite linkage in a complex trait (33), with most of these linkage peaks being in the 2.0 to 3.0 range. It is also disturbing that the *MHC*, *CTLA4* and *PTPN22* loci, where association is unequivocally established, have not by-and-large, also been found to show linkage with GD or AH in these studies. This remains an unexplained anomaly, as one would anticipate linkage to be found at these loci. Furthermore, although linkages to the long arm of chromosome 5 (5q31-q33) and 8q24 have been replicated in two patient cohorts each (29,30), the vast majority of these linkage peaks remain unreplicated and hence unconfirmed. After the first linkage studies, involving 100 to 130 AITD families (20,28-31), this failure to replicate both linkage peaks and disease associations was felt to be owing to a lack of power in the linkage analysis, probably combined with allelic heterogeneity between individuals with GD and AH. However, the most recent study used 1119 AITD relative pairs from 558 AITD families and thus power alone is unlikely to be responsible for these discrepant results (32).

Squaring the linkage circle: A combination of several factors, including power, is likely to explain the failure to replicate both previous linkages and known associations. Firstly, an initial study reporting linkage of a genetic variant(s) with disease may be a relatively extreme result and the follow-up study will generally need to be much larger to stand a chance of replicating a true linkage (34). Secondly, it is implicit that with a genome-wide design that there will be a 'false-discovery rate' due to multiple testing and the threshold of over 2 for reporting 'suggestive linkage' has certainly proven to be insufficiently stringent (33), if judged by the criteria of replication. In addition, all of these genome-wide linkage studies have been carried out using microsatellite (short tandem repeat) markers, which are genotyped by measuring their size, and most have alleles every two base-pairs, which may be difficult to separate. Even with pedigree checking of inheritance, rejection of any incomplete genotype data and duplicate genotyping of a proportion of families, some spurious results may result from genotyping errors. It is notable that almost all research labs have abandoned the use of such markers for mapping over the last 5 years. Also, with families derived from a broad ethnic background (two of the studies used white families from 4 or more different countries) (20,28,32), there can be difficulty in defining the background allele frequencies from which the linkage scores are derived. In other words, there may be inflation of linkage scores when an apparently rare allele in the population is found to co-segregate with disease in a proportion of families who may be ethnically distinct. This may be a particular issue with autoimmune diseases where immune response alleles have been under recent natural selection and may have quite different frequencies even in populations that are superficially similar (e.g. *PTPN22*, ref 14). Lastly, there are theoretical reasons why linkage analysis might be a less sensitive method than association studies for detecting loci with relatively small genetic effects (35).

The future

Although candidate gene studies have had modest success, it remains disappointing that no novel AITD disease susceptibility gene has been robustly identified by reverse genetic approaches, despite a substantial amount of effort from several research groups over the last 10 years. The advent of SNP genotyping chips has made rapid high-fidelity genotyping of numerous markers in thousands of individuals a reality and genome-wide association studies are now underway using dense marker maps of 300,000 SNPs or more in substantial AITD cohorts. These studies may be the ultimate genomic experiment in AITD and will no doubt provide new information, which may be helpful in understanding pathogenesis, in disease prediction or in design of novel compounds to ameliorate disease. These genome-wide association analyses will have even greater issues with multiple testing than the equivalent linkage studies, and will also have practical limitations in being insensitive to rare aetiological variants, or those variants in recombination 'hotspots' with little surrounding linkage disequilibrium (36). Furthermore, although copy number variation (CNV) is firmly established and mapped across the genome (37), its association with disease is just starting to become apparent. It is already clear that CNV has a role in other autoimmune and infectious disorders (38-40) and it could well also be important in AITDs.

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MALABSORPTION OF T4: NEW INSIGHTS ON ORAL THYROXINE TREATMENT

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Background

Levothyroxine is an effective and reliable drug, widely prescribed for replacement of defective iodothyronines synthesis and secretion (1-3), or to lower TSH in patients with non toxic multinodular goitre (1, 4, 5) or with thyroid cancer (1, 2). Serum TSH represents the best marker for assessing the appropriateness of thyroxine dose (1,2,6-8) both in replacement and in interventional mode. However, due to the lack of sensitive TSH assays, large doses of thyroxine were prescribed for many years (2), which were liable for iatrogenic clinical or subclinical hyperthyroidism. Chronic hyperthyroidism has detrimental effects on some tissues or functional pathways (9,10) such as cardiovascular system and bone remodeling process (1,10-12). Thus, even the appropriateness of thyroxine treatment, when given to lower the TSH below the normal range, has been questioned (1-5). On this ground, it is worth to note that a significant reduction of the described harmful effects has been observed by individually tailoring the dose (13,14). Anyway, these concerns and the availability of more sensitive TSH assays (8), led to a progressive general reduction of thyroxine dose on both interventional and replacement treatment (1). On the other side, growing evidence highlighted that even undertreatment and/or mild hypothyroidism have detrimental effects on several tissues and functions (15-20). Indeed, undertreatment during pregnancy has been described as harmful to the fetus as well as to the successful pregnancy (15-17). In adult patients, mild or subclinical hypothyroidism has been implicated in impaired lipid metabolism and atherosclerosis as well as in cardiovascular disease (18-20). Therefore, the need for an individually tailored dose potently emerges from these findings. The continuous search for an optimal daily dose has led to consensus that 1.5-1.6µg/Kg body weight/day is the dose able to restore TSH into the normal range in most hypothyroid patients (1,2) as well as 2.0-2.2µg/Kg body weight/day is the one that lower TSH under the normal range (7,21). Despite these efforts, a number of patients fail to show a clinical and chemical response to the expected dose of T4, leading to uncertainty about the optimal dose in every single patient (1-3). The consequence of that is the need for an increased dose and of care and monitoring as well as repetition of needless and costly diagnostic workup. While the lack of complete clinical effect of replacement T4 treatment deal with the appropriateness of serum TSH as marker of euthyroidism in all tissues (22-24), an impaired absorption of levothyroxine may explain most of the incomplete biochemical effects of T4 in both replacement (1-3) and interventional therapy (25).

The absorption of thyroxine

The daily dose required to obtain the therapeutic effect is not a linear function of the ingested dose of thyroxine which is the main, but not the only decisive event. The amount of absorption of oral thyroxine is, in fact, also key to obtain successful treatment. The absorption of oral thyroxine (70 to 80% of the administered dose) takes place at the intestinal level, is incomplete (26,27), and does not differ in euthyroid and hypothyroid patients (1). Noteworthy, conjugated iodothyronines are secreted, partially deconjugated and reabsorbed in different intestinal sites (26, 28). So far, the direct measurement of thyroxine malabsorption is difficult because several factors must be taken into account (26). The tissue specific metabolism of

T4 is a further problem for large investigations in humans (29,30). Hays MT (26), in elegant studies using double isotope equilibrium method, has shown that about 20% of T4 is absorbed at the level of duodenum, about 40% is absorbed in the upper and the remaining fraction in the lower jejunoleum. Studies in euthyroid subjects from Benvenega S et al. (31), showed that peak values of T4 absorption ($\Delta T4$) occurs between 30 and 60 minutes following drug administration and that most of the hormone absorption occurs within the first 90 minutes. In the same study, the interference of food has been proven to delay the absorption of oral thyroxine too (31). In fact, serum TSH was not lowered despite the high doses administered until ingestion of food was postponed from 15 minutes to at least one hour following T4 ingestion (31).

Increased need for thyroxine

The complexity of a direct measurement of thyroxine absorption makes serum TSH levels the best diagnostic tool to evaluate thyroxine treatment effectiveness (1-3,6,7). There are some circumstances in which patients fail to show a chemical response to thyroxine treatment and larger doses of thyroxine are required to attain the desired serum TSH concentrations (1,3,26). Psychological, nutritional, and pharmacological circumstances in which the T4 dosage should be adjusted are summarized in Table I. Interference appear to be mainly related to a) patient characteristics, nutritional habits and compliance with the drug; b) the pharmaceutical characteristics of thyroxine and c) the interference of other drugs. So far, mechanisms other than malabsorption of T4 may be responsible for the increased need for thyroxine in these patients (see 1,3 for review) and some of them deserve mention. The effect of pregnancy and of estrogen treatment is very well known and reviewed elsewhere (1-3). Certain patients, due to poor compliance with the prescribed regimen, do not assume thyroxine regularly, a condition known as pseudomalabsorption (32). Similarly, some patients assume T4 while having breakfast or with a minimal delay (15-20 min). According to the abovementioned studies (31), a lag time of one hour between oral T4 and food ingestion may improve the efficiency of T4 absorption. These observations have been recently confirmed in patients with non-toxic multinodular goitre (25). Using that treatment design, a mild suppression of TSH was obtained at a median dose (1.53 μ g/Kg body weight/day) which usually normalized serum TSH in most hypothyroid patients (21) and was lower than the one required to suppress thyrotropin (7). This suggests that more efficient thyroxine absorption occurred at the intestinal level in real fasting conditions. So far, delaying breakfast up to one hour following levothyroxine ingestion may reduce the amount of the required T4 dose in most patients (25). Increased need for thyroxine may also ensue from the different T4 bioavailability in areas where a number of generic and brand name thyroxine preparations are available (33). The occurrence of these differences in the bioequivalence of thyroxine preparations is probably infrequent, but switching from one preparation to another should be avoided anyway (33). Some drugs have also been shown to interfere with thyroxine dose (1,3) (Table I). Most of them seem to interfere by sequestering oral thyroxine into the intestinal lumen but some also interacting with the acid environment. In particular, calcium carbonate, aluminum hydroxide, sucralfate and proton pump inhibitors are all drugs known to interfere with gastric acid pH and/or secretion (25,34-36). In few circumstances the need for thyroxine may be also decreased, namely in elderly patients or when body weight decreases and finally in patients treated with androgens (1,3).

Table I – CONDITIONS ASSOCIATED WITH INCREASED NEED FOR ORAL LEVOTYROXINE

PATIENTS CHARACTERISTICS AND COMPLIANCE	<ul style="list-style-type: none"> •Pseudomalabsorption •Body weight o Mass Index (BMI) gain •Pregnancy
PHARMACOLOGICAL PREPARATION OF T4	<ul style="list-style-type: none"> •Altered bioequivalence of thyroxine preparation
NUTRITIONAL HABITS	<ul style="list-style-type: none"> •Ingestion of T4 along with food •Soy foods •Fiber supplementation
PHARMACOLOGICAL INTERFERENCES	<ul style="list-style-type: none"> •Proton pump inhibitors (PPI) •Aluminum hydroxide •Calcium Carbonate •Ferrous Sulfate •Ion exchange resins •Raloxifene and Oestrogens •Sucralfate

Malabsorption of thyroxine

Increased need for thyroxine not necessarily indicates T4 malabsorption, which also requires the presence of gastrointestinal disorders. Studies dealing with T4 malabsorption were in the form of case reports and mainly focused on intestinal disorders. Erratic but reliable reports indicated that celiac disease (37,38), lactose intolerance (39), short bowel syndrome (3),

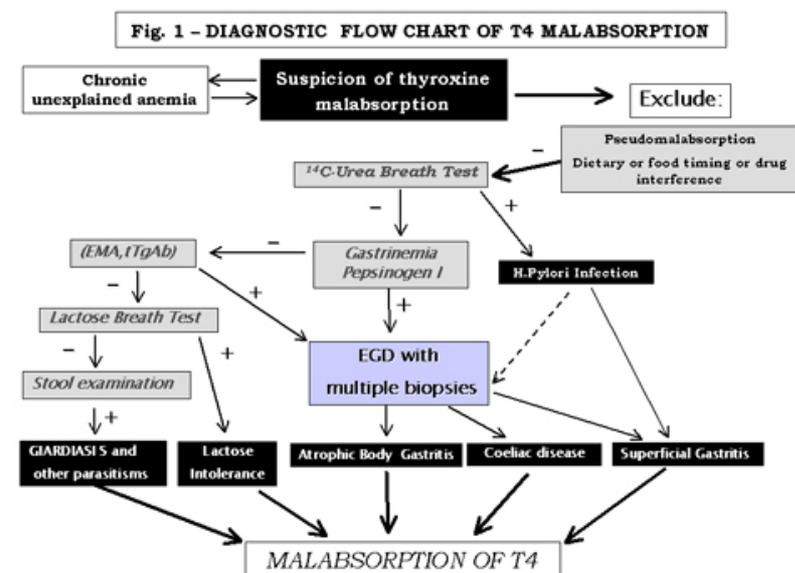
chronic giardiasis (40) are all associated with an increased need for thyroxine (table II). The recent report of an increased need for thyroxine in patients with impaired gastric acid secretion (25) highlighted also a novel role for the stomach in the subsequent intestinal T4 absorption (Table II). Indeed, the gastric acid producing machinery (i.e. the oxintic glands) is destroyed in patients with atrophic body gastritis (41), is blocked in those treated with proton pump inhibitors (PPI) (42) as well as partially destroyed and counteracted by NH₃ production in those with *Helicobacter pylori* infection (43,44). Daily T4 requirement was found to be almost 1/3 higher in patients with H.pylori-related gastritis, atrophic gastritis and maximal in those with both these conditions, than that observed in control patients (25). The prospective approach was consistent with these results and showed a reversible TSH increase in thyroxine-treated patients de novo-infected by H.pylori or simultaneously treated with PPI (25). The mechanism by which intestinal absorption of thyroxine may be impaired in hypo-achlorhydric patients remains, however, unclear but hypothesis can be made. The efficiency of intestinal T4 absorption may be altered by the ionization status and conformational characteristics of T4 molecule. The native lipophylic thyroxine enters target cells through both passive diffusion and in a carrier-mediated, inhibitable way (45). Instead pharmaceutical T4 preparation is a hydrophilic sodium salt that may remain undissociated under hypochlorhydric gastric environment and thus less efficiently absorbed through the intestinal lipid bilayer. Whatever the mechanism would be, the meaning of these findings is not restricted to the addition of further causes of increased need for thyroxine (Table II). In fact, while atrophic gastritis is a rare disease (41), H. pylori infection is widespread all over the world (43) and proton pump inhibitors are among the most prescribed drugs in the world (42). Therefore, the estimated optimal daily dose of T4 may be biased in a relevant number of patients taking oral T4, due to the large number of unaware infected or treated patients. Also, that occult variable may perhaps explain the large range of T4 dose reported by several authors (2,6,7). Conversely, malabsorption of T4 may also represent a tool to diagnose occult gastrointestinal diseases. In fact, once established that malabsorption represents a major disrupter of the relationship between the oral T4 dose and the expected TSH, patients treated with thyroxine may be divided in responders and non-responders. While the responders show a positive proof of not having the described gastrointestinal problems, the non-responders represent a group at risk for having these problems. In these latter, careful exclusion of pseudomalabsorption as well as of food and drug interference is needed, but then the presence of gastrointestinal diseases must be investigated. This is particularly true when an iron-deficient anemia, often associated to celiac disease (46), H. Pylori infection (47) and atrophic gastritis without pernicious anemia (48), is concurrently present. Based on these evidence and considerations, a diagnostic flow chart of T4 malabsorption, shown in Fig 1, may be proposed. Active H. pylori infection must be screened as a first line as it is the most frequent (43). The assay of fasting gastrinemia and pepsinogen I should be assayed following a negative response from ¹⁴C-Urea breath test (41,42). Increased gastrinemia and/or reduced pepsinogen I in serum should lead to EGDS with multiple biopsies of gastric body and antrum and duodenal mucosa (48). A third line of investigations may be proposed when previous steps would have been negative and should include lactose breath test to detect lactose intolerance (39) and parasitic search in the stool with special attention to the presence of giardia lamblia (40). A successful diagnostic workup may uncover the vast majority of gastrointestinal cause of poor response to thyroxine treatment, but would also be useful to diagnose and treat subclinical or silent, but not harmless, gastrointestinal disorders. A further issue to be taken into account is the pathogenesis of some of these gastrointestinal diseases: in fact both the atrophic body gastritis (48) and celiac disease (46) are of autoimmune origin and frequently associated to autoimmune thyroiditis, in turn the main cause of hypothyroidism in adult patients (11). Characteristic of these associations is the putative common pathogenic mechanism and the significant risk to have further occult immunoendocrinopathies in the patient and in its relatives (49). The complex of these diseases has been classified as autoimmune polyglandular syndromes, a definition, however, which is insufficient to describe all these associations and still in waiting for updated classification. The disclosure of these often silent diseases by using the screening described in fig. 1, may help to better characterize the immunological status of patients and of their relatives.

Table II - GASTROINTESTINAL DISEASES AND MALABSORPTION OF ORAL THYROXINE

- *Helicobacter Pylori Infection*
- *Atrophic Body Gastritis*
- *Celiac Disease*
- *Giardiasis and other parasitic disease*
- *Short Bowel Syndrome*
- *Other Intestinal Chronic Inflammatory Diseases?*

Gastrointestinal malabsorption of oral thyroxine is quite common and may be a major determinant of the T4 daily requirement for both replacement and interventional therapy.

Conversely, an unexplained increased need for thyroxine should be trigger for gastrointestinal diseases diagnostic workup.



LEGEND TO FIGURES

Fig.1- Diagnostic flow chart of T4 malabsorption

EMA = serum anti–endomisium antibodies; tTg = serum anti-transglutaminase antibodies;

EGD = esophagogastroduodenoscopy

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Potential new pharmacologic targets of benign thyroid nodules

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Introduction

Ultrasound investigations, in iodine-deficient areas reported a frequency of thyroid nodules as high as 1.3 – 52.4% in women and 4.7 – 28.8% in men depending on the age of the patients [1]. 85% of these benign nodules are scintigraphically cold, 10% are hot, and 5% cannot be distinguished by scintigraphy [2;3]. Whereas the ablative treatment of hot thyroid nodules (HTNs) is straightforward, the treatment of cold thyroid nodules (CTNs) with thyroid hormones results in a significant volume reduction in only 17% of the patients [4]. While the molecular aetiology of hot thyroid nodules is mainly characterized by constitutively activating thyrotropin receptor (TSHR) and G_s mutations [5-7], the molecular background of cold thyroid nodules is so far largely unknown. However, the recent identification of a defective sodium iodide symporter trafficking [8], a differential expression of several G_q-PKC-pathway associated genes [9], increased oxidative stress due to iodine deficiency causing DNA damage [10;11], and of insufficiently iodinated thyroglobulin [12] will contribute to the discovery of the molecular aetiology of CTNs. Moreover, these insights into molecular pathways will be instrumental in designing more efficient pharmacologic therapies for benign thyroid nodules.

Hot thyroid nodules

In different studies the prevalence of thyrotropin receptor (TSHR) and G_s mutations in hot thyroid nodules (HTNs) has been reported to vary from 8 to 82% and 8 to 75%, respectively [5;6;13-20]. In 75 consecutive hot thyroid nodules using the sensitive denaturing gradient gel electrophoresis, we observed a frequency of 57% TSHR mutations and 3% G_s mutations [21]. Similar frequencies were detected in iodine replete and iodine deficient areas [22]. These results raise the question of the molecular aetiology of TSHR and G_s mutation negative nodules. Because most of these nodules are characterized by a clonal origin, a neoplastic process with a mutation in a gene other than the TSHR or the G_s protein as the starting point of nodule development, can be anticipated [23]. In a recent study [24] analyzing the gene expression patterns of HTNs harbouring a somatic TSHR mutation and TSHR mutation negative HTNs, we could show that a number of signal transduction genes (e.g. p21/Cdc42/Rac1-activated kinase (*PAK*)1 and 2, regulator of G-protein signaling (*RGS*)4 and 6, Janus kinase (*JAK*)1, and G-protein coupled receptor kinase (*GRK*)2) are characterized by a different expression pattern in these two subgroups of HTNs. These findings argue for strong differences in the signal transduction of HTNs caused by constitutively activating mutations in the TSHR compared to HTNs without somatic TSHR mutations. Thus, these findings can give a lead for the elucidation of their molecular aetiology.

While the molecular background of TSHR and G_s mutation negative hot thyroid nodules still needs to be discovered, there are also open questions concerning the HTNs harbouring a somatic TSHR- or G_s mutation: they lack a clear genotype/ phenotype correlation [25]. A similar finding is evident for germline TSHR mutations [26]. The different phenotypes, which are associated with the same TSHR mutation could result from unknown signalling events, like G protein coupling, negative feedbacks (e.g. leading to receptor desensitization and internalization) or a cross-talk with other signalling cascades, as well as influences on the signalling downstream of the TSHR.

Negative feedback mechanisms

Microarray investigations of HTNs and TSH stimulated primary thyroid cells showed a remarkable induction or activation of negative feedback mechanisms, e.g. an up-regulation of phosphodi-esterases [27-29], which is in line with previous findings of Persani et al. [30]. Moreover, the presence of negative feedback mechanisms in HTNs is further supported by an increased expression of G-protein coupled receptor kinases (*GRK*) in hot thyroid nodules and their ability to desensitize the TSHR as shown in *in vitro* experiments [31]. Interestingly, while microarray studies of TSH stimulated primary thyroid cell cultures revealed an increased expression of the regulator of G-protein signalling (*RGS*)2 [28;29], which has been shown

to reduce the TSHR signalling via inositol-3-phosphate [32], *RGS2* is characterized by a decreased expression in HTNs [28;29;33]. Such a difference can most likely be explained by defects in the RGS regulation pathway or by additional counter-regulatory mechanisms, which occur only in the chronically proliferating HTNs.

Sialylation of the TSHR

Sialylation is known to be one kind of protein modification influencing the biological properties of its targets. For the TSHR it has been shown that the formation of the TSH-binding site and its cell surface expression is dependent on complex posttranslational modifications in which carbohydrate residues play an important role [34]. Therefore, the prominently increased *SIAT1* expression pattern in our microarray study of HTNs [24] prompted us to further investigate the physiological meaning of this finding. The data of this subsequent study add a new aspect of protein modification (i.e., sialylation) that has not been studied for functional consequences so far. We could demonstrate for the first time that the transfer of sialic acid to carbohydrate residues of the TSHR can improve and prolong the cell-surface expression of a transmembrane receptor, thereby regulating the availability of the receptor for ligand signalling [35].

Effect of TSHR signalling on TGF β signalling

As constitutively activating TSHR mutations disturb the coordinated signal transduction network of the thyroid in a drastic way, subsequent changes in the signal transduction network can be expected. Particularly the TGF- β signalling cascade is characterised by strong changes in its gene expression pattern in HTNs when compared to their normal surrounding tissues: the type III transforming growth factor receptor (TGFBR3), *SMAD* 1, 3, and 4, as well as p300, a transcriptional coactivator, showed a decreased expression in HTNs, whereas the inhibitory *SMAD* 6 and 7 showed an increased expression in HTNs [24]. Moreover, a decreased expression of TGFBR3 and TGFBR1 could also be shown at the protein level [24;36]. Because TSH stimulated primary thyroid epithelial cells [28] also showed significant differences in the gene expression profiles of the TGF- β signalling cascade, the findings in the HTNs are most likely due to the constitutively activated cAMP cascade. Moreover, in the context of an increased b-arrestin 2 expression in HTNs [37], findings of a b-arrestin 2 mediated endocytosis of the *TGFBR3* and a subsequent downregulation of its signalling by Chen et al. [38] support and further elucidate this interpretation.

Cold thyroid nodules

In contrast to thyroid malignancies (such as follicular and papillary thyroid carcinoma) where several molecular alterations have been shown to be associated with the pathogenesis of these lesions [39;40], and in contrast to HTNs, which are mainly associated with constitutively activating TSHR and G α_s mutations, the knowledge concerning the molecular aetiology of cold thyroid nodules constituting the most abundant thyroid lesion with a frequency of approximately 85% [2;3], is so far very limited.

Sodium-iodide-symporter (NIS)

Due to the decreased iodide uptake in CTNs, a defective iodide transport and/or iodide organification can be anticipated. Indeed, a decreased expression of the sodium-iodide-symporter (NIS), which is most likely caused by a hypermethylation of its promoter [8], has been shown in several studies [8;41-43]. Whereas there is no correlation between the mRNA and protein expression of the sodium-iodide-symporter and NIS promoter methylation [8], a defective cell membrane targeting of the sodium-iodide-symporter [8;44;45] could be a more likely mechanism for the defective iodide uptake of the CTNs.

Disturbed hormone synthesis

The intrathyroidal iodine deficiency of CTNs is accompanied by an up-regulation of enzymes involved in the thyroid hormone synthesis such as thyroid peroxidase (TPO) and thyroid oxidase (ThOX) 1 and 2 [12]. However, although these enzymes are characterized by an up-regulation, they cannot insure sufficient iodine supply since it is known that in CTN thyroglobulin is poorly iodinated [12].

Interestingly, the analysis of microarray data of CTNs revealed a significantly altered expression pattern in the group of G protein signaling molecules, which is mainly based on a differential expression of several protein kinase C (*PKC*) isoforms and an increased expression of the G α_q protein [9]. This expression pattern is important because it has been shown

that thyroid cells undergoing a long-term PKC stimulation are characterized by a general loss of thyroid specific functions (e.g. loss of iodide transport and thyroglobulin iodination) which is reminiscent of CTNs [46-50]. Regarding possible reasons for PKC stimulation no activating mutations were found in benign and malignant thyroid tumours. However, PKC activation may be due to stimulation by other growth factor receptors [51] or other causes.

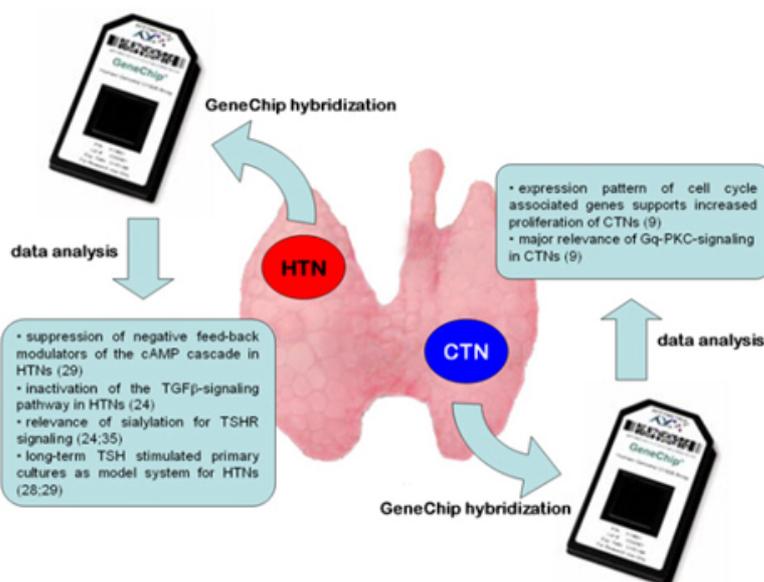
Oxidative stress

An increased expression of H₂O₂ detoxifying enzymes (glutathione-S-transferase, peroxiredoxins) has been shown in CTNs [12]. However, the increased formation of 8-oxo-guanidine DNA adducts in CTNs, which is an indirect marker of oxidative stress, gives evidence that the up-regulation of the antioxidative system does not sufficiently counterbalance the increased H₂O₂ generation [12].

In a recent study Maier et al. [10] could show a strikingly high spontaneous mutation rate, an increased level of oxidative DNA damage, and high levels of 8-oxo-guanidine in the normal mouse and rat thyroid in comparison to other organs. Therefore, the high spontaneous mutation rate in the thyroid could be due to oxidative DNA damage caused by the specifics of thyroid hormone synthesis, which involves generation of free radicals and reactive oxygen species [10]. In the case of CTNs, which are characterised by oxidative stress and increased proliferation [9;12;52], this finding might suggest a further increase of mutagenesis resulting in a dedifferentiation of the thyrocytes.

Increased proliferation

A recent microarray study [9] could further define the molecular pattern of the increased proliferation in CTNs [52]. This increased proliferation is much more prominent in CTNs compared to HTNs [52;53]. Regulation of gene expression was most consistent for a number of histone mRNAs and of gene sets containing cell cycle-associated genes, like cyclin D1, cyclin H/cyclin dependent kinase (CDK)7, and cyclin B. Furthermore, these expression data also revealed that contrary to papillary thyroid carcinomas, altered expression of components belonging to the RAS-MAPK cascade is of minor importance for the development of CTNs, since gene sets representing this pathway did not show differential expression in comparison to the surrounding normal tissue. This is in line with findings of Esapa et al. [54], who showed that a general relevance of RAS mutations for the development of follicular adenoma is unlikely. Moreover, these results are supported by findings of Krohn et al. [55] and a recent *in vitro* study indicating that the dedifferentiated phenotype of CTNs is unlikely to be the result of an activated RAS signalling [56]. However, in contrast to the gene expression data of CTNs, which did not reveal any evidence for RAS-MAPK signalling, we could recently show a specific expression pattern of RAS-MAPK associated genes in papillary thyroid carcinoma [57]. This expression pattern is most likely due to activating mutations or chromosomal rearrangements of *BRAF*, *RET*, or *RAS* (i.e., effectors along the MAPK pathway), which have been identified in about 70% of all PTCs [58-61], and have been shown to be essential for the transformation of thyroid epithelial cells in PTCs [62].



Conclusion

Although the molecular aetiology of about 60% of HTNs has been characterised by constitutively activating TSHR mutations, the molecular background of TSHR and G_sα mutation negative HTNs still needs to be discovered. Furthermore, the molecular aetiology of CTNs needs further investigation, recent findings showing increased oxidative stress and differences in the expression of G_q-PKC pathway associated genes in CTNs could be a basis for a pharmacologic enhancement of antioxidative mechanisms and a selective inhibition of G_q-PKC signalling in CTNs in the future. However, a more detailed knowledge about the molecular mechanisms of disturbed thyroid hormone production in CTNs is necessary to achieve these goals. For CTNs the underlying genetic defect at present is unknown.

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Thyroid Cancer Diagnosis by PET Scan

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Introduction

¹⁸F-deoxyglucose(FDG) is an analogue of glucose and is distributed in the glucose-utilizing cells by membrane glucose transporters which are usually overexpressed in cancer cells. Unlike glucose, FDG does not undergo further metabolism and is trapped in the cell, which permits visualization by positron emission tomography(PET). FDG-PET is regarded as a very powerful tool in cancer imaging, including thyroid cancer.

The role of FDG-PET in postoperative follow-up of thyroid cancer

Up to 20% of patients with differentiated thyroid cancer will develop recurrences, and rarely some of them will eventually die from their disease. Differentiated thyroid cancer runs a relatively indolent course compared to other malignancies, nevertheless, it is a lethal illness in some cases that requires a vigorous search for recurrence. Traditionally radioiodine has been used to detect recurrence or distant metastasis of well-differentiated thyroid cancer. High dose radioiodine was used to treat recurrent or metastatic disease when the lesion showed active iodine uptake on the diagnostic whole body scan (WBS). This approach is a still valid and very helpful diagnostic and therapeutic modality, especially in high risk patients with functioning residual disease after surgery. However, recent findings, namely that the stimulated thyroglobulin (Tg) is the most important tumor marker and an undetectable Tg after surgery and remnant ablation is the accepted criteria for cure, have highlighted the diagnostic dilemma of patients with elevated Tg with negative WBS (1).

Pacini et al found that when stimulated Tg measurement was combined with neck ultrasonography, the diagnostic sensitivity was 96.3% and the negative predictive value (NPV) was 99.5%; however, for stimulated Tg and iodine WBS, the sensitivity was only 21% with a NPV of 89% (2). It is certain that modern ultrasonographic techniques are extremely sensitive and can detect small lesion at a reasonable cost. Moreover ultrasonography-guided aspiration can be done simultaneously, facilitating cytological confirmation. In this regard, ultrasonography is considered as the first line imaging modality in the follow up of low risk patients with differentiated thyroid cancer. However, ultrasonography can detect only loco-regional recurrences. Distant metastases, which occur up to 15% of the patients, are not to be found with ultrasonography and another systemic imaging modality is required which can detect lesions regardless of the site of recurrence.

Moreover during carcinogenesis, dedifferentiation occurs and thyroid follicular cells may lose their ability to concentrate iodine. In this case, iodine WBS is not helpful to detect the non-functioning lesions compounding the need for other imaging modalities. Various imaging techniques have been applied in this condition and recently FDG-PET was very successfully utilized in patients with elevated serum Tg and negative WBS (3, 4), with a very high sensitivity and specificity ranging 75 to 95%. The sensitivity and specificity of FDG-PET was superior to MIBI SPECT and I-131 post treatment scan (5) and tetrofosmin SPECT (6). FDG-PET plays a complementary role with the conventional radioiodine WBS due to the flip-flop phenomenon, which is uptake of radioiodine with no FDG uptake and vice versa (7). This means that FDG uptake is relatively increased in less well differentiated thyroid cancer which has lost the normal characteristics of thyroid follicular cells, namely iodine uptake, during cancer development and is therefore not visualized by conventional WBS. In this regard, it seems quite natural that FDG-avid metastatic thyroid cancers are resistant to treatment with high-dose radioactive iodine (8).

FDG-PET is a very sensitive and specific method to detect recurrent/metastatic disease in patient with thyroid cancer, however, it is not clear whether it should be the first-line diagnostic method during the follow up of the patients, because of concerns over the very high costs and limited availability. Recurrent disease is usually diagnosed by the finding of an elevated Tg and after the diagnosis of recurrence localization of the recurrent disease is necessary for further therapy. Usually ultrasonography is done as the first line technique in low risk patients. In low risk patients FDG-PET may be performed to detect recurrent disease elsewhere in the body when neck ultrasonography is negative or to exclude the possibility of distant metastasis when loco-regional disease is considered for surgical management.

FDG, although a good tracer, is not perfect and there is a physiologic uptake in muscle, adipose tissue, and lymphoid tissue. Moreover increased uptake may occur in various benign conditions such as inflammation. In this regard, false positive cases may occur. Most of the physiologic FDG uptake in the neck occurs in the laryngeal muscles, eye muscles, tongue and various muscles in the neck due to tension. This can be avoided by relaxing the patient or administration of a benzodiazepine before study (9). Postoperative changes including abscess and lymphoid hyperplasia or second malignancy can also be a source of false FDG uptake and any FDG uptake should be confirmed by biopsy (10, 11).

An interesting finding is that with increasing Tg levels, the detection rate of the remaining disease by FDG-PET is increased. As shown in Table 1, the scintigraphic sensitivity of FDG-PET increased as serum Tg levels increased (12, 13). Tg levels roughly correlate with tumor burden in patients with differentiated thyroid cancer (14) and it seems that FDG-PET may not detect very small tumors. Menzel et al showed that a stimulated Tg of 1.9 ng/ml was a cut-off value, above which patients had detectable lesions by FDG-PET (15). It seems clear that like many other diagnostic modalities, FDG-PET has the highest value when tumor burden is high. From the above findings, FDG-PET examination is not recommended in patients with a Tg below 10 ng/ml as an initial evaluation, due to unacceptably low sensitivity. Thyrotropin stimulation, either endogenous after thyroid hormone withdrawal, or exogenous with recombinant human thyrotropin injection, may yield better results in patients with relatively low Tg levels (16). Thyrotropin stimulation is usually recommended for higher yield, however, in patient with Tg levels above 100 ng/ml, thyrotropin stimulation may not be necessary (17).

Thyrotropin stimulation, either endogenous with thyroid hormone withdrawal, or exogenous with recombinant human thyrotropin injection, may yield better result in patients with relatively low Tg levels (16). Although some investigators reported no difference of diagnostic performance of FDG-PET according to serum thyrotropin level (4, 11), most investigators agree that thyrotropin stimulation has some influence on the FDG uptake by tumor cells (16, 18). Thyrotropin stimulates FDG uptake by human thyroid cells in vitro (19). Thyrotropin stimulation may be recommended for a higher yield in selected patients with very low Tg levels; however, in these patients with relatively low tumor burden, it has to be determined whether the added cost of recombinant human TSH or discomfort of thyroxine withdrawal is justifiable rather than adopting a wait and see policy with yearly US examination.

Table 1. Positive rate of FDG-PET uptake according serum thyroglobulin levels in differentiated thyroid carcinoma with negative iodine uptake

Author of reference	TSH status	Number of patients	Thyroglobulin levels (ng/mL)			
			<10	10-20	20-100	>100
Schluter (12)	stimulated	43	17%	50%	67%	90%
	suppressed	17	11%	50%	100%	100%
Giammarile (13)	suppressed	45	46%*	43%*	67%*	86%*

*according to reclassified thyroglobulin levels from published raw data

PET/CT

The combination of PET and CT imaging equipment seems to be very helpful for evaluation of anatomical lesions in the neck region and yielded sensitive result at low Tg levels. In one recent study, the sensitivities of FDG-PET/CT at serum Tg levels of less than 5, 5-10, and more than 10ng/ml was 60%, 63% and 72% respectively (11). In another study, PET/CT findings modified the original PET diagnoses in 77% of the patients (18). FDG-PET/CT seems promising, and, PET/CT using other tracers, such as I-123, may be also utilized, permitting individual dosimetry (17, 20).

Thyroid PET incidentaloma

Widespread use of FDG-PET in various malignant and benign diseases has disclosed some patients who have unexpected FDG uptake in the thyroid, the so-called "thyroid PET incidentaloma". Several groups have reviewed the nature of thyroid PET incidentalomas from a large number of PET examinations (21-24). Thyroid PET incidentalomas were found in approximately 2% of subjects in all series, and the rate of malignancy ranged from 14% to 50%. Most of these were primary thyroid malignancies and some were metastatic tumors to the thyroid. Diffuse thyroid uptake was noted in patients with thyroiditis. The prevalence of malignancy in thyroid PET incidentaloma is high. Therefore, every focal FDG uptake in the thyroid has to be properly investigated and often operated on. When FDG-PET/CT was applied, focal thyroid uptake was noted in 4% of subjects and 36.7% of the cases with focal uptake were malignant (25).

The role of FDG-PET in preoperative diagnosis and staging of thyroid cancer

Some investigators reported that FDG standardized uptake values (SUVs) of PET imaging might predict malignancy and malignant tumors had higher SUVs (22, 23, 25). However, others have found that maximum SUVs in benign thyroid nodules were as high as in malignant tumors and SUVs alone were not helpful in differentiating malignancy from benign nodules

in thyroid PET incidentalomas (21, 24). There are two recent prospective studies concerning the value of maximum SUVs predicting malignancy in cytologically undetermined thyroid nodules. In one, the subjects had thyroid nodules with inconclusive cytology (26) and in the other the subjects had a cytological diagnosis of follicular neoplasm (27). In both studies, SUVs were not helpful in differentiating malignant from benign nodules. However, in one study all the malignant nodules accumulated FDG, whereas only about one third of the benign nodules had FDG uptake and the remaining two thirds did not have any FDG uptake. This finding may help in differentiation between malignancy and benign nodules in patients with cytologically inconclusive results (26). In another study, all patients with a cytological diagnosis of follicular neoplasm had vivid FDG uptake and the maximum SUVs between malignant and benign nodules were similarly high (27), indicating that FDG-PET does not help to differentiate malignancy from benign thyroid nodules.

The efficacy of FDG-PET in preoperative staging of thyroid cancer has also been evaluated (28, 29). For thyroid papillary microcarcinomas, SUVs from FDG-PET imaging had a correlation with tumor size only and did not predict extrathyroidal extension: ultrasonographic findings gave better information (28). Diagnostic accuracy of FDG-PET/CT for the preoperative evaluation of the cervical lymph nodes was compared with that of ultrasonography and conventional contrast-enhanced CT (CECT). The overall sensitivity, specificity and diagnostic accuracy of FDG-PET/CT was 30%, 96% and 87%. The corresponding values for US and CECT were 41%, 97%, 89% (US) and 35%, 96%, 87% (CECT) respectively. The diagnostic value of FDG-PET/CT, ultrasonography and CECT were similar and PET/CT did not provide any additional benefit for the preoperative evaluation of patients with thyroid cancer (29).

FDG-PET may be performed as an initial work up in high risk patients. PET may alter the therapeutic approach. In one study, initial preoperative FDG-PET result was an independent prognostic factor for survival (30).

Summary

FDG-PET is a very useful imaging modality in the postoperative follow-up of the patients with differentiated thyroid cancer. When tumor recurrence is diagnosed with elevated Tg levels and diagnostic iodine WBS is negative, FDG-PET can localize recurrent/metastatic disease in 70 to 90% of the patients. However, considering the very high cost of FDG-PET examination and its availability only at some referral centers, neck ultrasonography should be done first. If ultrasonography is negative, FDG-PET or PET/CT may be the diagnostic procedure of choice. In the case of those with localized recurrent disease, FDG-PET may help excluding distant metastases. Some patients show incidental FDG uptake in the thyroid during FDG-PET or PET/CT. Since many of these thyroid PET incidentalomas are malignant, they need to be thoroughly investigated and properly managed by surgery. However, maximum SUVs by FDG-PET do not differentiate malignancy from benign thyroid nodules. FDG-PET does not have any beneficial effect in the initial preoperative evaluation of the patients with thyroid cancer, especially for the assessment of extrathyroidal extension and regional lymph node metastasis.

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PAPILLARY THYROID CANCER IN THE TWENTY FIRST CENTURY: A MIDATLANTIC PERSPECTIVE ONE YEAR AFTER THE PUBLICATION OF THE AMERICAN THYROID ASSOCIATION CLINICAL MANAGEMENT GUIDELINES

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Thyroidologists on both sides of the Atlantic and their patients with papillary thyroid carcinoma (PTC) are living in interesting times, since during the past decade the topic of thyroid malignancy has emerged from being, formerly, a lesser topic for discussion at the annual gatherings of both the American (ATA) and the European Thyroid Associations (ETA) to now being the number one topic for submitted abstracts. How has this change come about so recently?

The emergence of thyroid cancer as a “hot topic” in thyroidology

With the explosion of information derived from genomic unraveling and the increased emphasis of international funding bodies on awarding research grants almost exclusively to projects of a cell biologic and molecular genetic nature, we live in a time when goitrogenesis, deiodination and thyroid hormone metabolism have reluctantly accepted a shift of the spotlight to studies of thyroid cancer. Consequently, acknowledged authorities in those former fields now espouse a great interest, and are fast acquiring a recent experience, in the management of thyroid cancer patients. Moreover, the desks of busy clinical thyroidologists are cluttered with, often largely unread, endocrine journals which overflow with preliminary communications related to new insights into the pathogenesis of sporadic and radiation-associated PTC, aided and abetted by the intellectual fallout from the Chernobyl reactor accident, studies touting the value of novel markers for the diagnosis and outcome prediction of follicular cell-derived cancers (FCDC), others defining yet more prognostic scoring and staging systems, in addition to both in vitro and in vivo animal studies of potentially new pharmacologic agents, that appear active in cell culture and could theoretically in future years be used in the management of advanced (recurrent and metastatic) FCDC.

Additionally, the recognition that kinase inhibitors may play a role in the pathogenesis of PTC has led for the first time to increasing interest by the powerful multinational pharmaceutical companies to conduct, in centers of academic endocrinology on both sides of the Atlantic, therapeutic trials in FCDC patients of novel agents that have shown promise in patients with non-thyroid cancers. In my own life, after more than three decades of treating FCDC patients with rarely a meaningful conversation with a medical oncologist, I was amazed to be approached within the past year by one of the most senior oncologists in our institution to enquire about the numbers of thyroid cancer patients I personally saw per year. When asked why he sought for the first time such information, I was told that, after recent discussions with a number of pharmaceutical consortia, the ascertainment of such numbers would be an essential prerequisite to determine whether our institution could be eligible to participate in the lucrative pursuit of future clinical trials, especially now that our medical oncology group had decided to become enthusiastically involved in "the business" of thyroid cancer!

Influence of rhTSH and sonography in contemporary clinical thyroidology

It is well recognized that the role of a clinical thyroidologist is often to order appropriate studies of thyroid structure and function and to interpret the results “for the benefit of the individual thyroid patient”. In the management of patients with FCDC, the role of the endocrinologist in the multidisciplinary team (MDT) appears now in both European and American settings to be pivotal. Obviously, in the politics of internal medicine the powerful “nations” of cardiology and gastroenterology in part owe their power base to the financial impact of procedures. The cardiologists have their potpourri of echocardiography, angioplasty and ablation procedures, while the gastroenterologists have their diagnostic and therapeutic endoscopies. Certainly, in the United States, the endocrinologist in private practice is increasingly performing in his/her own office-based laboratory endocrine function tests, supervising within the same office bone mineral density measurements, and performing an increasing number of fine needle aspiration (FNA) biopsies of ever smaller examples of nodular thyroid disease (NTD). And in the past decade, there have been two areas of “hot thyroidology” that have significantly contributed to the groundswell of interest in patients with NTD and FCDC: these are the introduction of recombinant human TSH (rhTSH) and the increasing use by endocrinologists of high-resolution real-time thyroid sonography (HRTS).

For years the clinical thyroidologist had been satisfied with a basic repertoire of endocrine testing that often consisted of fT4, fT3, TSH, Tg, anti-TPO, anti-Tg antibodies and TSH

receptor antibodies. With the coming of increasingly sensitive TSH assays, the era of the TRH stimulation test was soon gone for ever. However, since the introduction in 1999 to the USA of rhTSH, this has heralded an explosion of interest in studies fueled by new financing, a huge increase in the number of meetings devoted to the topic of managing FCDC, and for the endocrinologist in practice the advent of a lucrative new tool, as well as a new thyroid function (and stimulation) test to interpret or misinterpret, perhaps not always for "the benefit of the individual thyroid patient". And sadly, despite our recognition for decades that thyroglobulin (Tg) is a specialized complex protein made naturally by thyroid follicular cells, both benign and malignant, there now seems to be an almost acceptance that, in the post-thyroidectomy and ablation setting, a detectable serum Tg level (even the most tiny amount) should be viewed as a surrogate for the presence of FCDC. Moreover, a FCDC patient with a (presently) undetectable Tg on adequate thyroid hormone suppressive therapy (THST,) who has a detectable increment of Tg (no matter how small) after rhTSH stimulation, is now generally viewed with suspicion in the clinical thyroidology community!

And what of HRTS...formerly the domain of the diagnostic radiologist, but often a very poor "Cinderella" to the much more affluent "ugly sisters" of computed tomography (CT), magnetic resonance imaging (MRI) and positron emission tomographic (PET) scanning? What of ultrasound-guided biopsy (USGB): a procedure that the Radiological Society of North America (RSNA) described in their recent annual meeting as a "procedure run amuck"? Well, at least in the United States we are now observing a "turf war", (not being fiercely defended by the radiologic community), where increasingly it is non-radiologists who are wielding in their hands the ultrasound probes, which are unmasking the occult malignancies and swelling interest in the management of papillary thyroid microcancer (PTM). As Terry Davies has recently said, "the recommendation not to perform biopsy on any thyroid nodule less than 1 cm in size leaves me bewildered. Don't all thyroid cancers start out as small nodules? Take a look at the microcarcinoma literature and I am not sure you will be as confident as you now feel" (1).

In recent years, no annual scientific meeting in the United States of the AACE, AAES, ATA, TES or ACS has been considered complete without a rapidly over-subscribed CME-creditable satellite session devoted to "hands-on" experience of HRTS. Fellows in North American endocrine fellowship training programs now expect to leave their training equipped to do competent thyroid ultrasound (US) and to be proficient at USGB of patients with NTD. In 2005 an Academy of Clinical Thyroidologists (ACT) was formed at the AACE meeting in Washington, DC to allow a gathering together of the expertise of those specialist endocrinologists, whose mission it is to "take charge" of the patient with NTD and FCDC, to the point of supervising "in-house" the thyroid function tests, the radioiodine studies, the diagnostic US, the USGB and sometimes even to the interpretation of the cytology, in addition to the selection and supervision of subsequent management. And even Terry Davies, a lifelong academic, an acknowledged expert in thyroid autoimmunity and the present Editor-in-Chief of *Thyroid*, the official journal of the ATA, last month proclaimed that "The only thing that has significantly changed my practice of thyroidology in recent years has been my thyroid ultrasound. I am in love with it...It gives me almost everything I want...Frankly, I do not know how anyone can see thyroid patients without their own ultrasound by their side" (1). Certainly, I would suggest, that this represents a very strong endorsement for clinical thyroidologists to take the ultrasound probe into their own hands!

Impact of Sonography on PTC recognition

But has this renewed interest in thyroid sonography, both in private and academic practice, made a difference to the detection of patients with FCDC? Between 1975 and 2001, the incidence of thyroid cancer in the United States rose by 52% (2) and, in a retrospective cohort evaluation of patients with thyroid cancer using the National Cancer Institute's Surveillance Epidemiology and End Results (SEER) program, Davies and Welch (3) also demonstrated that the incidence of thyroid cancer has increased from 3.6 per 100,000 in 1973 to 8.7 per 100,000 in 2002: a 2.4 fold increase. Virtually, the entire increase was attributable to an increase in the incidence of PTC, which increased from 2.7 to 7.7 per 100,000: a 2.9 fold increase. However, mortality from thyroid cancer was stable between 1973 and 2002 (approximately 0.5 deaths per 100,000). These authors concluded that "these trends, combined with the known existence of a substantial reservoir of subclinical cancer and stable overall mortality, suggest that increasing incidence reflects increased detection of subclinical disease, not an increase in the true occurrence of thyroid cancer" (3).

These authors are in no doubt that "increased diagnostic scrutiny" explains the apparent increased PTC incidence, and that the case for "overdiagnosis", a term more often applied to prostate cancer, is strengthened because "almost all the increased incidence is attributable to the detection of small cancers best discovered by use of the new technologies: ultrasound and fine needle-aspiration". Noting that HRTS is "increasingly performed in the physician's office", Davies and Welch went on to caution that "if ultrasound continues to grow as an office-based adjunct to physical examination, there could be a dramatic increase in the number of nodules, and ultimately, cancers, identified", and they advised that "further

studies will be needed to determine if a more cautious diagnostic approach, perhaps simply providing follow-up for symptomatic thyroid nodules, is worthwhile". They concluded that "in addition, papillary cancers smaller than 1 cm could be classified as a normal finding", a conclusion remarkably similar to those derived from the meticulous pathologic anatomical studies of Fransilla and colleagues in Helsinki, which had been published in *Cancer* during 1985 (4). And reminiscent too of the observations on "benign metastases from thyroid malignancies" published twenty one years ago by TB Schwartz in the *Lancet* (5).

Pursuit of consensus in the presence of potential conflict of interest and in the absence of presently published prospective controlled therapeutic trials for FCDC

And into this present era of FCDC patients being hotly pursued by both endocrinologists and medical oncologists, and during a time when it could appear that the notion of a management program, consisting of surgery followed by thyroxine supplementation and increasing use of radio-iodine remnant ablation (RRA), has not much changed in almost four decades, and no prospective multicenter controlled trials have ever been performed, would come in the year of 2006 the apparent "voices of authority" from the Thyroid Associations of both the United States (6) and Europe (7).

As stated by the ATA Guidelines Taskforce in their February 2006 Thyroid contribution, other groups (the AACE, AAES, BTA, RCP, NCCN and SSO) had previously developed guidelines "which have provided somewhat conflicting recommendations because of the lack of high-quality evidence from randomized controlled trials" (6). The ATA taskforce used a strategy similar to that used by NIH for its Consensus Development Conferences and "developed a series of questions pertaining to thyroid nodule and thyroid cancer diagnosis and treatment". They admitted that "given the paucity of randomized controlled trials in the treatment of thyroid cancer, the panel relied on all the available published evidence." The taskforce described in their methods section their meetings in January through June 2005, and their painstaking attempts to review and categorize "all English-language papers published between 1995 and December 2004", while also being supplied with "relevant review articles, book chapters, and pre-1995 articles". However, they were forced to admit that "when evidence was judged to be insufficient, the taskforce members also relied on their experience and judgment to answer the questions that had been posed" (6). After categorizing the published data using modified criteria adopted from the U.S. Preventive Services Task Force (USPSTF), the task force then "made specific recommendations using the schema proposed by the USPSTF". Under this scheme, a rating of A was based on "good evidence that the service or intervention can improve health outcomes", a B rating was based on "fair evidence", while C was a recommendation "based on expert opinion".

Perhaps symptomatic of the time in which we live, the third page of the resulting 33 page document documented that seven (70%) of the ten authors, selected by the ATA, had "financial interests, arrangements, or affiliations" with either the manufacturers of rhTSH or levothyroxine. When the ATA group considered and gave a rating B for a recommendation (R37 out of a total of 85) of a role for rhTSH stimulation, as an alternative to thyroxine withdrawal, in preparing patients for RRA, (a role that to date is not yet approved by the FDA in the USA), it was noted in the text that "because of varying degrees of involvement with the manufacturer of rhTSH, five (of 10) authors recused themselves from the discussion of this recommendation" (6).

Initial invited British and European editorial responses to the American Guidelines

Jayne Franklyn (8), in an invited accompanying editorial to the ATA Guidelines publication, noted that in the ATA manuscript "what is striking is the relative paucity of new (and old) evidence enabling the Taskforce to give grade 'A' recommendations (i.e. strong recommendations based on consistent results from well designed and conducted studies)". Indeed, of the 65 recommendations for the treatment of FCDC, only 6 (9%) were awarded an 'A' rating. Professor Franklyn further commented that "the lack of prospective evidence means that there is inevitably heavy reliance on carefully documented and detailed, but often retrospective evidence, and reliance on consensus opinion and experience" (8). Commenting on the ATA recommendation (at level 'B') that HRTS "should be performed in all patients with one or more suspected nodules" (6), she noted that "while intuitively it would seem important to detect (and treat) early-stage disease, proof of this principle is still awaited in the context of this generally slow-growing and relatively 'benign' malignancy" (8). She contrasted the taskforce's recommendation with the UK guidelines, which "presently recommend against initiation of thyroid imaging (including ultrasound) by the primary care practitioner before referral to the specialist (in order to avoid delay) and recommend against routine use of ultrasonography in the specialist setting [in favor of immediate use of fine needle aspiration cytology]" (8).

In his invited commentary on the ATA Guidelines, Furio Pacini (9), himself the lead author of the subsequently published ETA Guidelines, opined that "in the last ten years or so, the clinical presentation of differentiated thyroid cancer has been changing from advanced cases requiring intense treatment and surveillance to sub-clinical cancers detected by fortuitous neck ultrasound requiring less aggressive treatment and follow-up". He also noted that "these considerations dictate the need for applying the more appropriate, less invasive, and less

expensive procedures able to guarantee the best management and the best quality of life for a disease that in view of its intrinsic low mortality has to face with life-long follow-up" (9). In describing the 2006 ATA Guidelines, he concluded that the result of their considerations was a "text clear, concise, easy to read and, most of all, easy to be applied in daily practice" (9). In the following overview of the ATA Guidelines, commentary will be restricted to those recommendations that are directly relevant to the initial management and follow-up of PTC patients, who now represent on both sides of the Atlantic the vast majority (80-95%) of FCDC patients. And, as Pacini (9) has commented, it will be of relevance to determine in future years whether such guideline documents, published in 2006, will prove to have served the dual objectives of "improvement in healthcare outcomes....and promotion of cost-effective strategies" .

ATA recommendations for initial surgical management of PTC

The ATA manuscript consists of 20 printed pages of text, 301 references, 2 tables and 4 figures. The taskforce made 85 recommendations, 65 of which related to FCDC management. Given the immensity of this text described by Pacini as "concise" (9), one has to marvel at the remarkable skill of our forefathers in so briefly summarizing the so-called Ten Commandments! As stated above, in considering the ATA guidelines, this overview will be restricted to the initial therapy and the broad principles of the postoperative surveillance of patients with papillary carcinoma. Indeed, with relevance to the ATA guidelines, the following discussion, for reasons of required length restriction, will be limited to only 13 (20%) of the 65 recommendations made for differentiated thyroid cancer.

Further to the point raised by Dr Franklyn (8), in relation to HRTS and NTD patients, it is perhaps of relevance that the first recommendation of the ATA in the initial management of FCDC is to recommend (R21) with a B rating that preoperative neck HRTS be performed in "all patients undergoing thyroidectomy for malignant cytologic findings on biopsy"! Although at Mayo we strive unsuccessfully to achieve this in all operated PTC cases (10), such a procedure is certainly not being performed regularly in most North American institutions during 2006-7. The A-rated recommendation (R26) of a "near-total or total thyroidectomy for most patients with thyroid cancer" seems hardly surprising, although recommending lobectomy alone as "sufficient treatment" for "small low-risk isolated intrathyroidal" node-negative PTC seems rather extraordinary in the face of prior published reports (11, 12, 13) and the aggressive nature of the ATA taskforce's subsequent recommendations.

It seems likely that the AAES representative on the taskforce (13) was responsible for the B-rated recommendation (R27) that "routine central compartment (level VI) neck dissection should be considered" for PTC patients. This is thought currently at Mayo to represent optimal surgical care (15), but in most academic centers of surgical excellence in the USA this is not the presently expected standard of care. Indeed, one wonders whether many North American academic thyroidectomists would be willing or able to add a central neck dissection to their routine initial surgical treatment of PTC! Less controversial was the recommendation (R28) of lateral neck compartment dissection (16) for biopsy-proven nodal metastases, "especially when they are likely to fail radioactive iodine treatment based on lymph node, size, number, or other factors" (6). The final recommendation relevant to initial surgery (R30) advised a second surgical procedure, namely, a completion thyroidectomy, after an initial lobectomy for all PTC patients "except those with small (<1cm) intrathyroidal node-negative low-risk tumors".

With regard to postoperative staging, the ATA recommended (R31) with a B-rating the use of AJCC/UICC staging, while also recognizing the utility of "postoperative clinicopathologic staging systems to improve prognostication and to plan follow-up". The taskforce acknowledged that schemes such as AGES, AMES, and MACIS (17) permit accurate identification of the majority of patients who are at low-risk of death from PTC, "allowing the follow-up and management of these patients to be less intensive than the higher risk minority, who may benefit from a more aggressive management strategy".

Remnant ablation for patients with papillary thyroid carcinoma

With regards to postoperative radioiodine remnant ablation (RRA), the ATA taskforce stated that the reported advantage of reducing tumor recurrence (TR) and cause-specific mortality (CSM) in PTC "appears to be restricted to patients with larger tumors (>1.5 cm) or with residual disease after surgery, while lower risk patients do not show evidence for benefit". However, rather than advocating a selective use of RRA for only higher-risk patients, the ATA recommended with a B-rating (R32) that RRA be performed in "patients with stage III and IV disease (AJCC 6th edition), all patients with stage II disease younger than age 45 years (Any T Any N M1) and most patients with stage II disease 45 years or older (T2N0M0), and selected patients with stage I disease, especially those with multifocal disease, nodal metastases, extrathyroidal or vascular invasion, and/or more aggressive histologies" (6).

Franklyn (8) has pointed out that the UK guidelines presently argue for "almost routine" RRA for FCDC, and she considers the ATA approach, when compared to the UK, to be "more conservative, especially in selected patients with stage I disease". Since studies from Mayo (18, 19) and the NTCTCSG (20) have convincingly demonstrated a lack of efficacy in terms

of reducing either TR or CSM in low-risk PTC patients, and a European consensus report recommended in 2005 that RRA “should be selective, given that uncertainty persists concerning its benefits” (21), it seems disappointing that the ATA taskforce did not advocate a more selective use of RRA, confining its use to patients in the high-risk category.. Under the proposed ATA guidelines, the only PTC patients avoiding RRA would be those stage I patients with disease confined to the neck (M0), who were either aged <45 years with unifocal (any tumor size) intra-thyroidal node-negative (T1 or T2N0) PTC and favorable histology, or were aged 45 or more with a PTC of 2cm or less (T1), that would be unifocal, intra-thyroidal and node-negative. Thus, of patients with PTC having an initial near-total or total thyroidectomy with curative intent, one would estimate that approximately 70% would be submitted to RRA, although all current staging and scoring systems would identify the high-risk minority, who could potentially benefit from RRA, to be only about 15 to 20% of PTC cases!

Such a draconian recommendation seems all the more extraordinary, since three members of the ATA taskforce presented both in 2001 and again at the 2nd annual ATA Spring Meeting in 2005 the results of a registry study (with data gathered during 1986-2001 from 11 North American institutions, including Johns Hopkins, NIH, MGH, MD Anderson and University of Colorado) that was “unable to show any impact, positive or negative, of specific therapies in stage I patients” (20). Indeed, these same authors published in *Thyroid*, ten months after the Guidelines were distributed, the remarkable conclusion that “postoperative RAI therapy does not provide significant benefit in stage I patients, and could even be harmful” (20)!

Perhaps somewhat less controversial were the B-rated recommendations (R35) that the “minimum activity necessary to achieve successful remnant ablation (30-100 mCi) should be chosen” in low-risk patients, that (R38) patients should have a low iodine diet for 1-2 weeks prior to RRA, and (R39) that after RRA a posttherapy scan should be performed at 5-8 days after the therapeutic dose is administered. And, as was discussed above, the ATA (R37) did recommend with a B-rating that RRA could be performed “following thyroxine withdrawal or rhTSH stimulation”, despite the fact that in the United States, as against Europe, rhTSH was not at that time approved for such an indication.

ATA recommendations for followup of patients with DTC

Possibly the most interesting question posed in the ATA Guidelines manuscript was “what is the appropriate method of following patients after surgery with or without remnant ablation”? Under this heading comes a listing of criteria for absence of persistent tumor relevant to patients undergoing total or near-total thyroidectomy and RRA, which reads as: “Disease free status comprises all of the following: no clinical evidence of tumor, no imaging evidence of tumor (no uptake outside the thyroid bed on the initial posttreatment whole body scan, on a recent diagnostic scan or neck ultrasound), and undetectable serum thyroglobulin levels during TSH suppression and stimulation in the absence of interfering antibodies (Figs. 2 and 3).” Figures 2 and 3 in the Guidelines document depicted algorithms for initial (1-3 months after surgery) and longer term (6-12 postoperative months) follow-up of DTC patients.

These complex algorithms, along with fig 4 on empiric radioiodine therapy, occupy three full pages (approximately 20%) of the portion of the ATA guidelines paper devoted to thyroid cancer, and apparently were “modified and reprinted with permission” from the July 2005 issue of the *Journal of Nuclear Medicine*. Curiously, neither the author nor the title of this paper was identified on the accompanying twenty line Fig. 3 legend, which does give full details of the authors and titles of four other papers!

My curiosity thereby piqued, I searched through the 301 references of the guidelines manuscript to try to identify the full details of this paper, where I found it to be unlisted. Subsequent web searching led to the discovery that these three algorithms were derived from an “Invited Perspective” contribution, written by one of the taskforce members in April 2005 to accompany a Chinese meta-analysis, and entitled “Empirically treating high serum thyroglobulin levels” (22). This review described surveillance paradigms that the author noted to have changed substantially in the past five years, “sparked mainly by the introduction of rhTSH into clinical practice” (22). The author stated in his review that “the most common scenario for referral to my clinic is an rhTSH-stimulated Tg level of >2mcg/L with negative findings for tumor”, and he went on to define what he described as “my approach to treating patients with high serum Tg levels and negative imaging studies” (22).

Considering that this ten person taskforce prided itself on mastering the 1995-2004 literature, and grading the strength of the panelists’ recommendations according to “all the available published evidence”, it would seem somewhat extraordinary that the only management algorithms in this ATA document (6) would prove to be “modified and reprinted” from a non peer-reviewed “invited perspective” (22)!

Serum Tg measurements and appropriate imaging in followup of PTC

With regards to the role of Tg in follow-up, the taskforce (R43) advised with an A-rating that serum Tg should be measured every 6-12 months in patients who had undergone near-total thyroidectomy and RRA. The recommendations were less clear (awarded only a C-rating) for patients who had less than total thyroidectomy, or had a total thyroidectomy but no

RRA. The panel suggested that “periodic serum Tg measurements should be considered”, but they intimated that “the cutoff levels to detect tumor during TSH suppression or stimulation are not known, but unstimulated or stimulated levels greater than 2 ng/mL that increase over time may represent recurrent disease” (6).

The final recommendation (R45) relevant to Tg measurement in low-risk ablated patients with “negative cervical ultrasound and TSH-suppressed thyroglobulin 6 months after treatment” seems a little puzzling, as it states: “serum thyroglobulin should be measured after thyroxine withdrawal or rhTSH stimulation approximately 12 months after the ablation to verify the absence of disease. The timing or necessity of subsequent stimulated testing is uncertain for those found to be free of disease”. This is certainly not a practice that I will be performing in upcoming years, as I find the notion of measuring Tg after thyroxine withdrawal quite outmoded and barbaric. Moreover, for years our group have accepted that, akin to the prediction of TRH-induced TSH increments using high sensitivity thyrotropin assays, little relevant information is gained from rhTSH- stimulated Tg testing if the patient has no Tg antibodies, and one has familiarity with a highly sensitive Tg assay, with a functional sensitivity around 0.1 ng/mL (23). The definition of an “undetectable” Tg is forever changing, but a Tg <0.1 ng/mL in a low-risk PTC patient on adequate thyroid hormone suppressive therapy certainly provides reassurance to most of the endocrinologists and surgeons at our institution (24).

The penultimate A-rated recommendation related to the role of diagnostic whole body radioiodine scanning in low-risk patients. Here the ATA recommend (R46) that “after the first RxWBS performed after radioiodine remnant ablation, low-risk patients with negative TSH-stimulated thyroglobulin and cervical ultrasound do not require routine DxWBS during follow-up.” The ATA taskforce do admit that “cervical metastases occasionally may be detected by neck ultrasonography even when TSH-stimulated serum thyroglobulin levels remain undetectable” (25). Not surprisingly, therefore, they recommended (R48) that “after surgery, cervical ultrasound to evaluate the thyroid bed and central and lateral cervical nodal compartments should be performed at 6 and 12 months and then annually for at least 3-5 years, depending on the patient’s risk for recurrent disease and thyroglobulin status” (6).

Thyroid hormone suppression and management of regional and distant metastases

For the degree of thyroid hormone suppression, the ATA had three recommendations (R49-51). For persistent disease, a TSH below 0.1 mU/L indefinitely was advised (B-rating). For high-risk patients presently free of disease, a TSH between 0.1 and 0.5 mU/L for 5-10 years was recommended (C-rating). And for low-risk patients presently free of disease, the taskforce recommended with a C-rating that the serum TSH “may be kept within the low normal range (0.3 to 2 mU/L)”. For the next 34 recommendations, mainly relating to the management of patients with locoregional recurrence and distant metastases, and the role of radiotherapy and chemotherapy, there was only one last A-rated recommendation. This related to the treatment of lung metastases, particularly those with pulmonary lesions smaller than 1 cm diameter. Such “micrometastases should be treated with radioiodine therapy, repeated every 6-12 months as long as disease continues to respond, as the highest rates of complete remission are reported in these subgroups”.

The taskforce concluded their report with a statement regarding directions for future research. In discussing small cervical lymph node metastases, they conceded that cervical nodal metastases did comprise the majority of all tumor recurrences. Their final statement was that “future research must be directed to develop techniques to identify small cervical metastases, which in a substantial number of cases progress to overt, clinically significant metastases” (6).

Practical implications for PTC patients and providers

Now, the real question is: will these ATA Guidelines help us to steer the best course and provide “benefit for the individual patient” with PTC, that we may encounter in our clinics and hospitals in future days during 2007 and beyond? Clearly, more ultrasound neck exams are going to be performed, and more total thyroidectomies and central neck dissections will be attempted. For sure, more radioiodine will be administered, more serum thyroglobulins will be measured, and more and more rhTSH will be prescribed! There will inevitably be a significant cost to such proposed changes in practice, and one cannot be at all certain that there will be a resultant decrease in tumor recurrence and cause-specific mortality rates from PTC. But the recommendation that approximately 70% or more PTC patients will be advised to have RRA should be of particular concern.

Table 1. Actual and Proposed Rates of RRA after Successful Near-Total or Total Thyroidectomy for Localized Papillary Thyroid Cancer

	ACTUAL		PROPOSED	
	Mayo 2000 (n=50)	1970-1999 (n=1,269)	ATA (2006)	BTA / RCP (2001)
Ablated	36%	45%	69%	71%
Not Ablated	64%	55%	31%	29%

If we consider PTC patients who have undergone an optimal primary surgery (near-total or total thyroidectomy) and at the procedure's end there is no gross residual disease, then, at the Mayo Clinic, Rochester, during three decades (1970-99), only 45% of such patients received RRA within 6 postoperative months (18,19). In 2000, the minority ablated during that year was only 36%. If one applies the exclusion guidelines proposed by the recent Guidelines of either the ATA (6) or the British Thyroid Association (8), [who would ablate all PTC patients with a primary tumor > 1cm diameter], to our 1970-2000 cohort of optimally treated PTC patients, one would see the rates for RRA as outlined in Table 1. Compared to Mayo's past prescribing habits, almost 100% more patients would be ablated currently, if the new Guidelines were closely followed. Since neither the Mayo (18, 19) nor the NTCTCSG (20) data can demonstrate improvement in either tumor recurrence or cause-specific mortality rates with RRA, especially in low-risk patients, such an escalation of aggressive postoperative adjunctive therapy can hardly be justified. Indeed, one must seriously doubt whether the proposed increased use of RRA and the increasing evaluation of rhTSH-stimulated thyroglobulin levels will either be cost-effective, or lead in future years to improved outcome results for patients with PTC, the commonest endocrine cancer.

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APPLICATION OF RECOMBINANT HUMAN TSH IN THE DIAGNOSIS AND TREATMENT OF THYROID DISEASES

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Production of rhTSH

TSH is a pituitary glycoprotein composed of an α -subunit common to gonadotropins and a hormone-specific β -subunit. Once the β -subunit of the human TSH gene has been cloned, the encoded protein could be over expressed in a cell system (Chinese hamster ovary cell), by transfection with human α - and β -subunits of complementary or genomic DNA (1,2). With this technique, high quantities of highly recombinant human TSH (rhTSH; Thyrogen ®) can be obtained with the same biological properties as native TSH (3).

In vitro model systems were initially used to test the effects of rhTSH on thyroid function. In a human fetal thyroid cell system, rhTSH is able to activate TSH receptor and to induce Tg secretion and thyroid epithelial cell proliferation (4). Several preclinical studies were conducted in animals to evaluate the pharmacological and toxicological potential of rhTSH. The trials included single-dose and repeat-dose studies in primates and rodents (5, 6). Subsequent studies examined the effects of rhTSH on normal human thyroid function. A single injection of rhTSH was a potent stimulator of the release of T₄, T₃ and thyroglobulin (Tg) and was able to increase thyroid radioactive iodine uptake in normal subjects (7-9).

Clinical use of rhTSH

After its development, rhTSH has been extensively tested in several clinical indications. The main applications are reviewed below.

1. Follow-up of differentiated thyroid cancer
2. rhTSH-aided post-surgical thyroid ablation with radioiodine
3. rhTSH-aided radioiodine therapy for the treatment of metastatic disease
4. Use of rhTSH for improving radioiodine therapy of nontoxic multinodular goiter
5. Other potential use

1. Follow-up of differentiated thyroid cancer (DTC)

The availability of large quantities of rhTSH prompted clinical trials in patients with differentiated thyroid carcinoma. Phase I-II dose-finding and pharmacokinetic studies were conducted (10) and followed by two phase III studies (11,12). A first study (phase I/II) was completed in 1994 in 19 patients after a recent thyroidectomy for differentiated thyroid cancer. The study protocol compared the stimulation of ¹³¹I uptake and the release of serum Tg after rhTSH administration (0.9-3.6 mg for 1-3 days) and after T₃ withdrawal.

The quality of thyroid scan and the number of sites of abnormal uptake coincided in rhTSH and in hypothyroidism scans in 12 (63%) patients. Serum Tg levels increased more than 2-fold in response to rhTSH in 73% but was significantly lower than that observed after thyroid hormone withdrawal (93%). The encouraging results of this study were confirmed in a larger multicenter phase III study conducted between 1992 and 1995 in the USA in thyroid cancer patients (11). The primary objective of this multicentric study was to compare ¹³¹I whole body scan (WBS) performed after rhTSH injection to that obtained after withdrawal hormone therapy. The study showed that rhTSH stimulated radioiodine uptake and resulted in significantly fewer symptoms of hypothyroidism. However, in 23% of patients the scan performed after rhTSH was less sensitive for detecting residual or recurrent disease than the scan performed in hypothyroidism. A second phase III multicentric trials including US and European centres (12) was designed to compare two different dose regimens of rhTSH (2 injection of 0.9 rhTSH for 2 consecutive days or 3 injections of 0.9 mg rhTSH, 3 days apart). The trial enrolled 229 patients and the results of the study demonstrated that when using rhTSH as an alternative to hypothyroidism, the combination of WBS and Tg testing had 100% sensitivity for detection of thyroid cancer metastases. No significant difference was seen between the two and the three dose arms of the study.

All together, these trials have clearly shown that rhTSH is an effective and safe alternative to thyroid hormone withdrawal during the post-surgical follow-up of differentiated thyroid cancer. As a result, rhTSH obtained regulatory approval in late 1998 as a diagnostic methodology in North America by the Food and Drug Administration and in 2001 in Europe by Evaluation of Medicinal Products. The recommendations are to administer rhTSH 0.9 mg for two consecutive days, followed by a tracer dose (4 mCi of ¹³¹I) 24 hours after the last injection of rhTSH and a diagnostic WBS and Tg measurement 48 hours after the tracer dose of ¹³¹I. After the approval, a number of investigators have published their clinical experience with rhTSH for diagnostic monitoring of patients with differentiated thyroid cancer (13-18) confirming the safety and efficacy of rhTSH.

Although serum Tg and diagnostic WBS have been routinely performed in the follow-up of DTC patients, some authors advocate monitoring the stimulated Tg alone for the detection of recurrent or persistent disease in low-risk patients, based on evidence that serum Tg levels is more sensitive for disease detection than diagnostic WBS (19-20). Pacini et al (17) reported similar results when comparing rhTSH-stimulated Tg levels with hypothyroid Tg levels and diagnostic WBS in DTC patients. Similar results were reported in subsequent studies (21,22). However, few patients with evidence of lymph node metastases have falsely negative undetectable rhTSH stimulated Tg (<1.0 ng/ml) but are identified by neck ultrasound. Thus, the combination of rhTSH-Tg plus neck ultrasound provides the best sensitivity (96.3%) and negative predictive value (99.5%) for the detection of persistent/recurrent cancer (18).

Recently, both American Guidelines and European Consensus for the management of DTC patients recommend that rhTSH stimulated Tg levels (in patients with negative Tg antibodies) plus neck ultrasound represent an adequate tool for the follow-up of low-risk patients (23,24).

2. rhTSH-aided post-surgical thyroid ablation with radioiodine

Initial treatment of DTC patients consists of thyroidectomy followed by ¹³¹I thyroid remnant ablation. The rationale for thyroid remnant ablation is to decrease the risk of clinical tumor recurrence (25) and improve the sensitivity and specificity of follow-up testing with periodic serum thyroglobulin measurement and radioiodine scanning (26). Traditionally, withdrawal of thyroid hormone has been used to optimize the trapping and retention of radioiodine by increasing endogenous TSH levels. Since 1995, rhTSH has been employed in clinical trials for post-surgical thyroid remnant ablation in DTC patients. Early studies, using a fixed dose of 30 mCi of ¹³¹I, suggested that the use of rhTSH in LT4 substituted subjects was less efficient than in hypothyroid subjects (27,28). A recent study, using a different protocol comparing the effect of ¹³¹I therapy in hypothyroidism or rhTSH in LT4 substituted subjects found no significant difference in the rate of successful remnant ablation between rhTSH stimulation (81.2%) and hypothyroidism preparation (75%) (29). An international, randomized, multicenter, controlled study was designed to investigate whether preparation of patients with rhTSH while on LT4 therapy was equivalent to preparation by LT4 withdrawal. This study demonstrated comparable remnant ablation rate (100%) in patients prepared for ¹³¹I remnant ablation with 100 mCi by either administering rhTSH or withholding thyroid hormone. rhTSH-prepared patients maintained a higher quality of life and received less radiation exposure to the body. In addition, this study demonstrated that the additional iodine content of pills did not produce interference with successful ablation (30). Based on these results, on February 2005, the use of rhTSH as a preparation for post-surgical thyroid ablation has been approved by European Medicine Agency (EMA) in low-risk DTC patients using 100 mCi of ¹³¹I.

3. rhTSH-aided radioiodine therapy for the treatment of metastatic disease

Although not approved for the use in the treatment of DTC metastases, rhTSH has been employed in over 100 patients with metastatic disease as preparation for ¹³¹I therapy. A systematic review (31) of these patients demonstrated that significant radioiodine uptake in post-therapy WBS obtained after rhTSH-aided ¹³¹I therapy was present in 75% of the cases and that 36% of them had positive response from this modality (complete or partial remission/stabilization). Although insufficient for final conclusion these preliminary data may be a rationale for future prospective studies.

4. Use of rhTSH for improving radioiodine therapy of nontoxic multinodular goiter

Patients with nontoxic multinodular goiter require treatment when compressive symptoms are present. The treatment of choice is usually thyroidectomy but radioactive iodine has been occasionally employed with significant effect in reducing thyroid volume and ameliorating symptoms and signs of compression (32). In recent years rhTSH has been used in patients with multinodular goiter with the aim of increasing the uptake of radioiodine in the goiter, particularly in the "cold nodules". Huysmans et al (33-35) demonstrated that administration of a single-low (0.01 or 0.03 mg) dose of rhTSH considerably increased the RAI uptake in patients with multinodular goiter and caused a more homogeneous distribution of radioiodine within the goiter stimulating ¹³¹I uptake in relatively "cold areas". One year after treatment mean thyroid volume was approximately

reduced by 40%. Other studies, using high dose of rhTSH demonstrated significant goiter reduction but also the occurrence of severe, transient hyperthyroidism after 131-I therapy (36-40). Based on these results, pre-treatment with rhTSH seems a promising alternative to thyroid surgery for the treatment of non toxic multinodular goiter. However the optimal dose and timing of both rhTSH and 131-I as well as the criteria for eligible patients remains to be determined in randomized controlled studies.

5. **Other potential use**

- *To increase diagnostic sensitivity of FDG-PET*

2-[¹⁸F] Fluoro-2-Deoxy-D-Glucose-Positron Emission Tomography Scanning (FDG-PET) is indicated in selected patients with biochemical evidence (elevated serum Tg levels) of metastatic disease but negative imaging (41). In vitro studies showed that rhTSH increases FDG uptake by enhancing glucose transport and glycolytic activity in cultured thyrocytes (42, 43). Subsequently Chin et al (44) confirmed these findings in vivo and showed that rhTSH can increase the sensitivity and specificity of FDG-PET for localization of metastatic disease in DTC patients.

- *To potentiate the cytotoxic effect of chemotherapy*

Chemotherapy represents the only therapeutic option in most poorly differentiated carcinoma, although its effect is limited and short lasting. The cytotoxic activity of chemotherapeutic drugs is maximal when tumor cells are proliferating but commonly DTC patients are treated with suppressive doses of LT4 to block the TSH-stimulated cell proliferation. In this condition, the efficacy of chemotherapy may be limited. An experimental trial in patients with poorly differentiated thyroid cancer provided preliminary evidence that delivering cytotoxic drugs in metastatic thyroid cancer patients under elevated levels of either exogenous (rhTSH) or endogenous TSH levels enhanced the rate of positive response to the chemotherapeutic regime (45).

- *rhTSH in differential diagnosis of congenital hypothyroidism (CH)*

Recently rhTSH has been successful tested in the differential diagnosis of different form of CH. Injection of rhTSH produce serum Tg elevation and radioiodine uptake in ectopic thyroid or dyshormonogenetic goiter while no results was observed in patients with thyroid agenesis. (46).

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The McCune-Albright syndrome: a short review

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Abstract

The McCune-Albright syndrome (MAS) is a rare, sporadic disease characterized by a classical triad of clinical signs: polyostotic fibrous dysplasia, skin hyperpigmentation and endocrine dysfunction. The disease is caused by postzygotic, somatic mutations at codon 201 of the *GNAS1* gene that results in cellular mosaicism, thus leading to a broad spectrum of clinical manifestations. The major endocrine disorders include autonomous hyperfunction of several endocrine glands, such as gonads, thyroid, pituitary and adrenal cortex, i.e. glands sensitive to trophic agents acting through cAMP dependent pathway. Since specific treatment is required, the prognosis depends on the severity of each individual endocrine and non-endocrine manifestation. As mutation detection rates may vary considerably according to the type of tissue analyzed and the detection method used, sensitive and specific molecular methods must be used to look for the mutation from all available affected tissues and from easily accessible tissues, particularly in the presence of atypical and monosymptomatic forms of MAS. This review will briefly summarize the clinical manifestations and the most recent data on genetics and molecular diagnostic of the disease.

Introduction

The McCune-Albright syndrome (MAS) was described independently by Fuller Albright and Donovan McCune in 1937 (1,2). The classical triad of MAS, firstly reported in 1937 and subsequently confirmed in several reports, consists of polyostotic fibrous dysplasia (FD), skin hyperpigmentation (cafe-au-lait spots), and endocrine dysfunction (3-5). Typical endocrinopathies are precocious puberty, especially seen in females, hyperthyroidism, growth hormone excess, hyperprolactinemia, and hypercortisolism. The onset of these manifestations is usually during infancy and childhood. Since specific treatment is required, the prognosis depends on the severity of each individual endocrine manifestation. Among non-endocrine manifestations, fibrous dysplasia of bone (FD) is mostly polyostotic and frequently causes fractures needing surgical and orthopedic treatment. Since previous studies have suggested the overall prognosis of patients with McCune-Albright syndrome to be non-fatal, recent data have drawn the attention to non-endocrine affections, including hepatobiliary dysfunction and cardiac disease, which are probably important risk factors for early death (6).

Patients with MAS display mosaicism of activating somatic mutations of the gene encoding the alpha-subunit of Gs (*GNAS1*) (7,8). Mutations of *GNAS1* have been detected in all affected subjects and Arg²⁰¹ is the only location so far reported. Mutant Gsa is expressed in the affected endocrine organs, as well as in tissues not classically involved in MAS, the highest proportion of mutant alleles being found in regions of abnormal proliferation. This mosaic distribution is consistent with the hypothesis that this syndrome is due to a somatic mutation in Gsa gene occurring as an early postzygotic event. Thus, the clinical presentation of each individual is dependent on the particular distribution of affected cells, causing a broad spectrum of endocrine and non-endocrine manifestations, ranging from one or two mild clinical signs with excellent long-term prognosis to a severe life-threatening multiorgan

disease.

This review will briefly revisit the clinical manifestations of the disease (summarized in table 1), as well as recent data on genetics and molecular diagnostic.

Table 1

Disorder	Prevalence in MAS	F:M
Precocious puberty	64-79% (F), ~15% (M)	F>M
Thyroid dysfunction	2.7-21.9%	F>M
GH/PRL hypersecretion	up to 21%	F=M
Cushing's syndrome	up to 5%	F=M
Fibrous dysplasia	46-98%	F=M
Skin hyperpigmentation	53-92%	F=M
Hyperphosphaturic hypophosphatemia	~ 50%	F=M

Endocrine dysfunction

Precocious pseudopuberty

Precocious puberty is the most frequent endocrinopathy in females with MAS and it was described in the first reports as a hallmark of the disease (1,2). It is estimated that its prevalence ranges from 64 to 79% in girls, whereas it is much rarer in boys (15%) (reviewed in ref. 5). Pubertal signs often onset before the age of 4 years and, typically, they are characterized by alternate phases of sexual maturation progression and regression. Ovaries of affected girls show multiple autonomous follicular cysts that secrete estrogen and are therefore accompanied by pre-pubertal levels of serum gonadotropins. Curiously, these cysts spontaneously enlarge and regress, thus explaining the atypical clinical course of hyperestrogenism. It is also frequent to observe a precocious shift from a GnRH-independent puberty to a GnRH-dependent puberty. Independently from the cause, precocious puberty often leads to definitive short stature (5,9-12).

In boys, precocious activation of Leydig cell androgen secretion results in pubertal spermatogenesis, leading to testicular enlargement, and in the development of secondary sex characteristics. However, sexual precocity is rare in MAS males while isolated testicular enlargement is frequently observed. In a boy with macro-orchidism and signs of Sertoli cell hyperactivity but no signs of hyperandrogenism, microdissection of a testicular biopsy demonstrated that the mutated GNAS1 allele was present only in Sertoli cells, resulting in isolated Sertoli cell hyperfunction (13,14). Lack of occurrence of the mutation in Leydig cells may explain why sexual precocity is rarely observed in boys with MAS.

Thyroid disorders

Nodular and diffuse goiter, with or without hyperthyroidism, has been reported in association with MAS and, taken together, these abnormalities represent the second most endocrinopathy associated with MAS. Pathophysiology consists of autonomous production of thyroid hormones due to GNAS1 mutations that activate the TSH-mediated intracellular signaling, thus determining both thyroid hyperfunction and growth. Thyroid disorders are generally more common in girls than in boys, and the spectrum of clinical presentation ranges from asymptomatic subclinical hyperthyroidism to thyrotoxic crisis. Thyroid dysfunction, like that of the ovaries, is associated with structural abnormalities in the gland itself, together with suppressed levels of the respective stimulating hormone. Its prevalence has been overall estimated from 2.7 to 21.9% (5). The largest review of thyroid disorders in MAS includes all the cases reported in the literature since the first report in 1936 up to 1997, and it includes 64 cases, 41 females and 23 males, with a median onset age of 10 years (range: birth to 48 years) (15). Ultrasonography revealed thyroid abnormalities in most patients, with nodular goiter in 19 subjects (14 with and 5 without hyperthyroidism), diffuse goiter in 23 patients (20 with and 3 without hyperthyroidism), and 22 cases with subclinical or clinical hyperthyroidism without thyroid enlargement (15). Overall, the prevalence of a real goiter is still controversial, ranging from very low rates to 71.4% (5,15,16). The reason why the same GNAS1 mutation, that presumably affects all thyrocytes, may determine such variable clinical manifestations either in thyroid function and in thyroid growth is still unknown.

The differential diagnosis between MAS-related thyropathies and other forms of neonatal/juvenile hyperthyroidism are summarized in Table 2 (17).

Table 2:

	MAS-related Hyperthyroidism	TSH-receptor Mutations	Neonatal autoimmune hyperthyroidism
Familiarity	No	Yes	Yes (mother)
Age of onset	Neonatal/Infancy/Adulthood	Neonatal	Neonatal, transient
Autoimmunity	Absent	Absent	Present
Associated syndrome	Present: MAS	Absent	Absent
Thyroid size	Normal/Diffuse goiter/ Multinodular goiter	Diffuse goiter	Diffuse goiter
Hyperthyroidism	Absent/Present	Present	Present

Interestingly, a report by Feuillan and colleagues described 8 girls with hyperthyroidism and MAS in whom only tri-iodothyronine was elevated in the presence of normal thyroxine levels, postulating a high deiodinase 2 activity in MAS (16). This hypothesis has not been demonstrated so far and the pathophysiology of this phenomenon does not seem clear anyway. There are no data on the natural history of the disease, since treatment is always started in symptomatic disease. A definitive therapy such as thyroidectomy or radioactive iodine is often required, since hyperthyroidism tends to recur after antithyroid drug withdrawal (15,18).

Finally, two cases of thyroid carcinoma arisen from a nodule bearing the *GNAS1* activating mutation have also been reported in patients with MAS (19).

Growth hormone (GH) and prolactin (PRL) excess

GH hypersecretion in MAS was first described by Scurry and colleagues in 1964 (20). Since then, most of the data on GH excess in MAS come from single case reports, with the exception of one large series including 12 patients (21), with therefore non-definitive data on this aspect of the disease (22). According to the largest study, the incidence of GH excess among patients with MAS has been assessed as up to 21% with an equal sex ratio (21). The age of onset is usually adolescence, before the age of 20 years, thus leading to either acromegaly or gigantism depending on bone age. The clinical expression of GH excess can be masked because of precocious puberty or craniofacial fibrous dysplasia, indicating the necessity for routine screening in all MAS patients. Hyperprolactinemia is an accompanying finding in most patients (>80%), whereas it has never been reported as an isolated form (23). The pathogenesis of GH hypersecretion in MAS is not completely understood as many findings, such as GH responsiveness to GHRH and preserved nocturnal GH increase, seem to indicate abnormal hypothalamic GHRH release rather than primary pituitary dysfunction. Moreover, pituitary tumors have been found in some patients on CT scans or MRI but much less frequently than in non-MAS patients with GH excess. In 1984, Kovacs and colleagues reported mammosomatotropic hyperplasia in the excised pituitary from a 11-year-old girl with MAS (24). The transition from hyperplasia to adenoma could explain the variety of sellar radiographic findings in such patients.

Medical treatment is often the only option in MAS patients with GH excess, as trans-sphenoidal surgery is usually restricted due to both massive thickening of the skull base and the frequent absence of documented pituitary tumors. The use of bromocriptine, cabergoline and octreotide, or the combination of these, has shown variable results, whereas pegvisomant, a GH receptor antagonist, is a new promising option, having been recently tested in a subset of patients with MAS (25,26).

Hypercortisolism

ACTH-independent Cushing's syndrome may occasionally occur in patients with MAS, its prevalence being quite low (around 5%). Cushing's syndrome is diagnosed in most cases during the neonatal period or infancy, often being, when present, the first clinical sign of the disease (27). Patients have the typical clinical and laboratory signs of Cushing's syndrome, including severe effects on bone density, with frequent spontaneous fractures and delayed bone maturation (28). There is evidence of autonomous cortisol secretion due to activating *GNAS1* mutations in the adrenal gland, leading to macronodular adrenal hyperplasia (27). Despite occasional reports on spontaneous resolution, most patients require early adrenalectomy. Recently, treatment with metyrapone has been proposed as an alternative option in these patients (29).

Non-endocrine dysfunction

Fibrous dysplasia (FD)

FD is a focal and benign fibrous bone lesion that was first described in 1942 (30; reviewed in refs. 4&5). While most patients with isolated FD have a single bone lesion, FD associated with MAS is predominantly characterized by multiple lesions (polyostotic fibrous dysplasia) (4,31). Prevalence of FD in MAS ranges from 46-98% with an onset of symptoms mostly during infancy (5). FD lesions are typically found in the long bones of the extremities, the femur being affected in virtually all cases, the ribs, and craniofacial bones while they are rarely found in hands, feet, or spine (31-33). FD lesions may be asymptomatic but often lead to bone deformity, pain and pathological fractures with a peak incidence between 7 and 12 years age (31-33). In addition, cranial nerve compression syndromes due to progressive FD lesions have been reported (34).

FD is a lesion composed mainly of fibrous tissue that originates in the medullary cavity and expands concentrically outward into the surrounding cortical bone through the bone-resorbing activity of osteoclasts, which are present in greater numbers in the periphery (35,36). Most of the cells are immature mesenchymal cells with a spindle-shaped fibroblastic

appearance and express alkaline phosphatase and other osteoblast-specific proteins (36). The involvement of the proto-oncogene *c-fos* and cytokines such as IL-6, with subsequent hyperproliferation and incomplete differentiation of osteoblasts together with osteoclast activation, have been suggested to play a role in the biochemical pathways leading to FD (37,38).

Bisphosphonates have been shown to have therapeutic benefit, presumably due to their antiresorptive activity (reviewed in ref. 39). Rarely, these lesions expand beyond the normal boundaries of the cortical bone or undergo malignant degeneration (4). In any case, there is a tendency of extension and progression of FD lesions, with deformities and physical impairment that often lead to the need of a wheelchair (33).

Skin hyperpigmentation

The café-au-lait spots are one of the most obvious signs of MAS and present as single or multiple tan-brown hyperpigmented flat macules with irregular ("coast of Maine") borders developing during infancy and becoming even more obvious with age or with sun exposure (1,2). Their prevalence in MAS has been estimated from 53.1-92.5% (5). These lesions are often limited to one side of the body, which usually corresponds to the side with bone involvement and generally do not cross the midline. Moreover, they are typically arranged in a segmental pattern, which follows the developmental lines of Blaschko (40). Malignant transformation of these lesions has not been described. Melanocytes cultured from these lesions show increased intracellular cAMP levels, increased numbers of dendrites and melanosomes, and increased levels of tyrosinase, the rate-limiting enzyme for the production of melanin (41).

Hyperphosphaturic hypophosphatemia

Hyperphosphaturic hypophosphatemic rickets or osteomalacia has been associated with MAS and polyostotic FD, with a prevalence of renal phosphate wasting up to 50% of patients (42,43). Increased intracellular cAMP levels in renal proximal tubules leading to decreased phosphate reabsorption, even in the absence of hyperparathyroidism, has been proposed as one potential mechanism for hypophosphatemia in MAS patients, but this hypothesis has not been confirmed (4). In addition to increased phosphate clearance, patients have other evidence of renal tubular dysfunction, including aminoaciduria and mild proteinuria (42). Recently, the factor causing hypophosphatemia in MAS was indicated to be fibroblast-growth factor 23 (FGF-23), although the possibility of some other humoral factors has not been excluded (44,45). Serum levels of FGF-23 are increased in MAS patients with FD compared to normal age-matched controls, and are significantly higher in MAS patients with renal phosphate wasting compared to those without (44).

Phosphate loss leads to profound skeletal effects, such as weakening of the mechanical resistance of bone and promotion of bowing and deformity. Thus, evaluation of phosphate metabolism is mandatory in all patients with MAS.

Other non-endocrine manifestations

The abnormalities in MAS patients are generally restricted to bone, skin, and endocrine organs, and therefore there is little effect on mortality (3,32). However, some patients also develop one or more non-endocrine abnormalities that may markedly increase morbidity and mortality (6). Non-endocrine organs that may be affected in MAS include the liver, heart, thymus, spleen, bone marrow, gastrointestinal tract, and brain (5-7). Liver abnormalities associated with MAS include severe neonatal jaundice and persistently elevated liver enzymes, with histological findings ranging from normal to giant cell hepatitis (6,45). Cirrhosis with liver failure and need for transplantation has been reported in one case, but it was in association with hepatitis C (6). Cardiac abnormalities perhaps represent the major risk factors for long-term survival, their clinical spectrum including hypertension, cardiomegaly associated with atypical myocyte hypertrophy, persistent tachycardia, and sudden infant death (SIDS) (6).

Other rare features of MAS include thymic hyperplasia, myelofibrosis, gastrointestinal polyps, pancreatitis, breast cancer, microcephaly, and other neurological abnormalities (1,2,6). Very recently, testicular microlithiasis in boys affected with MAS was also reported, but the pathogenesis of this defect remains unclear (47).

Genetics and molecular diagnosis

Genetics

Activating mutations in *GNAS1* (the so-called *gsp* oncogene) (48) are known to be involved in the pathogenesis of different human endocrine diseases, such as sporadic endocrine tumors, in particular GH-secreting pituitary adenomas and autonomous thyroid adenomas, and of course MAS. *GNAS1* activating mutations are missense mutations leading to amino acid substitution of either residue Arg201 or Gln227 (Arg201 only in MAS). These two residues are catalytically important for GTPase activity; therefore, these mutations cause constitutive activation by disrupting the signalling turn-off mechanism. Growth and hormone release in many endocrine glands are stimulated by trophic hormones that activate Gsa-

cAMP pathways. As a matter of fact, in MAS endocrine manifestations affect those glands sensitive to trophic agents acting through cAMP-dependent pathway, leading to autonomous hyperfunction of gonads, pituitary, thyroid and adrenal cortex (7,8). Somatic *gsp* mutations are also present in FD lesions from patients with or without other features of MAS (49,50). MAS is virtually never inherited, and germ-line *gsp* mutations are considered lethal (40), although a possible germ-line mutation was reported in one patient with severe manifestations (51).

The human $Gs\alpha$ gene maps on a locus under complex imprinting control with multiple maternally, paternally and biallelically alternatively spliced transcripts (reviewed in ref. 4). Recent reports demonstrated that in the thyroid, the gonad and the pituitary *Gsa* transcription mainly derives from the maternal allele (52-55). Moreover, it has been demonstrated that in most *gsp* positive GH-secreting pituitary tumors the mutation occurs on the maternal allele (52,53), most likely indicating that the same mutations on paternal allele are clinically silent. As a matter of fact, in MAS, gigantism/acromegaly seem to occur only in those patients carrying the activating mutation on the maternal allele, while *gsp* mutations may indifferently lay on the paternal or the maternal allele in all the other patients (56). It is therefore conceivable that paternal mutations in tissues such as thyroid and gonad with variable, but not negligible paternal contribution to *Gsa* expression (about 20-30% in our laboratory, ref. 53), are still able to have clinical significance.

Molecular diagnosis

Accuracy and sensitivity in the molecular diagnosis of MAS is mandatory for optimal therapeutic strategy and adapted follow-up, especially for incomplete clinical forms of MAS. The somatic nature of mutations in the *GNAS* gene in McCune-Albright syndrome and isolated fibrous dysplasia makes their identification often very difficult. Conventional methods for the detection of mosaic mutations of *GNAS* have required polymerase chain reaction analysis of genomic DNA from affected tissues or multiple rounds of tandem polymerase chain reaction and endonuclease digestion to enrich for mutant alleles DNA from other tissues (57). Most recently, a new diagnostic method using peptidic nucleic acid (PNA) primers that specifically block synthesis from the wild type allele, has been tested to detect low copy numbers of mutant *GNAS* alleles in DNA from peripheral blood cells from patients with MAS and FD (58,59). More than 100 patients have been screened so far with promising results, indicating PNA clamping as a rapid, reliable, and economical method to diagnose MAS.

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Pendred's syndrome: from genotype to phenotype

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Introduction

Pendred's syndrome (PS) is an autosomic recessive disease accounting for 4-10% of congenital hearing losses. It was firstly described in 1896 as the combination of deafness and goiter (1). More recently, the phenotype of PS has been better defined. The constant feature of PS is the severe/profound sensorineural hearing loss (SNHL), invariably associated with malformations of the inner ear such as the enlargement of the vestibular aqueduct (EVA), the enlargement of the endolymphatic duct and sac (EED and EES) and, in some cases a cochlear malformation known as Mondini cochlea (2). In about half of the patients goiter of different sizes and subclinical hypothyroidism are observed, whereas in the remaining cases the thyroid has a normal volume and function. In about 80% of patients a partial iodide organification defect has been documented (3). One hundred years after the recognition of PS, the disease gene (SLC26A4 or PDS) has been cloned and mapped on the long arm of chromosome 7 (4). The putative encoded protein, pendrin, belongs to a superfamily of exchangers of chloride and other anions, such as bicarbonate and formate (5). It is characterized by intracellular N-terminus and C-terminus and by 12 transmembrane domains.

Pendrin expression

In humans the highest pendrin expression has been found in the thyroid, but it is also expressed in the kidney, in the endolymphatic duct and sac of the inner ear, in the breast and in the testis (6-9). Pendrin is also expressed in the endometrium, where it seems to have a different localization during the menstrual cycle (10), and in the placenta, where the expression increases during gestation.

PDS expression is significantly different in rats and mice, where the expression at the kidney level is higher than that of the thyroid (8). This is consistent with the lack of functional and/or microscopic thyroid alteration in the Pds KO mouse (11). Therefore, a difference between humans and rodents in the function of pendrin itself or the presence of other regulatory factors that may influence pendrin expression, can be hypothesized.

Pendrin function

- Thyroid

At the thyroid level pendrin has been found to be located at the apical membrane of the thyroid cell facing the lumen of the follicle (6, 12). Pendrin is thus believed to transport iodide from the cell to the colloid space, where iodide will be organified. An impaired function of pendrin at this level could result in a defect of iodide transport. From a clinical point of view this is predicted to result in goiter, total iodide organification defect (TIOD) and hypothyroidism. Surprisingly, the thyroid picture is very variable. Indeed, goiter is not a constant feature and can range from a slight thyroid increase to a large multinodular goiter. Furthermore, most patients are euthyroid or subclinical hypothyroid. Moreover, the perchlorate test shows only a partial organification defect (PIOD). In accordance with this "mild" thyroid phenotype, it has been hypothesized that in the absence of pendrin function, an iodide flux into the colloid space may still occur through one or more transport systems. Thus, the role of pendrin in the thyroid is still not defined. Indeed, since the first study in *Xenopus* oocytes and insect cells indicating that pendrin mediates chloride and iodide transport (13), other observations have been obtained. In particular, it has been shown that in mammalian cells, pendrin is able to transport iodide only at high concentrations and that the function is independent from chloride (14). Furthermore, it has been suggested that chloride concentrations in thyroid cells are too high for iodide to be transported against it (15). Recent studies from our group, are in favour of a role of pendrin in iodide transport. Indeed, by means of experiments in which chloride was substituted by iodide, a transport of both ions in the same cellular system (Hek 293) by pendrin was demonstrated, revealing a Cl⁻/I⁻ exchange with a 1:1 stoichiometry (16,

17).

- Kidney

Much more is known about pendrin function at the renal level. Many studies have shown that pendrin plays a critical role in bicarbonate secretion and regulation of acid-base transport (8, 18, 19). Pendrin is localized in the connecting tubule and in the collecting duct of the kidney cortex and in particular at the apical membrane of a subpopulation of intercalated cells (type B and non-A non-B) (8). These cells carry out a fine regulation of acid-base excretion through bicarbonate-transport processes (18). Experiments in mouse and rat confirm a role of pendrin in these processes. Indeed, in basal conditions, pendrin has an apical membrane localization and a bicarbonate loading leads to an increase in pendrin expression. On the contrary, an acid loading induces a reduction in pendrin expression which seems to be shifted to the cytoplasm. Bicarbonate secretion is thus supposed to be regulated by the trafficking of pendrin between apical plasma membrane and the cytoplasm (19-21). The reduction of urinary bicarbonate excretion and the development of a metabolic alkalosis found in *Pds*-knockout animals, further strengthens this hypothesis (8). Intercalated cells are also known to participate to chloride reabsorption. Accordingly, pendrin expression is inversely correlated to urinary chloride excretion; indeed it is increased when urinary excretion of chloride is low, and decreased when the urinary chloride excretion is high (22). In mice pendrin has also been found to be critical in the pathogenesis of mineralcorticoid-induced hypertension (23).

Despite its critical role in bicarbonate secretion, an impaired function of pendrin at this level is not associated with disturbances of renal function and particularly in the regulation of electrolytes and acid-base balance. Indeed, no renal abnormalities have been never recorded neither in patients nor in *Pds*-knockout animals, when studied in basal conditions. This is likely due to the redundancy of secretion-reabsorption mechanisms in the kidney or to the reduced expression of other transporters, which likely attenuates the rise in intracellular and systemic pH expected for pendrin impairment (24). However, careful studies of renal function after basic and acid loading in PS patients could reveal abnormal handling of anions transported by pendrin.

- Inner ear

In 1999 the expression of pendrin in the endolymphatic duct (ED) and sac (ES) of the mouse was demonstrated (25). The ED is a part of the membranous labyrinth and connects the hearing (cochlea) and equilibrium (vestibular apparatus) organs to the endolymphatic sac which is located in the posterior cranial fossa. They separate 2 different compartments filled with different fluids: perilymph with a composition similar to extracellular fluids and endolymph with a potassium and protein rich, sodium low composition. It has been postulated that pendrin could be involved in the maintenance of endolymph homeostasis promoting the ionic transports (11). Recently, the importance of mitochondria-rich cells (MRC) of the ES in the transcellular transport of ions and water has been shown. Interestingly, striking similarities in ultrastructural characteristics between MRCs of the ES and renal intercalated cells have been found. In particular, the subtype B of the MRC, that are believed to function as Cl⁻/bicarbonate exchangers, are the most likely candidates to be affected in PS. These cells are activated after induced endolymph volume decrease and deactivated after injection of artificial endolymph and are thus believed to be specifically involved in endolymph homeostasis (26). It has been thus hypothesized that an impaired function of pendrin at this level could result in a progressive endolymph volume increase followed by the enlargement of the membranous labyrinth and of the surrounding bony structures and to a damage of the neuroepithelium leading to SNHL (Fig. 1).

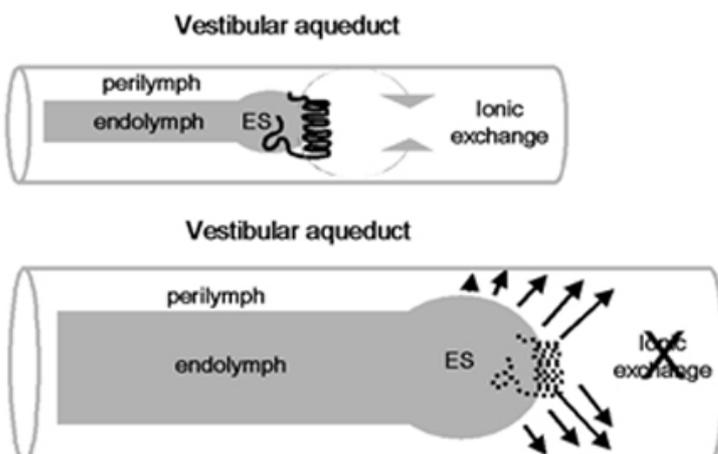


Fig.1: In normal conditions, pendrin maintains the ionic exchanges between perilymph and endolymph in the membranous labyrinth which is contained in the bony structure named vestibular aqueduct. If pendrin function is lost, the endolymph volume increases resulting in the enlargement of the membranous labyrinth and of the surrounding bony structures, such as the vestibular aqueduct and the cochlea. ES: endolymphatic sac.

This mechanism is consistent with what observed at the inner ear level in the *Pds* KO mouse. Indeed, at ED15, the inner ears of *Pds* KO mouse begins to develop an enlarged ED and ES. Progressively, also the cochlea, that is normal in the heterozygous mouse, and the entire membranous labyrinth enlarge. At electron microscopy, a degeneration of the sensory cells of the inner ear is also observed, resulting in SNHL and vestibular dysfunction (11).

This endolymphatic swelling corresponds to the malformations detected radiologically in PS patients (Fig. 2). These inner ear abnormalities and the derived SNHL are a constant feature of PS.

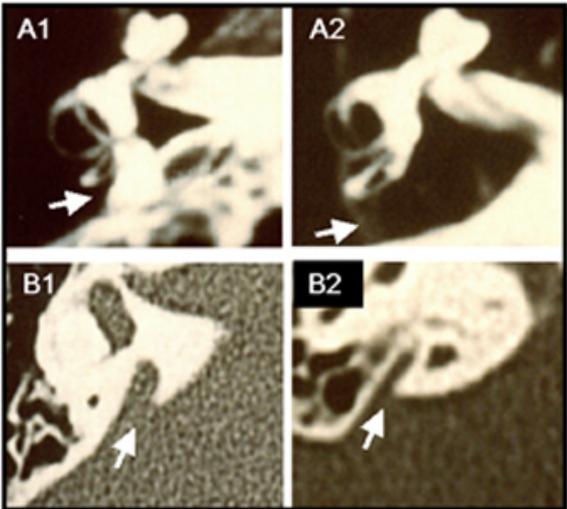


Fig. 2: high resolution MRI section in a patient with Pendred syndrome (A1) and in a control (A2). Note the cochlea and the semicircular canals, and an enlarged endolymphatic sac that results, as expected, not visible in the normal control. The enlargement of the vestibular aqueduct at the CT scan of the petrous bone in one Pendred patient (B1) is shown, in comparison to the normal finding in a control (B2)

From genotype to phenotype

Up to date, nearly 100 different mutations of the PDS gene have been reported in the literature, spanning the entire gene, without hot spot regions (Fig. 3). However, it is worth of note that the great majority of the mutations are localized in the intracellular N- and C-terminus (<http://www.medicine.uiowa.edu/pendredandbor>).

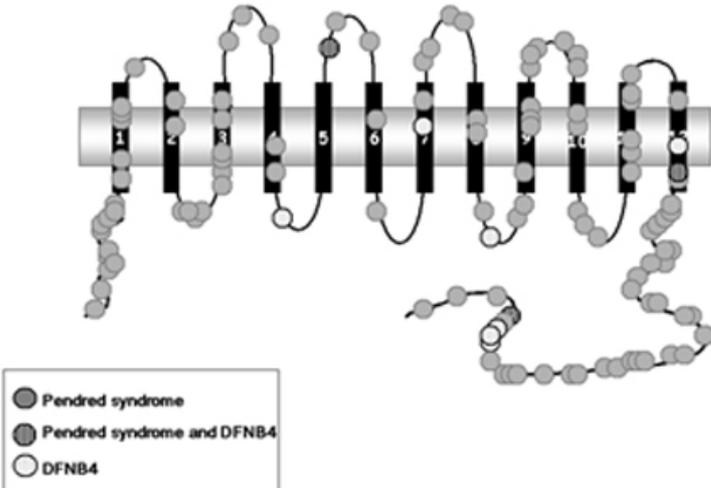


Fig. 3: Schematic representation of all PDS mutations reported to date and associated with Pendred Syndrome, with the non-syndromic hearing loss DFNB4 or with both diseases.

Interestingly, PDS mutations are associated not only to PS, but also to a non-syndromic deafness (DFNB4, enlarged vestibular aqueduct syndrome), and in some cases the same PDS mutation can be associated with PS or with DFNB4 in different families. Functional analysis of the mutations associated with PS or DFNB4 demonstrated the complete loss of chloride/iodide pendrin-mediated transport, whereas those associated with DFNB4 still allow a residual transport of both chloride and iodide, even if at a lower level with respect to the wild-type (27). Moreover, it has been recently reported that two mutant PDS alleles are associated with PS, while a single mutated allele is frequently found in DFNB4 (28). Very recently, the intracellular trafficking of PS mutants has been studied. As above mentioned, WT pendrin is located at the plasma membrane whereas natural mutants of pendrin do not reach the plasma membrane since they are retained in the endoplasmic reticulum probably due to improper folding (29). The mutant protein has been also shown not to interfere with the arrival of WT pendrin at the plasma membrane, in accordance to the recessive mode of inheritance of the disease (30).

No genotype-phenotype correlations have been described in PS. Indeed, as described above, a great interfamilial and intrafamilial phenotypic variability has been reported (31-33). The degree of iodide deficiency could affect the clinical manifestation of the disease, but other environmental or genetic factors are likely involved.

The accurate clinical and genetic analyses on several Italian families led us to a precise characterization of the PS phenotype (3, 34). In accordance with the literature, the SNHL is invariably present, of a severe/profound degree, and it is always bilateral. The onset of deafness is congenital, bilateral and fluctuating in about 80% of cases, while develops suddenly during childhood in a minority of patients. The enlargement of the membranous labyrinth (EVA, EED and EES) is always present, whereas the Mondini cochlea has been found only in 20% of our patients. The coexistence of a vestibular disorder is rarely found, but strongly affects the quality of life of these patients. Differently from what reported in the literature, a goiter of different sizes is present in 95% of our patients and in all cases the discharge after perchlorate ranged 35-60%, indicating a PIOD. About 80% of our cohort of patients is euthyroid and a minority has a subclinical hypothyroidism. The TSH suppressive treatment with L-thyroxine has no effect on thyroid volume reduction and the patient with the largest goiters always need thyroidectomy. No renal function alterations were never found in our patients in basal conditions. Normal menstrual cycles and at term pregnancies were recorded in female patients.

Concluding remarks

Pendrin is an interesting protein with a critical function in the inner ear. However, its role at the thyroid level is still debated and it seems not to be crucial to renal function, at least in basal conditions. The phenotype of PS is extremely variable, being the only constant feature the severe/profound hearing loss and the inner ear malformations. The differential diagnosis between PS and Pseudo-Pendred should always be done and should be based on these data. Indeed, as shown in the schematic flow-chart of Fig. 4, clinical picture such as moderate or unilateral deafness and clinical hypothyroidism argue against the diagnosis of true PS. Similarly, the absence of inner ear malformations definitely excludes PS, while the presence of these alterations strongly indicates the genetic involvement of the PDS gene.

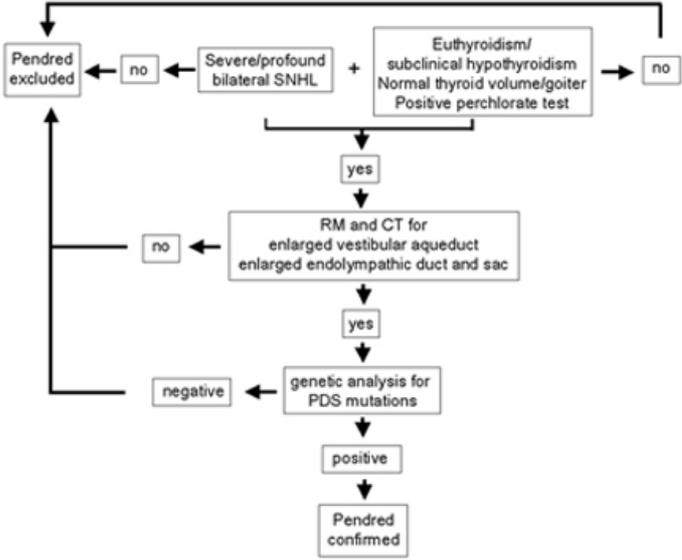


Fig. 4: schematic flow-chart for the differential diagnosis between Pendred and Pseudo-Pendred.

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Thyroid transcription factors and congenital hypothyroidism

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Introduction

Primary congenital hypothyroidism (CH) is the most frequent endocrine-metabolic disease in infancy, with an incidence of about 1/3-4000 newborns. In about 85% of the cases, CH is caused by an alteration in the morphogenesis of the thyroid (thyroid dysgenesis, TD) (2). In 5-16% of cases TD it is associated with other major birth defects, mostly cardiac (Table 1) (3).

Most of the critical events in thyroid morphogenesis take place in the first 60 days of gestation in man or the first 15 days in mice. For this reason, thyroid developmental abnormalities result from morphogenetic errors during this period.

The regulation of formation, migration and proliferation of the thyroid gland are still largely unknown. Several genes, including those encoding thyroid specific transcription factors (TITF1, TITF2, PAX8), thyrotropin (TSH) and its receptor (TSHR), and/or other genes, have been demonstrated to play a role (1). Alterations in any of these genes can be responsible for thyroid dysgenesis.

Mutations in the genes involved in thyroid development give rise to animal models with TD, and mutations in the same genes have been identified also in a small number of patients with congenital hypothyroidism associated with TD.

In this review we will briefly describe the role of thyroid transcription factors and their involvement in the pathogenesis of TD.

NKX2-1/TITF1

NKX2-1, also known as TITF1 (Thyroid Transcription Factor-1) is a homeodomain transcription factor that was initially identified in a rat thyroid cell as a nuclear protein able to bind to specific sequences in the Tg promoter. TITF1 belongs to the Nkx2 class of transcription factors and is encoded by a gene, located on chromosome 14q13 (Table 1). The gene is formed by at least 3 exons and encodes for 42 kDa protein that is phosphorylated. During human development, the gene is expressed in the ventral diencephalon and in the telencephalon; in the lung bud and in the thyroid primordium (1, 4).

Studies in mice demonstrated that *Titf1* is required for the survival and subsequent differentiation of the cells.

TITF1/NKX2-1 disease

TITF1/NKX2-1 disease is characterized by a variable spectrum of neurological, thyroid and lung abnormalities with incomplete penetrance and the variability of the phenotype (Table 1).

A heterozygous homozygous deletion and a loss of function mutations in TITF1/NKX2-1 gene were initially identified in an isolated infant (5) and in two siblings (6) respectively. All the patients were affected by respiratory failure, hypotonia and thyroid dysfunction, without apparent TD. Later reports (7, 8) have demonstrated the association between mutations in TITF1/NKX2-1 genes and a syndrome characterized by choreoathetosis, respiratory distress and a thyroid phenotype ranging from a normal gland to athyreosis.

When tested in vitro, the mutated forms of *Titf1*/*Nkx2-1* show neither functional activity nor a dominant negative effect on the wild type form, suggesting that the haploinsufficiency is responsible for the pathological phenotype.

After these reports, several other mutations in TITF1/NKX2-1 have been shown to be responsible for this syndrome characterized by the presence of TD, benign familial chorea with or without pulmonary distress (1).

PAX8

Pax8 (Paired Box gene 8) is a member of a family of transcription factors characterized by the presence of the paired domain (Prd), a 128 aminoacid-long domain that recognizes and

binds to specific DNA sequences. The gene encoding Pax8 (called PAX8 in humans) is located on chromosome 2 (Table 1). It consists of 12 exons encoding for a 450 aminoacids protein. Pax8 is expressed in the adult and developing thyroid from the early stages of morphogenesis. In addition, during embryonic life Pax8 is transiently expressed in the myelencephalon and in the neural tube. Expression is also present in the developing and adult kidney (1, 4).

Experiments in Pax8 null mice (9) demonstrated that, during morphogenesis, Pax8 is required for the survival of the thyroid precursor cells and to maintain the tissue-specific gene expression program.

In adult thyroid cells Pax8 promotes transcription from the TPO and the Tg promoters (10).

PAX8 disease

The involvement of PAX8 has been described in sporadic and familial cases of CH with TD (1, 11). All affected individuals are heterozygous for the mutations and in the familial cases transmission is autosomal dominant with a variable penetrance and expressivity. In humans, both PAX8 alleles are necessary for correct thyroid morphogenesis and a reduced dosage of the gene product (haploinsufficiency) causes dysgenesis (Table 1); in contrast, the Pax8^{+/-} mice display a normal phenotype (9).

Of note, in mice the combination of partial deficiencies in the Ttf1 and Pax8 genes results in a small thyroid gland, elevated TSH, reduced thyroglobulin biosynthesis, and high occurrence of hemiagenesis (12).

Foxe1/TTF2

Foxe1 (also called TTF-2 for Thyroid Transcription Factor–2) was originally identified as a thyroid specific nuclear protein that bind to a sequence present on both Tg and TPO promoters (1).

Foxe1 belongs to the winged helix/forkhead family of transcription factors. The gene encoding Foxe1 (called FOXE1) in humans is located on chromosome 9q22 and consists of a single exon encoding for a 42 kDa protein that is phosphorylated and contains an alanine stretch of variable length (13-15).

During development, Foxe1 is expressed in the thyroid, in the tongue, in the epiglottis palate and in the esophagus as well as in the Rathke's pouch, which gives rise to the anterior pituitary. In adult, Foxe1 is expressed in the thyroid, in the tongue, in the secondary palate, in the choanae, and in the whiskers and hair follicles.

Analysis of Foxe1 null mice revealed that, during embryonic life, Foxe1 has a specific role in controlling the migration of thyroid follicular cell precursors.

The role of Foxe1 in adult thyroid follicular cells was only partially clarified, and functional studies in cell culture systems have shown that Foxe1 can act as a promoter-specific transcriptional repressor. The transcription of the Foxe1 gene is regulated by TSH and insulin or IGF-1 (reviewed in (1)). These data suggest that Foxe1 plays an important role in the hormonal control of gene expression in thyroid cells.

FOXE1 disease

Bamforth syndrome (16) is characterized by cleft palate, bilateral choanal atresia, spiky hair and athyreosis. The observation that Foxe1^{-/-} mice display thyroid defects and cleft palate (Table 1) (17) has led to the hypothesis that FOXE1 could be a candidate gene for this syndrome. Indeed, so far three mutations in FOXE1 gene have been identified in patients affected by this syndrome (18-20). The patient described in the last report presented the Bamforth syndrome phenotype, and congenital hypothyroidism without athyreosis (20). All the affected members carry homozygous missense mutations within the FoxE1 forkhead domain. The mutant proteins were tested in vitro and have shown a reduction in both DNA binding and transcriptional activity. In all the patients thyroid tissue is undetectable, while in the mice the absence of this factor causes either athyreosis or defects in thyroid migration. In humans ectopic thyroid associated with FOXE1 mutations has not yet described.

Nkx2-6, Nkx2-3 and Nkx2-5

In addition to Nkx2-1, other genes of the Nkx2 family are present in the primitive pharynx and the thyroid anlage.

Nkx2-6 is transiently expressed in the endodermal layer of the midline region of the pharynx (21). Nkx2-3 is strongly expressed in the developing thyroid and disappears at birth (21).

Nkx2-5 is expressed in the ventral region of the pharynx and in thyroid bud later Nkx2-5 transcript disappears from the thyroid bud, persisting in the heart region (21-23). The gene encoding Nkx2-5 (called NKX2-5) in humans is located on chromosome 5q34 and consists of two exons encoding for a 324 aminoacids protein (Table 1). In vitro studies indicate that Nkx2-5 is a potent inducer of the NIS promoter (24), that Nkx2-5 C-terminus interacts with the TTF-1 homeodomain and, moreover, that the expression of a dominant-negative Nkx2-5 isoform (N188K) in thyroid cells reduces TTF-1-driven transcription of several thyroid-specific genes, including pendrin and thyroglobulin (25).

NKX2-5 disease

NKX2-5 is essential for normal heart morphogenesis, myogenesis, and function (26), and several loss of function mutation in NKX2-5 have been described in patients with congenital heart diseases (Table 1) (27). Three heterozygous mutations (A119S, R161P, R25C) were found in four subjects with TD (three patients with thyroid ectopy and one with athyreosis) (23). Functional studies demonstrated that these mutants exhibited a significant functional impairment, with reduction of transactivation properties and dominant negative effect.

Hhex

Hhex (hematopoietically expressed homeobox) is a homeodomain-containing transcription factor. The gene (called HHEX in humans and located on chromosome 10q23.32 encodes for a 270 aminoacids protein that is expressed, in adults thyroid, liver and lung. Hhex is necessary for thyroid morphogenesis: studies in Hhex null embryos thyroid precursor cells demonstrated that, at early stages, Hhex is required to maintain the expression of these genes in the thyroid primordium. In vitro experiments demonstrate that Hhex is regulated by Ttf1 and its overexpression partly inhibits Tg promoter activity. These data suggest that Hhex act as transcriptional repressor in thyroid cells (Reviewed in (1)).

CONCLUSIONS

NKX2-1, FOXE1, PAX8 and NKX2-5 are transcription factors involved in thyroid development. Mutations in the genes encoding for those transcription factors cause alterations in thyroid morphogenesis with or without other congenital defects. However, despite the several studies completed to address the role of transcription factors in thyroid morphogenesis, mutations have been identified only in no more of the 5% of the cases. Such low frequency of mutation can be an underestimate because the molecular analyses to search for mutations in TITF1, FOXE1, PAX8 and NKX2-5 it has been limited to the coding region of these genes, and therefore alterations in regulatory non-coding regions can lead to a disease phenotype.

In addition, it should be considered that TTF1, FOXE1, PAX8 and NKX2-5 are transcription factors able to modulate target downstream genes that ultimately activate the organogenesis of the thyroid and that, in order to produce their biological effects, they may require the presence of cofactors. Some cases of TD could be due to mutations in the not yet identified gene targets for these transcription factors as well as in factors involved in the modulation of their action.

An other possibility is that TD is the consequence of combined defects (multigenic disease) as recently demonstrated in animal models (12), that make the identification of the candidate genes a much more complex process.

Finally, other genes seem to be important candidate genes in controlling of the thyroid development and therefore can be responsible for TD. In this group should be included Hoxa3 and Hoxa5 that have not been investigated as possible cause of TD in humans.

Table 1: Chromosomal localization, molecular features and phenotype produced by mutations of the genes described in the text.

Gene	Chromosomal localization	Features of the gene product	Expected human phenotype associated with gene mutations
<i>NKX2-1/TITF1</i>	14q13	Homeodomain transcription factor	Congenital hypothyroidism dues to thyroid hypoplasia with neurological and lung abnormalities
<i>PAX8</i>	2q12-14	Paired domain transcription factor	Congenital hypothyroidism dues to thyroid hypoplasia
<i>FOXE1/TITF2</i>	9q22	Forkhead domain transcription factor	Congenital hypothyroidism dues to athyreosis with cleft palate, bilateral choanal atresia, spiky hair
<i>NKX2-5</i>	5q34	Homeodomain transcription factor	Congenital hypothyroidism dues to ectopic thyroid or athyreosis, hearth disease
<i>HHEX</i>	10	Homeodomain transcription factor	Not described
<i>TSH-R</i>	14q31	G-protein coupled receptor	Congenital hypothyroidism dues to thyroid hypoplasia

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Autoimmune Polyglandular Syndromes

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Clinical presentation

Autoimmune polyglandular syndromes (APS) are rare endocrinopathies characterized by the coexistence of at least two gland diseases that are based on autoimmune mechanisms. Associations with non-endocrine immune diseases may occur. Two major subtypes of APS, types 1 and 2, are distinguished according to age of presentation, characteristic patterns of disease combinations, and different modes of inheritance. APS 1, also known as autoimmune polyendocrinopathy, candidiasis and ectodermal dystrophy or multiple endocrine deficiency autoimmune candidiasis syndrome, usually appears in childhood at age three to five years or in early adolescence and, therefore, is also called juvenile autoimmune polyendocrinopathy. It is defined by a persistent fungal infection (mucocutaneous candidiasis), the presence of acquired hypoparathyroidism, and Addison's disease. In most patients, candidiasis precedes the other immune disorders, usually followed by hypoparathyroidism. While first clinical manifestation occurs in childhood, the main component diseases develop in the first 20 years of life, and further associated diseases may not evolve until the fifth decade or later. The female-to-male ratio varies from 0.8:1 to 2.4:1. The highest prevalences of the rare APS 1 have been found in populations who are characterized by high degree of consanguinity or who are descendant of small founder populations, particularly in Iranian Jews and Finns. Genetic studies have shown an autosomal recessive inheritance in a single gene. APS 2 is more common and occurs in adulthood, mainly in the third or fourth decade. It is characterized by autoimmune thyroid disease and/or type 1 diabetes with or without Addison's disease. Disorders like autoimmune gastritis, pernicious anemia, vitiligo and alopecia may occur in type 2. The prevalence of APS 2 is estimated to be 1: 20,000, and females are affected three times more frequently than males. In contrast to type 1, family members of APS 2 patients are often affected. APS type 2 is believed to be polygenic, characterized by autosomal dominant inheritance (1-6).

Immunopathogenesis

Autoaggression in polyglandular autoimmunity is considered multifactorial. The principal antigen-specific immune response is initiated by antigen presenting cells (7). Ubiquitous dendritic cells are the most important APC's. Immature dendritic cells pick up antigen molecules in non-lymphoid organs, fragment the antigens, and migrate to the secondary lymphoid organs presenting their HLA class I or II associated antigen fragments. This activates antigen-specific T helper cells that stimulate by use of different cytokines the cellular immune response via cytotoxic T lymphocytes (Th1) and/or the humoral immune response via B lymphocytes (Th2). During the Th1 response, activation of mononuclear phagocytes also occurs, because Th1 cytokines comprise proinflammatory mediators. T suppressor cells regulate the immune responses; when immune tolerance is lost, autoaggression occurs.

Recently, a T cell population (CD4⁺CD25⁻) with potent regulatory properties that inhibit the activation of CD4⁺CD25⁺ T effector cells has been described (8-9). These T cells regulate autoaggressive T and B cells and may have profound influence on the control of human autoimmune diseases.

Animal models of the pathogenesis of APS are consistent with a viral infection theory as well as a suppressor effect theory. The viral infection-theory couples autoimmune disease with viral infection. The so-called "molecular mimicry" is characterized by an immune response to an environmental agent that cross-reacts with a host antigen, resulting in disease. In an animal model, mice infected with reovirus type 1 developed APS (10-11). Some of the resultant autoantibodies showed cross-species reactivity, recognizing similar antigenic determinants in mouse and human organs. With respect to the suppressor effect theory, administration of the immunosuppressive drug cyclosporine to newborn BALB/c mice caused a selective defect of the regulatory T suppressor cells (12). Thymectomy conserved the T-cell defect and produced autoimmune diseases in a wide spectrum of organs (thyroiditis, insulinitis, adrenalitis, oophoritis/orchitis, and gastritis) with pathology similar to that of human organ-specific immune diseases. These pathological processes lead to the pre-clinical phase of APS, with production of organ-specific antibodies and progressive immune-mediated destruction of endocrine tissues. In the clinical phase, major organ destruction occurs due to the autoimmune activity that is primarily characterized by chronic inflammatory infiltration of lymphocytes. Destruction of endocrine glands causes their secretory insufficiency. The role of apoptosis in immunodestruction has been associated with deregulation of apoptotic signaling pathways. Dysfunction of the Fas apoptotic pathway or production of soluble factors including soluble Fas and soluble Fas ligand may be involved in the pathogenesis of endocrine diseases. In the case of type 1 diabetes it has been postulated that increased susceptibility of islet cells to the induction of apoptosis by cytotoxic T cells – presumably through the cell surface receptor Fas pathway – may be responsible for facilitated death of islet

β cells (3, 13).

Immunogenetics

Significant associations of APS type 2 with HLA class I antigens were observed in various studies (14). In part, this may be explained by the observation that APS patients with HLA linkage showed a decreased HLA class I expression on the surface of their lymphocytes and a defective transcription of HLA class-I processing genes. APS type 2 is polygenically inherited, characterized by dominant inheritance. Several component diseases of APS have a common immunogenetic background, but the major genetic factor remains to be detected in the HLA region. One factor in the pathogenesis of APS may be an immunologic dysfunction that results from one or more genes on chromosome 6, in linkage disequilibrium with the HLA-B8 allele. APS type 2 is also associated with the HLA antigens DR3 and/or DR4, and DRw3, whereas HLA DR3 is associated with almost all immune endocrinopathies of APS type 2. Further detailed analyses of the HLA DR3 alleles showed that the HLA DR3-DQB1*0201 haplotype may be associated with multiple component diseases of APS, while the HLA DR4-DQB1*0302 haplotype is implicated in beta-cell autoimmunity only. Patients with APS may be highly selected for HLA-B8/DRw3 positivity. In comparison, for autoimmune thyroid diseases, a high percentage of family members of patients showed significant titers of thyroid autoantibodies and segregation analyses favored a dominant mode of inheritance.

Genetic transmission of autoimmune thyroid diseases seems to be complex and the familial pattern indicates a multigenic disease in which multiple genes may contribute to the clinical phenotype. Recent studies have proposed the cytotoxic T lymphocyte-associated gene that contributes to the genetic susceptibility to thyroid antibody production, located on chromosome 2q33. Susceptibility to APS type 2 diseases has further been associated with the major HLA class I chain-related MIC-A genes. Moreover, quantitative defects in the density of conformational correct HLA class I complexes on the surface of lymphocytes were found in patients with diverse HLA-linked autoimmune diseases (13-15).

Associations with HLA class II alleles also have recently been reported in APS type 1. An increased frequency of the HLA-DR3 allele was observed in these patients. In a study comprising patients with APS type 1 from 12 different countries, Addison's disease was found to be significantly and positively associated with the HLA-DRB1*03 allele (relative risk RR 8.8). Here, only one of 19 patients with HLA-DRB1*03, in contrast to 28 of 85 patients without this allele, had not developed Addison's disease (4). Moreover, in these patients with APS 1, the component disease alopecia was significantly and positively associated with HLA-DRB1*04 (RR 4.8) and DQB1*0302 (RR 6.6). In contrast, the most common protective alleles for type 1 diabetes (DRB1*15 and DQB1*0602) were similarly protective in APS 1 patients, as indicated by significant negative correlations (3, 15). However, in the immunogenetics of APS type 1, mutations of a single gene that is termed the autoimmune regulator (AIRE) gene, play an important role. The AIRE gene is assigned to chromosome 21q22.3 and has been cloned by two independent research groups. In the coding region of the AIRE gene, 45 different mutations have been reported. Mutations comprise nonsense and missense mutations, deletions, and small insertions (6, 25). A few mutations are responsible for the expression of a truncated regulator protein. AIRE encodes a 545-amino-acid protein of 57.5 kDa that contains structural domains characteristic for transcription regulators. AIRE is also an important DNA binding molecule that is involved in immune regulation. The AIRE gene is expressed in immunologically relevant tissues, particularly in the thymic medulla, as well as in lymph nodes and peripheral blood cells (CD14-positive monocytes), but not in CD4-positive T cells. Mutational analysis of AIRE helps identify patients with atypical phenotypes resembling to APS type 1, e.g. the AIRE mutation R257X was responsible for 82 % of APS 1 alleles in a Finn population (17-26).

Diagnostic recommendations

The clinical presentation of APS is often preceded by an asymptomatic latent period characterized by the presence of circulating disease-associated antibodies which are useful markers for the prediction of the development of APS (27-28). Absence of these antibodies does not exclude the disease, because not all patients show positive antibodies. In view of the possible long time interval between the manifestation of the first and further autoimmune endocrinopathies, regular and long-term observation of patients with autoimmune endocrine disorders is warranted. Moreover, if a patient has one endocrinopathy and a family member has another, it is likely that they also may have antibodies against other endocrine tissues. In view of the tendency of autoimmune diseases to associate with other disorders, of the metachronous manifestations of the component diseases, and of the subclinical course, it is necessary to suspect in all patients with one immune endocrinopathy the existence of a further autoimmune disorder, particularly in patients with positive family histories.

For patients with monoglandular autoimmune endocrinopathy, functional screening for autoimmune polyglandular syndromes is therefore recommended. If pathological findings, e.g. occurrence of a second autoimmune endocrine disease, are noted, measurement of organ-specific autoantibodies should be added. Furthermore, functional screening for autoimmune endocrine diseases of the first-degree relatives of these patients with newly diagnosed APS may be also done. Especially in the offspring of patients with type 1 diabetes, serological testing for the presence of diabetes associated antibodies should be considered. Genetic screening is especially useful in APS type 1. Thus, in subjects at risk, regular functional screening is warranted. If clinical disease is present, serological measurement of organ-specific antibodies should follow (29-30).

Table:	APS 1	APS 2
Prevalence	very rare	relatively common
Onset	Childhood	Childhood through adulthood
Inheritance	Monogenic (Aire gene)	Polygenic
HLA genotype	Risk decreased with HLA-DQ6	HLA-DQ2, DQ8, DRB1*0404
Phenotype	Hypoparathyroidism Hypogonadism Addison's disease	Autoimmune thyroid disease Type 1 diabetes Addison's disease
Non endocrine diseases	Candidiasis	Autoimmune gastritis Celiac disease

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CYTOSKELETAL ACTIONS OF IODOTHYRONINES

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Introduction

Thyroid hormone is required for the normal development of the mammalian brain where it regulates a diverse set of developmental programs that include i) cell proliferation and migration, ii) apoptosis, iii) neuronal integration, and iv) dendritic arborization (see [1-4] for reviews). The window of time for thyroid hormone-dependent regulation of these processes is limited to pre- and perinatal life in rodents. Despite such obvious biology, the molecular events mediating the morphogenic actions of thyroid hormone have proven elusive. While it is clear that many of the actions of thyroid hormone are mediated by T3-dependent regulation of gene expression managed by specific chromatin-bound thyroid receptors (TR) (see the following reviews [5-7]), recently, nongenomic actions of thyroid hormone have also been validated [8-10]. These latter actions of thyroid hormone have become more important with the discovery that the brain of the TR-null mouse shows few, if any, of the developmental defects observed in the hypothyroid animal. In this review, we will examine the evidence for a nongenomic mechanism of action of thyroid hormone in brain by considering: 1) what role the cytoskeleton and the extracellular matrix play in mediating the actions of thyroid hormone; 2) which iodothyronine(s) are responsible for regulated cell trafficking; and 3) the nature of the thyroid receptor(s) that modulates neuronal migration.

The cytoskeleton as a target for thyroid hormone in the brain

The influence of altered thyroid status on brain development was first documented in the late 1960s by the demonstration that altered thyroid status disturbed the maturation of the cerebellum and led to defects in granule cell migration, Purkinje cell arborization, the timing of apoptosis, and neuronal integration [11, 12]. The cell's cytoskeleton was one of the first choices as a target for thyroid hormone action [13-16] because of its central role in defining the architecture and motility of the cell. Analysis of microtubule polymerization in the developing cerebellum revealed that the expression of the tau family of microtubule-associated protein and at least 5 isoforms of tubulin were regulated by thyroid hormone. It is still not clear whether these changes in transcript abundance are due to a direct action on gene expression, are mediated by other genes, or are regulated by post-transcriptional mechanism(s) [17, 18].

Microfilaments are the other major component of the cell's cytoskeleton and is composed of fibrils and fiber bundles of polymers of the mechano-chemical protein actin. Both T4 and reverse T3 (rT3) dynamically regulate actin polymerization in astrocytes by a nongenomic process [19, 20]. It is this ability of thyroid hormone to rapidly reorganize the actin cytoskeleton in the developing cerebellum that provides a key component of a nongenomic process capable of regulating neuronal migration. *In vivo*, the actin fibers used for pathfinding and guidance of the migrating neurite/growth cone during neuronal maturation are disassembled in the cerebellum of neonatal hypothyroid rats [21-23], and this defect can be repaired by single injection of thyroxine [24]. Total cellular actin levels do not change with thyroid status; only the relative proportions of polymers (fibrous) actin to monomeric actin are influenced by T4 [25].

A second component of a nongenomic regulatory process that can directly impact neuronal migration is derived from the fact that neuronal migration/neurite guidance is directed by external cues from the extracellular matrix (ECM) protein laminin. Laminin is a product of astrocytes that is held in polymer arrays on the astrocyte cell surface by specific transmembrane receptors composed of integrin subunits. These integrin receptors cluster to form focal contacts that are anchored in place by actin filaments that bind to their cytoplasmic tails [26, 27], and integrin clustering is necessary to anchor laminin arrays on the cell surface. T4 regulates the organization of microfilaments in both astrocytes and neurons, especially those in neuronal processes, *in vitro* and *in vivo* [28]. One important consequence of loss of the microfilaments in the developing cerebellum of hypothyroid neonates is the temporal disruption of laminin deposition ~7-10 days [29, 30]. T4 replacement, given at least one day prior to the critical time period for granule cell migration (~7-10 days after birth), normalizes the timing and topological complexity of laminin deposition and, thereby, facilitates the orderly migration of granule neurons. Interestingly, a laminin receptor has recently been identified as a specific T4 binding protein on the cell membrane [31], although the contribution of such binding to the organization of the ECM is unknown. Thus, by modulating the organization of the actin cytoskeleton, thyroid hormone directly regulates the deposition and organization of a key guidance cue used by migrating granular neurons.

Which iodothyronine is responsible for regulating cell trafficking?

While T3 is widely thought to be the bioactive form of thyroid hormone, the T4-dependent regulation of actin polymerization is an exception to this rule. Comparison of the ability of individual iodothyronines to initiate actin polymerization in astrocytes revealed that T4, and its metabolically inert metabolite, reverse T3 (rT3)—two iodothyronines that do not regulate transcription—are at least 100-fold more potent than the transfactor activator, T3. Similarly, acute hormone replacement with either T4 or rT3 (6 hr treatment) completely restores microfilament organization to normal in the cerebellum of 14 day old, hypothyroid neonates, while acute T3 replacement fails to correct this defect [32]. These findings show that the effector protein(s) mediating the non-genomic regulation of actin polymerization have a ligand preference very different from that of the transcription regulating TRs. Using microfilament remodeling in astrocytes to evaluate iodothyronine potency, the thyroid hormone dependent effector(s) were shown to prefer iodothyronines with a fully substituted phenolic ring, i.e. two iodines (such as T₄ and rT₃) and an alanine side chain with a neutral or net positive charge [33]. Removal of one phenolic ring substituent or the presence of a net negative charge on the alanine side chain destroys the ability of the iodothyronine to regulate actin polymerization. Importantly, inactive analogs with negatively charged alanine chains were “reactivated” by blocking the free carboxyl group of the alanine side chain [33]. Thus, the effector molecule mediating thyroid hormone-dependent actin polymerization possesses a unique set of thyroid hormone binding properties that distinguish it from all the known TH binding proteins, including the TR and the cell surface receptor [31].

The role of thyroid receptors: neuronal migration

While the T4-dependent regulation of actin polymerization is clearly a nongenomic event, in mammals, most of the actions of thyroid hormone are mediated by chromatin bound T3 receptors. These T3 receptors are encoded by two genes—TR α (NR1A1) and TR β (NR1A2). The TR α encodes at least four gene products, the T3-binding TR α 1 and three that do not bind T3; TR α 2, TR $\Delta\alpha$ 1 and TR $\Delta\alpha$ 2. In the cerebellum, T3 binding, TR β gene products are found in the nuclei of oligodendrocytes and Purkinje neurons [34, 35]. On the other hand, granular neurons express TR α gene products suggesting that they may be responsible for thyroid hormone dependent granule cell migration [36].

The lack of overt developmental defects in the brains of most TR knockout mice was unforeseen and two possible explanations for this anomaly have emerged. The first is that gene repression by the unliganded TR α mediates the developmental defects observed in the developing brain of neonates; the unliganded TR α 1 appears to suppress hormone-induced tissue development in frogs [37]. A second is that a biology other than T3 regulated gene expression—a nongenomic action—mediates thyroid hormone's influence on the developmental program of the brain.

The first possibility is supported by the finding that selective deletion of the T3-binding gene product of the TR α 1 gene (TR α 1^{-/-}) eliminates the delay in granule cell migration observed after chemically induced hypothyroidism [36]. While it is generally presumed that the persistence of the external granule layer of the neonatal hypothyroid cerebellum is due to a delay in granule cell migration, a delay in the timing of the proliferation of the granule cell precursors also contributes to the preservation of a thickened external granule layer. Importantly, neonatal hyperthyroidism is associated with the premature termination of granule cell precursor proliferation [16, 38], while neonatal hypothyroidism is associated with a shift in the timing of granule cell proliferation by about 7-10 days. The role of the T3-binding TR α 1 in the regulation of granule cell precursor proliferation remains to be determined.

The unliganded TR is also thought to play a role in the survival of the developing neonate. Deletion of the entire TR α gene locus (TR α ^{0/0}) [39] greatly improved the survival of congenitally hypothyroid (Pax8^{-/-}) neonatal mice [40], although the growth retardation observed in these athyroidic neonates. It now appears that poor survival of the Pax8^{-/-} neonate is a consequence of impaired gut maturation [40]—a developmental defect shared by the TR α ^{-/-} neonate that lack the full-length TR α 1 and TR α 2, but express truncated TR $\Delta\alpha$ 1 or TR $\Delta\alpha$ 2 [41]. The TR α ^{-/-} shows a progressive fall in circulating T4 and T3 beginning 12-14 days after birth that leads to death between 3 and 5 weeks of life and like the hypothyroid Pax8^{-/-} mouse, T3 treatment beginning on day 21 normalizes gut maturation and improves survival. More recently, the role of the unliganded TR α 1 in the neonatal survival has been

questioned. Selective inactivation of the full-length TR α 1 and the truncated TR $\Delta\alpha$ 1 (TR α 1^{-/-}) gene products failed to improve the survival of the congenitally hypothyroid Pax8^{-/-} neonatal mouse [42]. This raised the possibility that TR α 2 and/or TR $\Delta\alpha$ 2 may be responsible for some of the developmental defects that lead to the poor survival of congenitally hypothyroid neonates.

Direct analysis of the role of the four TR α gene products TR α 1, TR $\Delta\alpha$ 1 TR α 2, TR $\Delta\alpha$ 2 on thyroid hormone dependent microfilament remodeling and laminin deposition by astrocytes was done with TR α 1^{-/-}, TR α 2^{-/-}, and TR α ^{0/0} mice and the data are summarized in Table I. None of these targeted gene deletions altered total cellular actin in astrocytes, but the actin cytoskeleton was disassembled in cells lacking TR α 1, TR $\Delta\alpha$ 1 (TR α 1^{-/-}) and in mice lacking all for TR α gene products, TR α 1, TR $\Delta\alpha$ 1 TR α 2, TR $\Delta\alpha$ 2 (TR α ^{0/0}). The TR α 2^{-/-} mouse lacking TR α 2, TR $\Delta\alpha$ 2 [43] showed a normal actin cytoskeleton. Similarly, astrocytes from the TR α 1^{-/-}, and TR α ^{0/0} mice did not assemble laminin arrays on their cell surface, while astrocytes from TR α 2^{-/-} or wild-type mice showed abundant arrays of extracellular laminin. Direct examination of the programmed deposition of laminin in the molecular layer of the neonatal cerebellum in TR α 1^{-/-}, TR α 2^{-/-}, TR α ^{0/0} mice revealed that the 7-10 day delay in laminin deposition observed in the hypothyroid cerebellum was also observed in the TR α 1^{-/-} and TR α ^{0/0}, but not the TR α 2^{-/-} animal [43]. These data suggest that the full-length TR α 2 and the truncated TR $\Delta\alpha$ 2 do not contribute to the organization of laminin on the astrocyte cell surface. While, both the full-length TR α 1 and its truncated partner TR $\Delta\alpha$ 1 appear to be candidate(s) for the effector mediating laminin deposition, the failure of T3 to modulate actin polymerization or to facilitate laminin deposition in astrocytes or cerebellar extracts [10, 33, 44] suggests that the full length TR α 1 does not participate in these biological events. Thus, these findings raise the possibility that non T3-binding TR α gene products, such as TR $\Delta\alpha$ 1 and/or TR $\Delta\alpha$ 2, are likely candidates for the effector molecule(s) that mediate T4-dependent regulation of cerebellar development and launch an important new avenue of research for the future.

Table I. Summary of the organization of the actin cytoskeleton and laminin deposition in TR knockout mice.

TR α	TR α 1	TR $\Delta\alpha$ 1	TR α 2	TR $\Delta\alpha$ 2	Actin cytoskeleton	Laminin deposition
WT	+	+	+	+	+++	Normal
hypothyroid	+	+	+	+	--	Delayed
TR α ^{0/0}	-	-	-	-	--	Delayed
TR α 1 ^{-/-}	-	-	+	+	--	Delayed
TR α 2 ^{-/-}	+	+	-	-	+++	Normal

Summarizing table:

Cytoskeletal actions of iodothyronines

A. The cytoskeleton as a target for thyroid hormone in the brain

1. Early development of the cerebellum

- Components of the microtubules, tubulin and MAPs, show hormone dependent changes in transcript and protein abundance
- The organization of actin fibers are regulated by thyroid hormone
- Cell migration requires an intact cytoskeleton

2. Astrocyte function

- The actin polymerization is regulated by thyroid hormone
- Integrin receptor clustering is requires an intact actin cytoskeleton
- Laminin deposition on the astrocyte cell surface is regulated by thyroid hormone

B. Iodothyronines responsible for regulating cell trafficking in the cerebellum

1. Astrocytes in culture

- T4, and rT3, but not T3, initiate hormone-dependent actin polymerization
- Iodothyronine specificity is determined by the charge on the alanine side chain and by the presence of two iodines on the phenolic ring.

2. In organ culture, and in vivo

- T4, and rT3 initiate hormone-dependent actin polymerization, and neurite outgrowth

- b. T4, and rT3 initiate laminin deposition in the molecular layer of the cerebellum
- c. pharmacological doses of T3 do not influence actin polymerization, neurite outgrowth or laminin deposition

C. The role of thyroid receptors neuronal migration

1. Astrocyte function

- a. TRalpha gene products are required for hormone dependent actin polymerization and laminin deposition
- b. deletion of three of the four potential TRalpha gene products, TRalpha1, TRalpha2 and delta alpha2 have no effect on hormone dependent actin polymerization and laminin deposition
- c. deletion of all TRalpha gene products, including delta alpha1, leads to the disruption of the actin cytoskeleton and eliminates laminin deposition.

2. Developing cerebellum

- a. the T3-binding TRalpha1, and the non-T3 binding TRalpha2 or delta alpha2 are not required for actin polymerization or laminin deposition
- b. the absence of delta alpha1 disrupts actin polymerization and laminin deposition

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GRAVES' OPHTHALMOPATHY PART1

PATHOGENESIS: IMPACT OF ENVIRONMENT ON THE ORBITAL DISEASE

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Clinically evident Graves' ophthalmopathy (GO) develops in 25-50 percent of patients with Graves' hyperthyroidism (1). While some patients experience only mild ocular discomfort, approximately 5% have severe ocular disease and are at risk for visual loss. Subclinical GO appears to be present in the vast majority of Graves' patients who are without clinically evident eye involvement; sensitive orbital imaging studies suggest that approximately 90% of patients with Graves' hyperthyroidism have orbital changes suggesting ocular involvement (2). It is not understood why some patients with Graves' disease develop severe ocular disease while others are spared this complication. While it is possible that there exist GO susceptibility genes, no unique gene associations, beyond those known to predispose to Graves' disease itself, have been convincingly identified in the subset with severe GO. In contrast, recent laboratory and clinical studies, to be discussed in this review, have begun to identify environmental factors that interact with the existing orbital autoimmune milieu to contribute to the progression of GO in some patients with Graves' disease.

THE ORBITAL AUTOIMMUNE MILIEU

Cellular constituents of the autoimmune response within the GO orbit include the myocytes of the extraocular muscles, connective tissue cells (fibroblasts, adipocytes, and intercellular matrix), as well as "professional" immune effector cells (3). Tissue histology shows largely intact muscle fibers and an expanded fat compartment containing macrophages, T lymphocytes, and to a lesser extent, B lymphocytes and natural killer cells. Further characterization reveals increases in both CD4⁺ and CD8⁺ T cells with restriction in the T cell receptor repertoire. The particular T cell-derived cytokines found within GO tissues appears to depend on the "stage" of disease; Th1 cells (secreting IL-2, IFN- β , TNF- α) are predominant in tissues from patients with early disease, while Th2 cells (producing IL-4, IL-5, IL-10) appear in later stages (4). Enlargement of the connective tissue compartment results both from an increase in the volume of the orbital fat and from tissue edema caused by the presence of hydrophilic glycosaminoglycans (GAG). The former is thought to result from de novo adipogenesis occurring within the orbital tissues (5), the stimulus for which is at present unclear. GAG production by orbital fibroblasts appears to be stimulated by inflammatory cytokines present within the orbit (6).

It is well accepted that an autoimmune response against the TSH receptor (TSHR) expressed on thyrocytes is responsible for the hyperthyroidism of Graves' disease. Because of the close clinical relationship between Graves' hyperthyroidism and GO, it has long been postulated that TSHR might also serve as an autoantigen within the orbit, thus linking Graves' hyperthyroidism with its ocular complications. Most investigators seeking evidence of orbital TSHR expression have reported finding at least low level TSHR gene expression in orbital fibroblasts, preadipocytes or orbital fat, and either intact TSHR protein, or an antigenically related protein within the cells (7-12). Further evidence that orbital TSHR expression may play a role in GO stems from studies showing higher levels of TSHR gene expression in orbital adipose tissues from patients with active GO than in normal orbital tissues or tissues from inactive GO patients (13). Recent studies by Terry Smith and colleagues have demonstrated autoantibodies against IGF-1 receptor in the sera of patients with GO, suggesting that this receptor may represent another important orbital autoantigen (14, 15).

ENVIRONMENTAL INFLUENCES

Mechanical factors and trauma

CT scans of the orbits of patients with Graves' ophthalmopathy (GO) show increased volume of orbital tissues. While this appears to be due to enlargement of both the orbital fat and the extraocular muscles in the majority of patients, some patients appear to have predominantly either adipose tissue or extraocular muscle expansion. (16). Because the bony orbital socket is unyielding in response to the pressure generated by increased tissue volume, forward displacement of the globe (proptosis) may ensue as a means of orbital decompression. The expanded orbital tissue volume may impact venous and lymphatic outflow from the orbit, leading to periorbital and conjunctival edema. Orbital tissue trauma generated by tissue expansion within the confines of the bony orbit might further aggravate the disease process by releasing inflammatory cytokines and factors that facilitate antigen presentation and T cell activation (17). In addition, it is possible that individual anatomic variability, such as the shape or size of the orbits, or variations in venous or lymphatic drainage, may aggravate the

intraorbital process and predispose to the development of severe GO. Similarly, it has been postulated that mechanical factors and trauma to soft tissues of the lower extremities is involved in the pathogenesis of the dermal complications of Graves' disease, termed pretibial dermopathy (18).

Tobacco Smoking

The association between smoking and GO is striking, representing the major risk factor known for this condition. The odds ratio, relative to controls, has been reported to be as high as 20.2 for current smokers, and 8.9 for ex-smokers, suggesting a direct and immediate effect of smoking (19). In addition, studies have shown that among patients with GO, smokers have more severe eye disease than non-smokers, that smoking is associated with aggravation of eye disease following radioiodine therapy, that that it adversely influences the course of GO during treatment with corticosteroids and orbital radiotherapy (19). That smoking is linked to other autoimmune diseases, including rheumatoid arthritis and Crohn's disease, suggests there may be a generalized stimulation of autoimmune processes in smokers. Although mechanisms underlying this association remain unclear, effects of orbital hypoxia, free radicals contained in tobacco smoke, or the low levels of interleukin-1 receptor antagonists found in smokers may be involved (19).

Therapy for Thyrotoxicosis

An area of considerable controversy in the past concerned the impact of the choice of therapy for hyperthyroidism on the subsequent course of GO. Several retrospective studies examined this topic, often with conflicting results. More recently, however, randomized, prospective trials have focused on this area and have helped to clarify the issues involved (20-22). A study by Bartalena and colleagues compared eye changes in 443 patients with moderately severe and active GO, prospectively treated with either radioiodine, methimazole, or radioiodine and prednisone (0.4 to 0.5 mg/kg body weight, starting two to three days after radioiodine therapy and continuing for one month, followed by a 2 month taper). Patients were monitored for 1 year and assessed by objective criteria, an activity score, and patient self-assessment. The groups were similar with regard to percentages of smokers or patients with preexisting GO. The investigators found worsening of eye disease within 6 months after radioiodine therapy in 15% of patients treated with radioiodine alone, in 2.7% of patients receiving antithyroid drugs, and in no patients receiving both radioiodine and corticosteroids. The majority (74%) of the patients who experienced worsening eye status after radioiodine therapy had preexisting GO; the eye changes that occurred were largely mild and returned to baseline within 2 to 3 months in 65% of cases. However, 8 patients (5%) in the radioiodine group required additional treatment for their GO, compared with 1 patient in the methimazole group. Patients with preexisting GO and smokers were more likely to have progression after radioiodine administration.

A recent study by Perros and colleagues examined the effects of radioiodine in patients with minimally active GO, and found no association between this treatment and ocular disease progression when post-radioiodine hypothyroidism is prevented (22). In composite, these studies suggest that patients with Graves' hyperthyroidism who have pre-existing and at least moderately active GO have a slightly increased risk of ocular disease progression following radioiodine therapy. When ocular worsening occurs, it is generally mild and may be prevented with concurrent steroid treatment. This does not appear to be the case in patients with minimally active GO. Mechanisms responsible for this mild worsening are unclear, but may be related to the hypothetical release of autoantigen from the thyroid gland, the elevated TSHR autoantibody production known to occur post-radioiodine, or to destruction of radiosensitive suppressor T cells in the thyroid. It is also possible that the effect is primarily due to the induction of hypothyroidism by radioiodine ablation of the thyroid, as ocular changes were not seen in the Perros study in which hypothyroidism was prevented.

SUMMARY

Ocular involvement see figure,

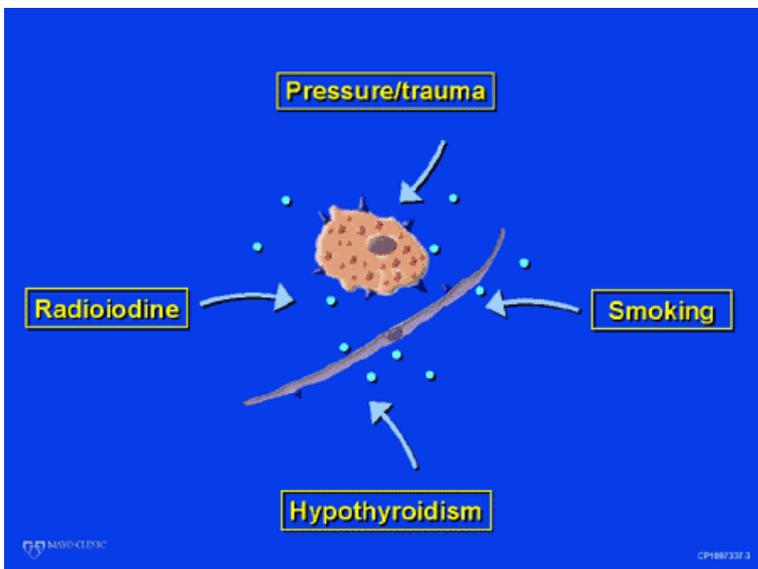


Figure: "Fibroblasts and adipocytes in the Graves' orbit; under attack on multiple fronts."

whether clinically evident or subclinical, appears to be almost ubiquitous in patients with Graves' disease. While studies from several laboratories have identified cells and cellular responses involved in orbital autoimmunity, it remains unclear why some patients with Graves' disease develop clinical GO. To date, no unique genetic associations have been identified in Graves' patients with severe GO. Recent studies suggest that environmental factors may interact with the orbital autoimmune milieu to worsen the ocular disease. Some of these, including variations in orbital anatomy and pressure-related trauma to intraorbital tissues, do not appear amenable to intervention short of orbital surgery. In this context, it would seem prudent to avoid intraorbital steroid injections. Attention to some of the other environmental factors may favorably impact the disease course. Certainly, the advice to stop smoking forms the centerpiece of patient counseling. In addition, some patients with active GO might benefit from a tapering course of prophylactic corticosteroids if radioiodine is to be given to treat hyperthyroidism. This could be considered especially in smokers or patients with severe thyrotoxicosis, weighing the potential benefit against the known side-effects of these medications. Further, it seems wise to avoid significant post-radioiodine hypothyroidism in these patients. Future investigations will focus on identifying therapeutic agents or clinical interventions that will either prevent the disease or interrupt disease progression at a point proximal to the development of serious ocular complications.

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Chernobyl beyond 20 years and thyroid cancer

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Abstract

April 26, 2006 was the 20th anniversary of the Chernobyl Nuclear Plant Accident. At that time there was no reliable information concerning the massive amount of radioactive materials that escaped from the reactor. We know however that Belarus, Russia and Ukraine, were exposed to huge amounts of radioactive materials. In exposed children and adolescents a dramatic increase in the incidence of thyroid cancer has been observed while the adult population does not seem to be affected by the radioiodine contamination. Yet 20 years of observation may not be sufficient to evaluate the full radiological consequences of this accident. Therefore, the current characteristics of so-called "Chernobyl thyroid cancer" need to be reviewed including long-term risk for thyroid cancer after radiation exposure at the level of clinical and molecular epidemiology.

Introduction

Thyroid cancer is the most common type of human solid tumors associated with external ionizing radiation exposure, especially if irradiation occurs in neonates, infants and children (1). The health impacts of the Chernobyl accident have recently been reviewed by the World Health Organization (WHO) (2). Since the Chernobyl accident, specific attention has been paid to an internal exposure of the thyroid gland and its close relationship with childhood thyroid cancer (3). The appropriate prophylaxis of iodine administration just after the accident like in Poland (4) could contribute to mitigate the increase of childhood thyroid cancer and also decrease the future risk of thyroid cancer occurrence. Iodine deficiency is another risk factor of radiation-induced thyroid cancer around Chernobyl (5).

Childhood thyroid cancers are originally quite uncommon and have a fairly good prognosis despite of the aggressive manifestations. Incidence of thyroid cancer in children dramatically increased around Chernobyl from 1990 until 2000, probably attributed to short-lived radioactive iodines. About 5000 childhood and adolescent cases of thyroid cancers have been diagnosed from 1990 until 2005 around Chernobyl with fewer than 20 deaths reported (6).

The knowledge gained in the last 20 years provides valuable information for the advancement of thyroid cancer research. Here, clinical and epidemiological data will be summarized and genetic and molecular aspects of Chernobyl thyroid cancer briefly discussed.

Clinical characteristics of Chernobyl thyroid cancer

Papillary carcinoma is the most common malignant tumor of the thyroid in both adults, adolescents and children. There have been already several reports of an association between radioactive iodine exposure and childhood thyroid cancer prevalence but the interpretation of data still needs some straightforward refining (7-11). Adult thyroid cancers include disease types that range from an indolent small-size solitary malignant nodule to the fulminant and lethal anaplastic carcinoma. Definitely, differences do exist between adult and childhood papillary thyroid cancers. For example, childhood thyroid cancers display a higher incidence of regional lymph node metastasis, extension outside the thyroid capsule and lung metastasis. The initial comparative study of post-Chernobyl thyroid cancer in Belarus and naturally occurring thyroid cancer in Europe clearly demonstrated that individuals 5 year-old or less at the time of accident accounted for the majority of thyroid cancer patients substantiating a necessity of careful monitoring of the subjects of younger age at radiation exposure (12). The prognosis of operated childhood thyroid cancer in Belarus is quite favorable so far (13). There is no clear evidence at a moment that the incidence of thyroid cancer has increased among those exposed who were adult in 1986 (14,15), however the role of adult radiation exposure, either by radioactive iodines or externally, remains to be clarified. In 1991, the Chernobyl Sasakawa Medical Aid Project was launched. Until 2001, nearly 200,000 schoolchildren were screened. The results point to the necessity of a cooperative multidisciplinary thyroid cancer research system of the long-term health care of exposed individuals (16,17). Along with a summary of clinical data on Chernobyl thyroid cancer, the project will include the current understanding of the molecular mechanisms of radiation-induced thyroid cancer in children and adolescents. It will focus of how to further assist the long-

term follow-up of the operated patients and will outline the approaches for the identification of high risk groups for the disease.

Age distribution of thyroid cancer morbidity after the Chernobyl accident

A dramatic increase of childhood thyroid cancer was observed in the early 1990s in Belarus (Fig1). The peak incidence of childhood thyroid cancer after the Chernobyl accident is now over, shifting from adolescents to young adult aged more than 20 year-old. Time trends of thyroid cancer incidence are similar among the three affected countries, supporting the concept that subjects of younger age at the time of radiation exposure had, and continue to have, an elevated risk of developing thyroid cancers. Despite of shortage of accurate dosimetry data for individual children, comparative studies in Gomel region, Belarus showed a significant effect of exposure to short-lived radioactive fallout after Chernobyl since, at the time of accident, the frequency of thyroid cancer in zero up to 3 year old children increased dramatically (9). Today, new cases are mainly found in 20 to 30 year old patients.

The difference between early- and late-onset thyroid papillary thyroid cancers after the Chernobyl accident, is under investigation but so far no clinical differences besides of age-related particularities of genetic background have been registered between childhood and adult papillary thyroid cancers.

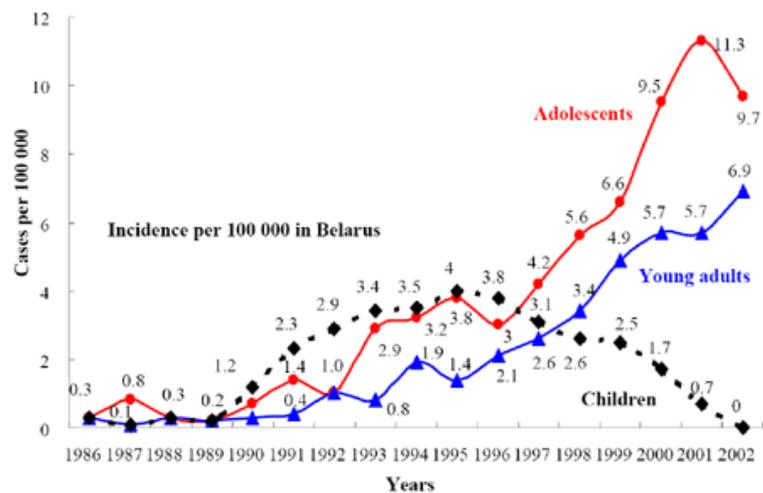


Figure 1: Annual incidence of thyroid cancer at the different age group in Belarus from 1986 until 2002. At the time of surgical operation, three aged groups are categorized from 0 to 14 (Children), from 15 to 18 (Adolescence) and from 19 to 35 year-old (Young adults). Courtesy of Dr. Yuri Demidchik.

How can we distinguish between radiation-induced and sporadic thyroid cancers?

High doses of ionizing radiation produce bulk damages in biological objects inducing cell death. In contrast, low doses induce mainly numerous DNA double strand breaks, deletions, point mutations and/or chromosomal instability. It is therefore reasonable to postulate that radiation induced papillary thyroid cancer could have specific molecular markers. Three major approaches for molecular discrimination between radiation-induced and sporadic thyroid cancers can be used: 1) mutational studies in radiation-induced and sporadic thyroid tumors, 2) comparative gene expression studies, and 3) genomic studies including molecular epidemiology in patients who developed radiation-associated thyroid cancers.

Gene analysis resulted in the discovery of fusions between RET located on chromosome 10q11.2 and other genes are specifically found in papillary thyroid cancer tissues. These are collectively called RET/PTC rearrangements and represent chimeric genes. Among 16 different types of RET/PTCs, RET/PTC1 and RET/PTC3 are the most common variants accounting for about 90% of all chimeric genes (18). The prevalence of RET/PTC rearrangements ranges from 11% to 43% in sporadic papillary thyroid cancers and 50-80% in patients with a history of radiation exposure. In children affected by the Chernobyl accident, RET/PTC3 was the most common type in tumors developed less than 10 years after the accident, whereas papillary thyroid cancers which occurred after a longer latency, had predominantly RET/PTC1 (19). However, the high prevalence all the RET/PTC rearrangements is characteristic of papillary cancer in young patients and not specific for irradiation. Another type of gene rearrangement, AKAP9-BRAF fusion, has been found in 11% of early onset papillary thyroid cancers but in 0% of tumors with the longer latency after the accident (20). Point mutation analysis of RAS-RAF-MAPK cascade genes, such as BRAF and RAS were also performed. They showed no significant difference of the mutational frequency between radiation-induced and sporadic thyroid cancers when similar age groups of patients were compared (21,22). The BRAF point mutation around Chernobyl is rare in childhood thyroid cancer and similar to other areas in comparison with adult papillary thyroid cancer (23).

As a whole, analysis of the mutational spectrum of the Chernobyl thyroid malignancies demonstrates that gene rearrangements leading to the activation of MAPK signaling pathway appear to play a perceptible role in radiation-induced papillary thyroid cancer. Yet, none of the cancer genes or impaired tumor suppressor genes has proved a marker of radiation etiology and gene expression patterns in radiation-related papillary thyroid cancers are similar to those in sporadic ones (24). Therefore at a moment there is no established "radiation signature" or any specific target gene has been identified.

Necessity of molecular epidemiology investigations

In view of the absence of genetic markers to distinguish between radiation-induced and sporadic papillary thyroid cancers, further genomic studies may give us critical hints of radiation sensitivity and tumor-prone susceptibility in man. Since our understanding is very limited as for why thyroid tumorigenesis occurs in a relatively small number of exposed individuals, large scale molecular epidemiology investigations in thoroughly designed cohorts around Chernobyl can potentially identify at the biochemical or molecular level specific exogenous and/or host factors which play a role in human cancer causation. Pilot studies suggest that molecular epidemiological methods targeting single nucleotide polymorphisms of DNA damage response and cell cycle control genes may be a promising tool in the area of radiation-induced carcinogenesis (25).

Chernobyl Tissue Bank

The considerable progress in our knowledge concerning radiation-induced leukemia mechanisms in children (26) may lead us to similar progress in radiation-induced thyroid cancer. One can surmise that the risk of radiation-induced thyroid cancer in a population may be largely attributable to a small number of predisposed individuals in whom clonally expanded translocation-carrying pre-cancer cells have accumulated. The high frequency of RET/PTC rearrangement has been predominantly seen in the early onset cancers in young age group of children after the Chernobyl accident; it seems to be declining gradually with patients' aging. The immature or precursor stem-cell like thyrocyte may be considered a preexisting initiated cell that might harbor a RET/PTC rearrangement. Indeed, RET/PTC rearrangement alone is unlikely to be sufficient to transform human thyrocyte. Thus, it is essential to elucidate genetic particularities of patients with radiation-induced thyroid cancers.

A research bank of biological samples and data has been established as an international cooperative project, the so called "Chernobyl Tissue Bank" (27) which is open to the scientific community. This is likely to favor markedly progress in this area of research.

Summary and conclusion

Today, 20 years after the Chernobyl accident, the large increase in thyroid cancer incidence among those exposed in childhood and adolescence continues. In contrast, no clearly demonstrated increase in the incidence of other cancers can be attributed to radiation exposure from the accident (28). Although radiation-induced thyroid cancer is a well-recognized medical phenomenon based on wide-ranged epidemiological studies, molecular signature(s) and other details of papillary thyroid cancer remain to be further clarified to pinpoint differential diagnostic criteria not only in childhood and adult thyroid cancers but also in radiation-induced and sporadic cancers (29). The latest study in Hiroshima and Nagasaki Atomic Bomb survivors in Japan has indicated that a biological effect from a single brief external exposure to ionizing radiation nearly 60 years in the past still occurs and can be detected (30). In childhood, once exposed even to low doses of ionizing radiation, either externally or internally, the cancer-prone cell damage within the thyroid gland can be preserved for a long time. Today, special attention should be paid to a high risk group of individuals who have been exposed to radioactive iodines just after the Chernobyl accident and who are now 20 to 30 year-old. Elucidation of the molecular mechanisms of radiation-induced thyroid cancer is expected to contribute to the disease prevention and treatment in the coming future.

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DEFINING THE ROLES OF THE CELL SURFACE RECEPTOR FOR THYROID HORMONE

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Introduction

The actions of thyroid hormone on gene transcription have been well-studied and involve a family of nuclear receptors for 3,5,3'-triiodo-L-thyronine (T_3) that are transactivator proteins (1, 2). The principal receptor, TR β 1, is usually found as a heterodimer with retinoid X receptor (RXR), another member of the superfamily of nuclear hormone receptors (1). In the repressed state, TR is associated with corepressor proteins, such as NCoR and SMRT. The binding in the cell nucleus of the natural ligand of the receptor, T_3 , is associated with shedding of the corepressors by TR, with recruitment of coactivator proteins, such as p300 (3) and, subsequently, with binding of the protein- T_3 complex to thyroid hormone response elements (TREs) of thyroid hormone-responsive genes. Transcription of the hormone-responsive genes results. Assumptions in this concept of nucleus-mediated thyroid hormone action include 1) few or no actions of the hormone at the plasma membrane and in cytoplasm, except for modulation of mitochondrial respiration, 2) predominance of T_3 as the active form of the hormone in the cell and the concept that L-thyroxine (T_4) is a prohormone, yielding T_3 by 5'-deiodination, 3) localization of TR to the cell nucleus, 4) residence in the nucleus as a small heterodimeric complex with either corepressors or with coactivators and T_3 . These assumptions, together with extensive studies of the structure-function relationships of domains of TR (1, 2, 4) have served to provide a clear understanding of the transcriptional activity of the hormone.

Integrin Receptor-Mediated Actions of Thyroid Hormone

Evidence that thyroid hormone can act primarily outside the cell nucleus has come from studies of mitochondrial responses to T_3 (5) or T_2 (6), from rapid onset effects of the hormone at the cell membrane (7-9) and from actions on cytoplasmic proteins (10, 11). The recent description of a plasma membrane receptor for thyroid hormone on integrin $\alpha V\beta 3$ (12-14) has provided some insight into effects of the hormone on membrane ion pumps, such as the Na⁺/H⁺ antiporter (9, 15), and has led to the description of interfaces between the membrane thyroid hormone receptor and nuclear events that underlie important cellular or tissue processes, such as angiogenesis (16, 17) and proliferation of certain tumor cells (18, 19).

Circulating levels of thyroid hormone are relatively stable; therefore, membrane-initiated actions of thyroid hormone on neovascularization or on cell proliferation or on membrane ion channels—as well, of course, as gene expression effects of the hormone mediated by TR mentioned above—may be assumed to contribute to 'basal activity' or setpoints of these processes in intact organisms. The possible clinical utility of cellular events that are mediated by the membrane receptor for thyroid hormone may reside in inhibition of such effect(s) in the contexts of neovascularization or tumor cell growth. Indeed, we have shown that blocking the membrane receptor for iodothyronines with tetraiodothyroacetic acid (tetrac), a

hormone-binding inhibitory analogue that has no agonist activity at the receptor, can arrest growth of glioma cells (19) and of human breast cancer cells in vitro (18). Tetrac is a useful probe to screen for participation of the integrin receptor in actions of thyroid hormone. In this review we will briefly summarize some of the known effects of thyroid hormone that are mediated by the integrin receptor and then concentrate on new directions to explore in the area of membrane receptors for the hormone.

Integrin $\alpha\text{V}\beta\text{3}$ binds thyroid hormone near the Arg-Gly-Asp (RGD) recognition site of the protein; the RGD site is involved in the protein-protein interactions linking the integrin to extracellular matrix (ECM) proteins such as vitronectin, fibronectin and laminin (13). The intact integrin is structurally very plastic (20). Its conformational changes in response to ligand-binding may underlie its ability to transduce cell surface signals into discrete intracellular messages, as well as the ability to expose new surfaces for interactions. The integrin also generates crosstalk with other cell surface receptors. The thyroid hormone signal at the integrin is transduced into mitogen-activated protein kinase (MAPK) activity via phospholipase C and PKC (21). MAPK (ERK1/2) activation is associated with increased Na^+/H^+ antiporter activity locally at the plasma membrane in response to thyroid hormone (15) and we speculate that hormone effects on other ion pumps at the cell surface relate to MAPK or PKC activation. Hormone-activated MAPK also is directed rapidly to the cell nucleus where it may phosphorylate TR β1 at Ser-142 (22), leading to disruption of the corepressor protein-TR complex and recruitment of co-activators. The fact that this can be achieved with agarose- T_4 that does not cross the cell membrane means that 'de-repression' of TR can be instigated from the cell surface without T_3 (or T_4) in the cell nucleus. However, only low ('basal') levels of transcription appear to be achieved in this manner and the natural ligand, T_3 , apparently must be present in the nucleus in order to achieve multiple-fold increases in transcriptional activity of TR.

Also initiated at the cell surface integrin receptor is the complex process of angiogenesis, monitored in either a standard chick blood vessel assay (16) or with human endothelial cells in a sprouting assay (S Mousa, PJ Davis: unpublished observations). This hormone-dependent process requires MAPK activation and elaboration of basic fibroblast growth factor (bFGF; FGF2) that is the downstream mediator of thyroid hormone's effect on angiogenesis (16). Tetrac blocks this action of T_4 and T_3 , as does RGD peptide and small molecules that mimic RGD peptide. It is possible that desirable neovascularization can be promoted with local application of thyroid hormone analogues, e.g., in wound-healing, or that undesirable angiogenesis, such as that which supports tumor growth, can be antagonized in part with tetrac.

Thyroid hormone can also stimulate the proliferation in vitro of certain tumor cell lines (13). Murine glioma cell lines have been shown to proliferate in response to physiological concentrations of T_4 (19) by a mechanism initiated at the integrin receptor and that is MAPK-dependent. In what may be a clinical corollary, a prospective study of patients with far advanced glioblastoma multiforme (GBM) in whom mild hypothyroidism was induced by propylthiouracil showed an important survival benefit over euthyroid control patients (23). We reported in 2004 that human breast cancer MCF-7 cells proliferated in response to T_4 by a mechanism that was inhibited by tetrac (18). A recent retrospective clinical analysis by Cristofanilli et al. (24) showed that hypothyroid women who developed breast cancer did so later in life than matched euthyroid controls and had less aggressive, smaller lesions at the time of diagnosis than controls. Thus, the trophic action of thyroid hormone on in vitro models of both brain tumor and breast cancer appears to have clinical support.

The cellular or tissue actions of thyroid hormone that are known to be initiated at integrin $\alpha\text{V}\beta\text{3}$ and that require transduction of the hormone signal via MAPK are summarized in Fig. 1.

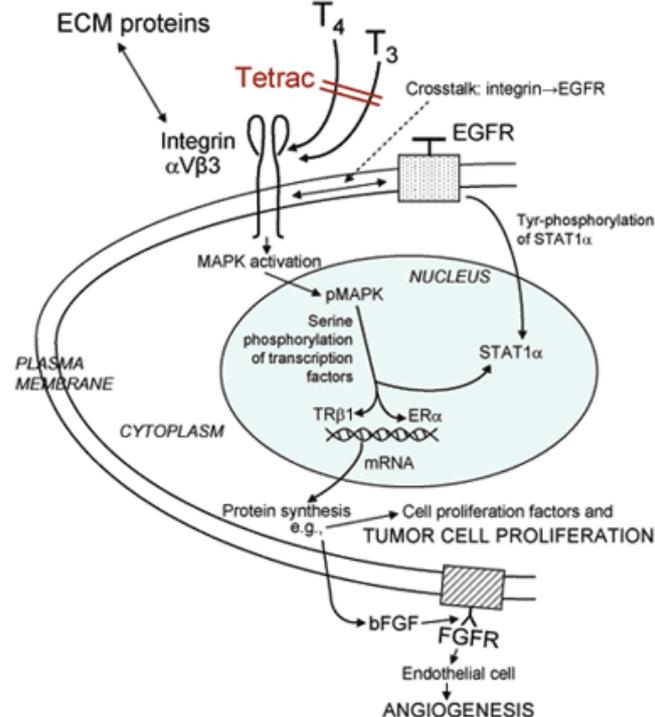


Fig. 1. Membrane-initiated actions of thyroid hormone that involve the hormone receptor on integrin $\alpha V\beta 3$. The integrin is a signal transducing protein connecting signals from extracellular matrix (ECM) proteins to the cell interior (outside-in) or from cytoplasm and intracellular organelles to ECM (inside-out). Binding of L-thyroxine (T_4) or 3,5,3'-triiodo-L-thyronine (T_3) to heterodimeric $\alpha V\beta 3$ results in activation of mitogen-activated protein kinase (MAPK; ERK1/2). Activated MAPK (phosphoMAPK, pMAPK) translocates to the cell nucleus where it phosphorylates transactivator proteins such as thyroid hormone receptor- $\beta 1$ (TR $\beta 1$), estrogen receptor- α (ER α) or signal transducer and activator of transcription- 1α (STAT1 α). Among the genes consequently transcribed are basic fibroblast growth factor (bFGF), that mediates thyroid hormone-induced angiogenesis) and other proliferation factors important to cell division of tumor cells. Depicted in this figure in red is the ability of tetraiodoacetic acid (tetrac) to inhibit the action of T_4 and T_3 at the integrin; tetrac blocks the binding of iodothyronines to the integrin receptor. Also shown is crosstalk between the integrin and epidermal growth factor receptor (EGFR). Here, the presence of thyroid hormone at the cell surface alters the function of EGFR to allow the latter to distinguish EGF from TGF- α , another growth factor that binds to EGFR.

When studied as an isolated heterodimeric protein and in contrast to TR, the integrin $\alpha V\beta 3$ thyroid hormone receptor has a higher affinity for T_4 than for T_3 (12). Consistent with this observation, T_4 may be more potent than T_3 in MAPK (ERK1/2) activation (25). But physiological concentrations of T_3 are active in MAPK-dependent models of angiogenesis (16) and, in contrast, T_4 is effective only when converted to T_3 in stimulating Na⁺/H⁺ antiporter activity (9). We know this action of the hormone is also MAPK-requiring (15). This spectrum of results suggest that affinities of the integrin for hormone analogues may be different when the integrin is studied as an isolated protein and when it is imbedded in the plasma membrane in experiments involving the intact cell.

New Directions in Characterization of Membrane-Initiated Actions of Thyroid Hormone

Definition of Thyroid Hormone Actions that are Initiated Outside of the Nucleus.

Although a cell surface receptor for iodothyronines has been described, this finding does not exclude the possibility that other mechanisms exist for actions of the hormone that begin or are consummated outside the cell nucleus. For example, plasma membrane transporters for thyroid hormone (27, 28) could conceivably be linked to specific intracellular events. In addition, TR is now appreciated to exist in the cytoplasm, as noted above (29, 30), and TR family members could bind cytoplasmic T_4 or T_3 to initiate effects that are exclusively extranuclear or a premonitory step to genomic actions.

It is also possible that more than one integrin contains a thyroid hormone-binding site. At least seven integrins include an RGD domain and could be candidate heterodimeric receptor proteins for thyroid hormone. We are currently pursuing the possibility that an integrin, clearly not $\alpha V\beta 3$, contains a receptor that binds T_3 preferentially. The issue here is whether such a site may support activation of phosphatidylinositol 3-kinase (PI 3-K) activation by T_3 . PI 3-K activation by T_3 has been reported by several laboratories (31, 32), but may be initiated by the hormone after it has achieved the cytoplasmic space.

Is there a Requirement for Membrane Integrin Receptor-Directed Posttranslational

Modification of TR prior to Genomic Action of T_3 ?

Current concepts of nuclear actions of T_3 include the shedding by TR of corepressor proteins and recruitment of coactivators as a consequence of intranuclear complexing of T_3 with TR, resulting in transcriptionally active TR- T_3 . As noted above, we have shown that T_4 at the cell surface can cause specific serine phosphorylation of TR and de-repression of the receptor that could be premonitory to the binding of T_3 and full activation of TR. Through knockdown experiments involving the integrin and pharmacologic inhibition of MAPK, we are investigating the possibility that T_4 and T_3 may work cooperatively to promote TR-based transcription.

Life Cycle of the Integrin Receptor for Thyroid Hormone.

Integrin $\alpha V\beta 3$ is recycled from the plasma membrane to endosomes by mechanisms that can involve protein kinase B (PKB)/Akt (33) or PKD1 (34) under the direction of platelet-derived growth factor (PDGF). It is not yet known if thyroid hormone can induce integrin recycling. Preliminary studies we have carried out of abundance of αV and of $\beta 3$ mRNAs in T_4 -treated CV-1 cells indicates no increase in either compared to untreated cells (12). This indicates that the hormone does not affect transcription of the monomeric genes, as do DNA microarray surveys of gene transcription in thyroid hormone-treated cells (35, 36).

Clustering of Growth Factor Receptors on the Cell Surface and the Integrin Receptor for Thyroid Hormone.

We have concluded that there is cross-talk between the integrin receptor and the epidermal growth factor receptor (EGFR), based on the ability of T_4 to potentiate the activation of MAPK by transforming growth factor- α (TGF- α), a ligand of EGFR (37), and the ability of tetrac to block the thyroid hormone effect. It will be useful to examine the possibility that signals of insulin-like growth factors and of PDGF are modified by iodothyronines.

Is Integrin Signaling to ECM Proteins Affected by Thyroid Hormone?

In a set of interesting studies a decade ago, Farwell and co-workers showed in vitro that the laminin-integrin interaction of astrocytes was affected by T_4 , but not T_3 (38). The process was shown to be RGD peptide sensitive at a time when the existence of an integrin receptor for thyroid hormone was not suspected. It is possible that the action of thyroid hormone on the integrin-laminin interaction required intracellular signal transduction concluding with an inside-out message, but we feel that it is just as likely that the binding of T_4 by the integrin directly induced a conformational change in the integrin that favored interaction with laminin. Such studies could be repeated today with tetrac and with inhibitors of MAPK or of PKC activities to 1) confirm that binding by the integrin of T_4 is the basis of the hormonal effect on laminin and 2) determine whether intracellular signaling is involved.

Actions of Thyroid Hormone on Cell Migration.

Farwell, Leonard and co-workers have recently reported that the rate of migration of neurons is increased by T_4 (39). It is not yet known where in the cell this action of the hormone is initiated. The same group has shown that fibrous actin content of glial cells is increased by T_4 treatment (40), as is that of neurons (39). This stabilization of contractile elements would support cell migration.

Conclusions

A recently-described cell surface receptor for thyroid hormone near the Arg-Gly-Asp (RGD) recognition site on integrin $\alpha V\beta 3$ transduces the hormone signal into mitogen-activated protein kinase (MAPK) activation. Consequent MAPK-dependent events occur at the plasma membrane and in the nucleus. At the cell membrane, basal activities or set points of certain ion pumps or channels may be regulated in part by thyroid hormone-directed MAPK. The nuclear thyroid hormone receptor (TR $\beta 1$) may be de-repressed from the cell surface by physiological concentrations of L-thyroxine (T_4) and complex cellular activities, such as angiogenesis, may be initiated at the integrin by T_4 or 3,5,3'-triiodo-L-thyronine (T_3). Proliferation of certain tumor cell lines may be stimulated by iodothyronines through the integrin receptor, notably, glioma cells and breast cancer cells. Tetraiodothyroacetic acid (tetrac) inhibits the binding of thyroid hormone to the integrin receptor and thereby blocks the pro-angiogenic effect of the hormone and actions of the latter on tumor cells. In this review we identify areas of further investigation regarding cell surface actions of iodothyronines. These include the possibility of additional receptors in the plasma membrane, proof of cooperativity between derepression of TR from the cell surface receptor by T_4 and important enhancement by nuclear T_3 of transcriptional activity of TR and crosstalk between the hormone receptor and polypeptide growth factor receptors on the cell surface.

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How many iodide transporters are there ?

How many true iodide transporter do we know ?

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How many iodide transporters are there ?

How many true iodide transporter do we know ?

Iodide entering thyroid gland and its functional units, the follicles, must sequentially cross two lipid bilayers: the basolateral and then the apical plasma membrane of thyrocytes (Step 1 and Step 2 – Fig.1) to reach finally the lumen of follicles. In this compartment, iodide is oxydized and used for the generation of iodothyronine residues inside thyroglobulin molecules.

Under certain circumstances such as an alteration of iodide oxidation...., iodide can leave the lumen of follicles by crossing the same plasma membrane domains but in the reverse direction (Step 3 and Step 4 – Fig. 1).

Follicle lumen

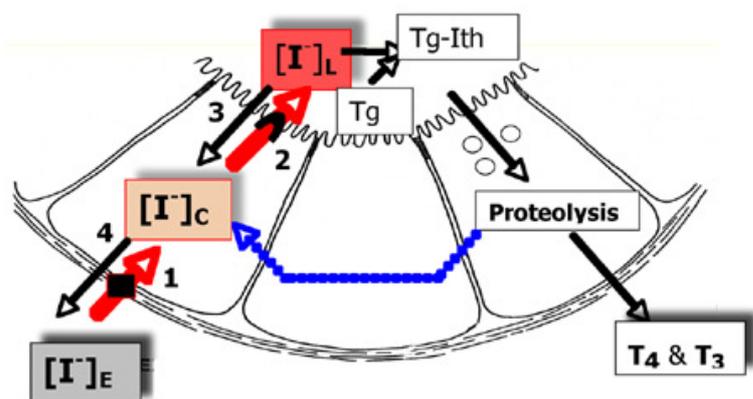


Fig.1: Schematic representation of iodide fluxes in the thyroid follicle. $[I]_E$, $[I]_C$ and $[I]_L$ are iodide concentration in extracellular fluid, cytoplasm and lumenal compartment, respectively. Numbers identify the different steps of the thyroid iodide transport. The dotted line illustrates the re-utilization of iodide coming from the deiodination of iodotyrosines generated by proteolysis of thyroglobulin (Tg).

1- Iodide transport – General considerations

As iodide and small charged molecules cannot easily diffuse through cell membranes, its transfer from one side to the other side of the plasma membrane requires a **membrane protein**. As the cell interior is negatively charged with respect to the extracellular milieu or to the follicle lumen, the transport of iodide inside thyroid cells (**iodide influx**) at either pole requires energy. Thus, the membrane protein involved in either Step 1 or Step 3 must be an active transporter i.e. an ATP-driven pump or a Na^+ gradient-dependent transporter. On the opposite, the movement of iodide from the cytoplasm of thyrocytes to either the follicle lumen or to the extracellular (extrafollicular) milieu (**iodide efflux**) is expected to be a passive process; it takes advantage of the favorable electrochemical gradient. Thus, Step 2 and Step 4 would require a membrane protein with a function of "permease" or a function of ion channel. From these considerations and experimental data, it is clear that the influx and efflux reactions occurring at a pole of thyrocytes (either basolateral or apical) are not mediated by a given protein capable of transporting iodide in either direction.

It is thus reasonable to think that thyrocytes could express four distinct membrane proteins with either a function of iodide transporter or a function of iodide channel ; a high selectivity towards iodide would only be required for some of them. At present, how many of these proteins do we know ?

2- Iodide transport – Step 1.

The nature and the main properties of the active iodide transport system located at the basolateral plasma membrane of thyrocytes allowing the uptake and the concentration of iodide

in the thyroid is known since several decades (1,2). It has formally been identified ten years ago (3) and named NIS for Na⁺/Iodide Symporter. Since that time, NIS expression and activity has been the subject of a large number of studies and reviews (4). NIS belongs to the solute linked carrier (SLC) transporter family and more precisely to the SLC5A subfamily of Na⁺-dependent transporters as the SLC5A5 member. NIS is characterized by a high selectivity for iodide especially towards chloride; it transports complex anions such as perchlorate which exhibits a size comparable to that of iodide. Thus, perchlorate ion is a competitive inhibitor of the iodide transport by NIS (5,6).

3- Iodide transport – Step 2.

The transport of iodide from the cytoplasm to the follicle lumen has been elegantly studied on polarized porcine thyrocytes cultured as tight monolayers in bicameral devices by Nilsson and his colleagues (7,8); they proposed that the apical efflux of iodide, which is rapidly increased in response to TSH, could occur through a perchlorate-insensitive, cAMP-regulated iodide channel. Independently, it was reported that an inhibitor of anion channels, DIDS, completely blocks apical iodide efflux from polarized thyrocytes (9). A study performed on thyroid membrane vesicles (10) also concluded to the existence of an iodide channel in the thyroid; however, the authors could not assign a precise location (in relation with the cell polarity) to this channel.

These data deriving from functional studies indicate that the membrane protein insuring the apical iodide efflux (expected to be a passive process) might be an anion channel.

Information about the molecular identity of the protein insuring apical iodide transport has been provided by genetic and genomic analyses. The discovery that pendrin, the protein encoded by the gene altered in the Pendred syndrome i.e. the PDS gene, is a membrane protein located at the apical pole of thyrocytes with an ion transport activity rapidly gave rise to over-interpretation (11). Indeed, the scientific community interested in the thyroid field was waiting for the identification of the apical iodide transporter ; it was claimed that pendrin might be an iodide transporter; pendrin was very rapidly considered as the apical iodide transporter despite the uncertainties and/or discrepancies which were apparent since the first reports. The initial proposal of pendrin as a sulphate transporter (12) was rapidly abandoned (13). Most subsequent studies performed on different experimental systems assigned a function of anion exchanger to pendrin exchanging chloride for OH⁻, for bicarbonate or for formate (14-17). Some studies concluded on an activity of chloride/iodide transporter(18,19) or chloride/iodide exchanger (20). If pendrin transports chloride and iodide, it is difficult to envisage a physiological contribution of pendrin to apical iodide efflux since the cytoplasmic chloride concentration is likely to be more than 1000- fold greater than that of iodide. An exchange of cytoplasmic iodide with luminal chloride on a 1 to 1 stoichiometry seems very unlikely considering the iodide and chloride concentrations. Still more confusing are the reports claiming that pendrin mediates iodide uptake (not efflux) by MCF-7 cells (21,22) or that pendrin has an activity of sugar transporter (23).

At present, there is no experimental data showing that pendrin activity accounts for the "transfer" of iodide from thyrocytes to the apical compartment in a polarized thyroid cell system. The result of the study performed on NIS expressing polarized MDCK cells transfected with the PDS gene (24) although interesting should not be extrapolated and considered as the demonstration of the function of pendrin in the thyroid. Several other arguments make the hypothesis of pendrin as an apical iodide transporter very uncertain. The knock out of the pds gene in the mouse, which causes functional alterations in the ear, does not induce any thyroid phenotype (25). It has often been quoted that many patients with the Pendred syndrome having a non-functional pendrin do not exhibit any thyroid alteration.

As already discussed by one of the pioneer and main contributor of the "iodide transport" field (26), it seems difficult to admit that a given protein could have different functions in different organs. As a simple suggestion, pendrin (SLC 26 A4 in the SLC transporter nomenclature) expressed in the thyroid, could exert (as in the kidney) a function of chloride/ bicarbonate exchanger (the most admitted function) and play, for exemple, a role in the control of luminal pH with a possible incidence on iodide oxidation.

Indecision about the identity of the apical iodide transporter : pendrin or nor pendrin ?, has been complicated by a report describing a potentiel "competitor" for the same function named AIT for Apical Iodide Transporter (27). As pendrin, AIT appears selectively located at the apical plasma membrane of thyrocytes and, as pendrin, was reported to cause iodide discharge in transfected cell systems. The first problem arose when it was found, using colon cell lines, that this protein, structurally related to NIS was a Na⁺-dependant transporter (28) belonging to the SLC5 family as the SLC5A8 member. Indeed, both previous studies and prediction (on biological considerations) do not point to a Na⁺ dependency for the apical iodide transport. Soon after, two independent groups (29-31) described SLC5A8 as a Na⁺- coupled transporter for short-chain fatty acids or as a Na⁺/ monocarboxylate co-transporter. Analyses of the recent studies on SLC5A8 indicate a consensus on this function (32,33) . Attempts by several investigators to confirm an activity of SLC5A8 in iodide efflux have been unsuccessful (personal communication).

At this stage, **one has to conclude that the identity of the protein(s) insuring the Step 2 of the iodide transport in the thyroid is not known.**

Interestingly, SLC5A8 appears to be endowed with a tumor suppressor function, which was convincingly demonstrated in colon cell lines. SLC5A8 expression is largely reduced (by

more than one order of magnitude) in colon cancers (28), gastric cancers (34), gliomas (35) but also in thyroid cancers (36,37). The silencing of SLC5A8 gene in thyroid cancer appears restricted to the group of papillary thyroid carcinomas of classical type (37). In thyroid tumors as in other cancers, the silencing of the tumor suppressor gene SLC5A8 results from an epigenetic event, the methylation of a promoter region (28,37).

4- Iodide transport – Step 3 and Step 4 – A statement of ignorance

These two steps should be operative to allow iodide to go out of follicles. There is often a confusion in the literature concerning iodide efflux from the thyroid. In the intact follicle, iodide efflux means Step 3 and Step 4. Using isolated thyroid cells, Step 4 occurs together with Step 2. Only thyrocytes cultured as polarized monolayer cells in bicameral devices allow to discriminate Step 3 (uptake from the upper or apical chamber) from Step 4 (release of iodide in the lower or basolateral chamber). Studies on this privileged model have generated important information on Step 1 and Step 2 (as already mentioned) but only few data on Step 3 and Step 4. In fact, measurements of apical iodide uptake by adding radioactive iodide in the upper chamber were made but uptake values were very low as compared to the uptake values obtained by adding radioiodide in the lower chamber (38); similarly, measurements of basolateral efflux after cell loading with radioiodide yielded very low values as compared to values of apical efflux (7,8). These observations indicate either that the reaction rates of Step 3 and Step 4 are low as compared respectively to those of Step 1 and Step 2 or that Step 3 and Step 4 mainly occur in certain circumstances which were not fulfilled in the experimental system.

As mentioned in the introductory part, the flux of iodide from the lumen to the cell cytoplasm (Step3) should be energy-dependent because of an unfavourable electrochemical gradient whereas Step 4 is probably a passive process. There is no NIS on the apical plasma membrane but a protein functioning as NIS could be operative; indeed, the Na⁺ concentration in the follicle lumen is close to that of the extrafollicular milieu. One way to learn about a biological process is to know whether it is regulated or not. Information about TSH action on Step 3 is scarce but, we can bring back to light rather old studies reporting the in vivo action of TSH on iodide fluxes (39) analyzed by measurements of the [T]/[S] ratio (or thyroid to serum iodide concentration ratio). TSH injection to rats led to the activation of iodide efflux before the activation of iodide influx (now known to result from TSH-induced activation of NIS gene transcription). Thus, thyroid iodide efflux, likely Step 3, is positively controlled by TSH.

Proteins involved in both Step 3 and Step 4 and mediating thyroid iodide efflux are completely unknown.

5- Concluding remarks

The bidirectional transcellular transport of iodide inside thyroid follicles brings into play several distinct membrane proteins endowed with a function of **ion transporter**, either active or passive or with a function of **ion channel**; at present, only one of these molecular species is identified, the Na⁺/ Iodide Symporter.

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TSH RESISTANCE**Paolo Beck-Peccoz**

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Introduction

TSH resistance (OMIM #275200) is a condition of thyroid refractoriness to TSH stimulation. The first description dates back to 1968 (1) when a child with congenital hypothyroidism, in situ thyroid gland of normal volume, and no increase of iodine uptake after exogenous TSH administration was reported. Since then, a number of other cases have been described. Beginning from the early reports, alterations of TSH receptor (TSHR), G_{α} or other downstream signaling elements were considered as possible causes of this particular disorder. However, it was only after the cloning of TSHR gene (2) that loss-of-function TSHR mutations were recognized as the molecular defect in most familial cases of TSH resistance (3-6). This review will focus on the clinical presentation, differential diagnosis and molecular genetics of this rare disorder.

Clinical and biochemical features of TSH resistance

High levels of serum TSH, normal/reduced levels of serum thyroid hormone and normal/hypoplastic thyroid gland characterize TSH resistance. Depending on the degree of TSH insensitivity, the presentation may be extremely variable, ranging from severe congenital hypothyroidism to only mild elevations of TSH in the absence of signs and symptoms of hypothyroidism.

Table 1. Clinical and biochemical phenotypes associated with inactivating mutations of TSHR depend on the degree of impairment in receptor function.

Resistance degree	Serum TSH	Serum FreeT4	Thyroid gland	Inheritance	Mutated alleles	Original references
Complete	↑↑↑	Low	Profound hypoplasia	Recessive	Two	Abramowicz et al (3)
Partial (moderate)	↑↑	Normal	Mild hypoplasia or normal	Recessive	Two	Sunthornthepvarakul et al (2)
Partial (mild)	↑	Normal	Normal	Dominant	One	Alberti et al (8)

As TSH is the major physiological stimulus of both thyrocyte function and proliferation, a profound reduction of thyroid sensitivity to TSH, as observed in patients with complete TSH resistance, leads to severe hypothyroidism with a hypoplastic thyroid gland. Because of reduced thyroid hormone feedback, TSH is markedly elevated. Such cases are typically diagnosed at the neonatal screening for congenital hypothyroidism. In the first description by Abramowicz et al. (4), thyroid hypoplasia was so profound and radioiodine uptake so impaired that thyroid agenesis was diagnosed at scintigraphy. However detectable serum Tg levels disclosed the presence of thyroid tissue.

When thyroid refractoriness to TSH is incomplete, a condition known as partial TSH resistance, TSH elevation can somewhat compensate for the reduced sensitivity of the thyroid and milder forms of hypothyroidism are seen. Patients typically have a thyroid gland of normal/reduced size, high TSH levels, but concentrations of free thyroid hormones in the normal range (3,5,6).

More recently, we described three families with only mild elevations of TSH levels and a normal thyroid gland, in which the defect was caused by heterozygous inactivating TSHR mutations (see below) (7). Similar alterations can be found in some heterozygous relatives of patients with homozygous TSHR mutations (8). Although such cases can be positive at neonatal TSH screening (7), they are generally diagnosed later on in life, when they can be easily confounded with the more prevalent condition of subclinical hypothyroidism due to autoimmune thyroid disease (AIT).

Differential Diagnosis

The occurrence of elevated TSH serum levels in the presence of low/normal free T4 concentration and thyroid volume is rather frequent in the general population. However, only a minority of these patients is affected with TSH resistance. So, the diagnostic workup should exclude other potential causes such as AIT, defects in TSH molecule (particularly those due to TSH beta gene mutation) and TSH biological activity or other forms of congenital primary hypothyroidism, including abnormalities in thyroid transcription factors (6).

Differential diagnosis with AIT, by far the most frequent cause of TSH elevations in the adult population, is based on clinical history, biochemical evaluation of anti-thyroid antibodies

(anti-TPO and anti-thyroglobulin) and thyroid ultrasound (7). The positivity of anti-thyroid antibodies and/or the presence of the typical heterogeneous hypoechoic pattern at thyroid ultrasound are strongly suggestive of AIT. Other elements in favor of AIT are diagnosis reached in adult/advanced age as well as progressive evolution from subclinical toward overt disease. The only exception is the very rare association of TSH resistance with AIT (9).

Some patients with central hypothyroidism may present with elevated serum TSH levels. These alterations are generally found in patients with hypothalamic (tertiary) hypothyroidism (10,11), but can also occur in the presence of TSH β gene mutations (12). In the former situation, despite high serum concentrations of the immunoreactive hormone, TSH biological activity is reduced, which explains the condition of hypothyroidism. In these cases, the presence of hypothalamic-pituitary disorders supports the diagnosis of central hypothyroidism and prompts to test the biological activity of circulating TSH molecules *in vitro*.

Complete TSH resistance due to loss-of-function TSHR mutations can lead to congenital hypothyroidism with thyroid gland *in situ*. Other known causes are defects in thyroid transcription factors, such as NKX2.1 (also named TITF1) or PAX8 (13). Due to the expression of these transcription factors in tissues other than the thyroid, complex phenotypes have been reported in these cases. Patients with NKX2.1 mutations may have neurological (choreoathetosis) and pulmonary alterations, while PAX8 mutations can be suspected in the presence of kidney abnormalities. NKX2.1 and PAX8 mutations can be excluded by genetic analysis.

TSH resistance can also occur in the context of the multiple hormone resistance syndrome (Albright's Hereditary Osteodystrophy) caused by inactivating mutations of the gene encoding Gs α protein. The presence of high PTH levels associated with hypocalcemia and hyperphosphatemia, as well as typical clinical features are suggestive of this disorder.

Molecular genetics

The congenital/childhood occurrence and the frequent family setting of TSH resistance suggest the genetic origin of the disorder. Therefore, after its cloning in 1989 (2), TSHR became the more obvious candidate gene. Shortly after, inactivating TSHR mutations were identified in a family with recessive transmission of elevated TSH levels and normal thyroid hormone secretion (3). Since then, 23 different TSHR loss-of-function mutations have been documented to be responsible for TSH resistance (5,6). An updated database of TSHR mutations can be found on the web (14). However, some cases of TSH resistance not associated with TSHR or GNAS (Gs α) mutations have been reported, suggesting the probable involvement of other not identified genes (7,15-17).

In the earlier studies, in which only probands with large TSH elevations were screened for mutations, the disease was linked to homozygous or compound heterozygous mutations and was described to follow a recessive pattern of inheritance. More recently, we described patients with a mild form of partial TSH resistance due to heterozygous TSHR mutations. In these cases, the defect had a dominant pattern of inheritance (7). Such genetic heterogeneity is well reflected by diversity of clinical presentation (Table I).

Natural mutations leading to resistance to TSH action are distributed all along the receptor backbone and affect either the extracellular or transmembrane domains. Almost all types of alterations (missense or nonsense mutations, deletions, insertions or alterations in intron-exon boundaries) have been reported. However, missense P162A and C41S mutations appear to be relatively more frequent. All of these mutations are associated with a defective cAMP response to TSH stimulation. This was shown by *in vitro* functional assays using different end points, such as determination of cAMP concentrations or using reporter genes (e.g., luciferase) under cAMP regulation.

The molecular mechanisms responsible for the loss of receptor function are probably multiple (5,6). Lack of receptor expression on the plasma membrane, ligand binding or coupling with G-proteins may be expected in case of mutations causing the synthesis of a truncated receptor. Similarly, the skipping of exon 6 as reported in two patients, which causes the deletion of one leucine-rich repeat in the aminoterminal hormone binding domain, is expected to impair TSH receptor binding. Initially, all missense mutations located in the extracellular domain were thought to alter ligand binding. However, several exceptions to this rule have now been reported (7,18). For instance, mutations C41S and I167N lead to a profound alteration of TSHR native conformation with hampers receptor targeting to the cell membrane (7,18). Another interesting exception is represented by mutation R310C which is instead associated with an unexpected increase in ligand-independent activity, possibly contributing to the euthyroidism observed in the affected patients. In contrast, mutations located in the transmembrane domain may result either in defective transmission of the stimulatory signal or in deranged routing of mutant TSHR to the cell plasma membrane. The mechanism of TSH resistance in patients with heterozygous TSHR mutations is less obvious. We recently demonstrated the presence of an *in vitro* dominant negative effect exerted by some of these mutants (C41S, L467P, C600R) at the level of wild-type receptor maturation and routing to the cell membrane. In fact, in cells co-transfected with wild-type and mutant TSHRs, we observed a reduction of both basal and TSH-stimulated cAMP production as compared to that of cell transfected with wild-type receptor alone.

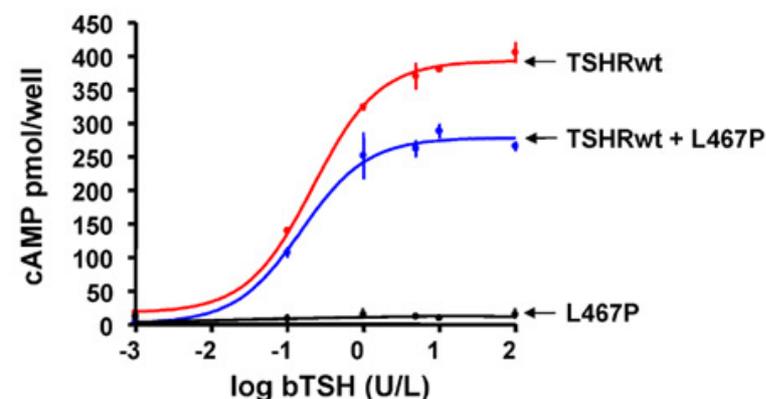


Figure. Dominant negative effect of TSH receptor L467P mutant. Plasmids encoding wild-type TSHR or L467P mutant were either single- or co-transfected in Cos-7 cells. A mutant to wild-type DNA ratio of 3:1 was used to enhance the effect of L467P. Cells were then stimulated with increasing concentrations of bovine TSH (bTSH). No cAMP accumulation was seen in cells transfected with L467P mutant. A partial impairment of cAMP production was observed in cells co-expressing wild-type and mutant receptor as compared to cells expressing wild-type receptor. Similar results were obtained with C41S and C600R mutants.

In addition, the co-expression of mutant TSHRs caused intracellular entrapment, mainly in the endoplasmic reticulum, of wild-type receptor. Finally, we documented, by fluorescence resonance energy transfer and co-immunoprecipitation, the existence of a physical interaction between wild-type and mutant receptors. Based on these data, the occurrence of TSH resistance in these cases appears to be due to a reduction of the amount of wild-type TSHR present at the cell membrane caused by oligomerization with intracellularly retained TSHR

Concluding Remarks

Several issues about TSH resistance remain to be addressed. First, some well-documented cases are not associated with mutations in TSHR coding regions. However, introns and 5' or 3' flanking regions of TSHR gene have not been thoroughly analyzed so far. In addition, other genes could be involved, as recently shown by a linkage analysis study (17). Another debated point is whether to treat or not patients with apparently well-compensated (i.e. partial) TSH resistance. Indeed, clinical evidence suggests that treatment may not be necessary. In fact, several of these patients were diagnosed in childhood or adult age and had quite a normal somatic and neurological development despite untreated since neonatal age. Moreover, one patient reported by our group was diagnosed at neonatal TSH screening, but was not treated with L-thyroxine because of thyroid hormone levels close to the upper limit of the normal range. He is also doing quite well without L-thyroxine supplementation. However, no general rule can be derived from these limited observations and a decision about starting or not therapy should be made in every single patient. In case no therapy is established, we advise a close clinical and biochemical follow-up, including pituitary MRI. In fact, conditions in which thyroid hormone needs are increased, such as puberty or pregnancy, as well as any insult to the thyroid, e.g. AIT, may easily turn euthyroid hyperthyrotropinemia into mild or even overt hypothyroidism.

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Transporter defects - a novel mechanism of thyroid hormone resistance with dramatic consequences

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Introduction

Thyroid hormone is important for brain development which is dramatically manifested by the severe neurological deficits caused by untreated fetal and neonatal hypothyroidism (1). In the brain, neurons are the major target cells for T3 during brain development, and this T3 is largely derived from outer ring deiodination of the prohormone T4 by the type 2 deiodinase (D2) located in neighbouring astrocytes (Fig. 1) (2-4). As in all T3-sensitive cells, the effect of T3 on neurons is largely initiated by its binding to nuclear receptors (TRs) associated with T3 response elements in the promoter region of T3-responsive genes, resulting in an altered transcription of these genes (Fig. 1) (5). In neurons, important T3-responsive genes are involved in the control of the migration, differentiation and arborization of these cells (1, 2). For the termination of T3 action, many neurons also express the type 3 deiodinase (D3) which catalyzes the inactivation of T3 via inner ring deiodination (Fig. 1) (2, 3). Normal brain development requires the coordinated time-dependent and tissue-specific expression of the deiodinases in the different cell types as demonstrated recently also in the developing human brain (6).

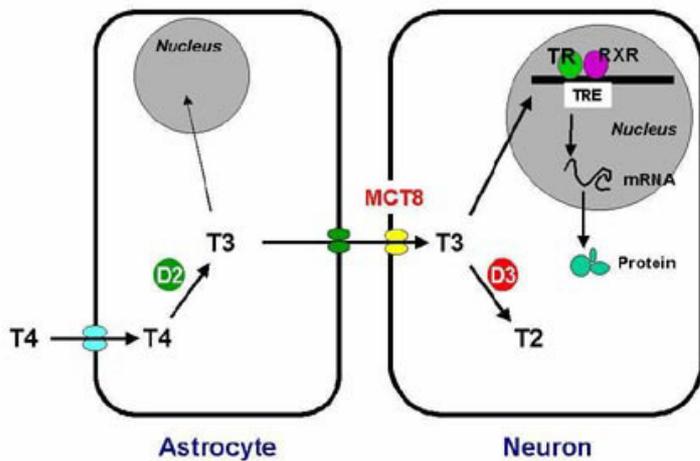


Fig. 1 Local regulation of thyroid hormone bioactivity in the brain in functional units of astrocytes, which express D2, and the major target cells for thyroid action during brain development, the neurons, which express MCT8 and D3. MCT8 is essential for neuronal T3 uptake and thus for T3 action and metabolism in these cells.

Not only the nuclear T3 receptors but also the deiodinase active centers are located intracellularly. Therefore, both action and metabolism of thyroid hormone require the transport of iodothyronines across the plasma membrane (7, 8). This transport does not occur by simple diffusion but is facilitated by transporters. In recent years several iodothyronine transporters have been identified, including organic anion transporters (Na/taurocholate cotransporting polypeptide [NTCP] and organic anion-transporting polypeptides [OATPs]), the L-type amino acid transporter (LAT), and fatty acid translocase (FAT) (7, 8). However, most of these transporters show low activity and specificity towards iodothyronines. A notable exception is OATP1C1 that shows strong preference for T4 as the ligand and is specifically expressed in brain (9-11). This transporter is particularly abundant in brain capillaries, suggesting that it plays an important role in the transport of T4 across the blood-brain barrier (4, 9-11).

Mutations in the MCT8 thyroid hormone transporter

Recently, we have identified rat and subsequently human monocarboxylate transporter 8 (MCT8) as an active and specific iodothyronine transporter (12, 13). The human MCT8 gene was already cloned in 1994 but its role has remained elusive until recently (14). It is located on the X chromosome (Xq13.2), consists of 6 exons and, depending on which of the two translation start sites (TSSs) is used, codes for a protein of 613 or 539 amino acids. The protein contains 12 putative transmembrane domains (TMDs), and both N- and C-terminal domains are located inside the cell. In particular the N-terminal domain is large (170 or 96 amino acids) and contains a PEST domain (rich in P [Pro], E [Glu], S [Ser] and T [Thr] residues) that is probably important for MCT8 turnover (14).

We have recently examined 9 unrelated young boys suffering from severe psychomotor retardation (Fig. 2), who also show strongly elevated serum T3 levels. Serum T4 and FT4 levels are low-normal to clearly decreased, serum rT3 is low, and serum TSH is normal or increased. In all patients this novel syndrome was found to be associated with different mutations in the MCT8 gene, i.e. 4 deletions of ~24 kb, 2.4 kb, 14 bp, and 3 bp (delPhe230 in TMD2); 3 missense mutations (Ala224Val in TMD2; Leu471Pro in TMD9; and Arg271His in the 2nd extracellular loop); a nonsense mutation (Arg245stop); and a splice site mutation resulting in the loss of 94 amino acids, including TMD4-6 (15, 16). Mutations in MCT8 have recently also been reported by Dumitrescu et al. in 2 boys with a very similar phenotype, and by Schwartz et al. and Maranduba et al. in 8 different families with the Allan-Herndon-Dudley

syndrome (AHDS), named after the authors of the paper describing one of these families in 1944 (17-20). In most families with AHDS, the affected males show again a very similar phenotype as in our patients (Fig 2), whereas in some families the phenotype is somewhat milder with some of the patients reaching old age and being capable of walking and speaking albeit with great difficulties. Only recently it has become clear that AHDS is also associated with elevated serum T3 levels (18, 19). None of the patients with MCT8 mutations has been recognized at neonatal screening for congenital hypothyroidism.

Age	1.5-16 years
Neurological findings	Central hypotonia Poor head control Spastic quadriplegia Inability to sit, crawl, stand or walk
Mental development	Severe retardation
Speech development	None
Social development	Poor communication skills
Physical	Reduced body length Very low body weight Microcephaly

Fig. 2 Most common clinical characteristics in patients with MCT8 mutations

Five of the 9 mutations identified in our patients are obviously deleterious for functional MCT8 expression. This is less obvious for the 3 missense mutations and the 3-bp deletion, resulting in the substitution or deletion of single amino acids. These mutations were therefore introduced in the cDNA coding for human MCT8 and their effects were studied by measurement of T3 uptake in cells transfected with wild-type or mutant MCT8. In addition, T3 metabolism was analyzed in cells transfected with wild-type or mutant MCT8 in combination with cDNA coding for human D3. In both systems, the single amino acid substitutions or deletion were found to result in an almost complete inactivation of MCT8 (16). Another interesting mutation was identified in one of the above-mentioned families with AHDS, namely a single nucleotide deletion in the 2nd last codon (18). This results in the by-passing of the natural stop codon until an alternative stop codon is encountered 196 nucleotides further downstream, resulting in the elongation of the protein with 64 amino acids which may include an additional TMD. Also this mutation was introduced in MCT8 cDNA and found to result in a major decrease in T3 uptake and metabolism in transfected cells, although the mutant showed significant residual activity (18). The genotype-phenotype relationship of the MCT8 mutations identified in the various families remains to be fully explored.

The neurological defect caused by mutations in MCT8 is at least as dramatic as that associated with iodine deficiency or untreated congenital hypothyroidism (1). It is hypothesized that inactivation of MCT8 blocks the neuronal entry of T3 and thus its access to its intracellular (nuclear) receptor and degrading enzyme D3 (Fig 1). As a consequence, T3 cannot exert its crucial action in the developing brain, resulting in an impaired neurological development. The reduced breakdown of T3 by D3 supposedly leads to an initial rise in serum T3 that subsequently stimulates the expression of D1 in liver and kidney with a further increase in T3 production. The secondary increase in hepatic D1 expression may also explain the low serum T4 and rT3 levels in patients with MCT8 mutations. This explanation is based on the assumption that the elevated serum T3 results in an increased T3 effect in the liver and kidneys despite the fact that MCT8 is also expressed in these tissues. However, liver and kidneys also express other thyroid hormone transporters that may be responsible for a relatively unhindered T3 uptake even if MCT8 is inactive. This is supported by the findings that serum SHBG levels are strongly elevated in patients with hemizygous MCT8 mutations, suggesting that hepatic SHBG production is increased because of an increased intrahepatic T3 concentration (16).

The effects of MCT8 mutations on the thyroid state of different tissues thus depends on the extent to which T3 uptake in these tissues requires a functional MCT8 transporter. MCT8 appears essential for T3 uptake in central neurons, and its inactivation will thus result in a hypothyroid state in different brain regions despite the high extracellular T3 levels. If MCT8 is only one out of many transporters facilitating T3 uptake in liver and kidneys, these tissues may well be thyrotoxic because of the high circulating T3 concentrations. In other tissues where MCT8 is an important but not the only T3 transporter, inactivation of MCT8 may result in a partial block of T3 uptake which in combination with the high serum T3 concentration could lead to relatively normal intracellular T3 concentrations. The heart which is known to express MCT8 may be an example of such a tissue which function appears to be normal in patients with MCT8 mutations.

Conclusion

Much remains to be learned about exactly how hemizygous mutations in MCT8 cause the severe X-linked psychomotor retardation in affected males. Obviously, mutations in thyroid hormone transporters represent a novel mechanism of thyroid hormone resistance with dramatic consequences.

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Multiple etiologies for reduced sensitivity to thyroid hormone

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Resistance to thyroid hormone (RTH) is a syndrome of reduced end-organ responsiveness to thyroid hormone (TH) that manifests as persistent elevation of serum levels of T_4 and T_3 with non-suppressed TSH. Following its clinical identification in 1967 (1) various potential mechanisms including transport, metabolism and action have been explored to explain the etiology of the defect (2). In 1989, three years after cloning of the nuclear TH receptor (TR) (3, 4), the first two mutations in the $TR\beta$ gene were identified as the cause for RTH (5, 6). The finding of mutations in other subjects with RTH has established the link between the syndrome and defects of the TR, a transcription factor whose principal action at the level of the nucleus is modulated by TH (7, 8). Nevertheless, in a broader sense, reduced sensitivity to TH encompasses all defects that can interfere with the expression of the biological activity of a chemically intact hormone supplied in normal amounts. These could be due to defects in 1) TH entry into the cell, 2) its intracellular metabolism and distribution, 3) cytosolic (non genomic) effects, 4) translocation into the nucleus, 5) association with the receptor and 6) abnormalities in co-regulators or other post receptor effects required for the proper mediation of TH action (Fig.1).

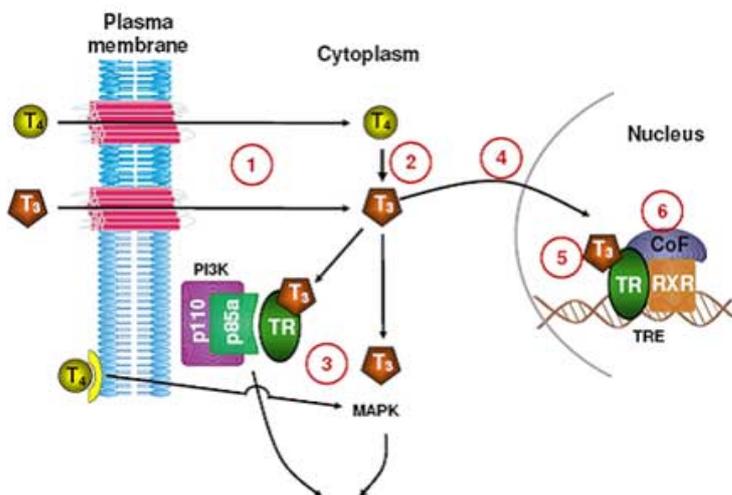


Figure 1. Thyroid hormone action: from entrance into the cells to the nuclear and cytosolic action. For details see text.

Non-TR RTH

Important for the understanding of the mechanism of TH action is the occurrence of RTH in the absence of $TR\beta$ mutations as 15% of families with RTH do not harbor mutations in TR.

The clinical manifestations and laboratory abnormalities in such subjects are not different from those with mutations in the TR β gene (9). Several lines of evidence suggest that cofactors involved in the TR-mediated TH action are likely candidates in the etiology of RTH (10) and this type of mechanism has been labeled as nonTR-RTH (10, 11). In humans, combined resistance to glucocorticoids, mineralocorticoids and androgens have been reported in the absence of mutations in the respective receptors (12-14) and a lack of a putative cofactor has been suggested (14).

Failure to identify mutations in the TR α gene, have led to speculations that either defects are innocuous or are lethal. Unexpectedly, mice deficient in all forms of TR α are more sensitive to TH (15), and the brain of mice deficient in TR α 1 is protected from the effects of hypothyroidism (16). In contrast, KI mice, heterozygous for a mutant TR α , have severe postnatal developmental and growth retardation, as well as reduced fertility, increase in body fat, insulin resistance and decreased cold-induced thermogenesis (17-19). Homozygotes do not survive, emphasizing the noxious effect of unliganded TR α 1.

Recent investigations have identified and explored the roles of cell membrane TH transporters (20-22) and the pathways of intracellular metabolism that lead to either TH activation by conversion of the secreted prohormone T₄ to T₃, or its inactivation by conversion to rT₃. However, the physiological importance of these protein molecules did not become apparent until the very recent discovery of genetic defects that produce complex clinical phenotypes and characteristic abnormalities in thyroid function tests.

TH transporter defect

Defects in the X-linked monocarboxylate transporter (MCT) 8, a transmembrane TH transporter have been reported (23-26). Affected males present a syndrome characterized by abnormal thyroid tests, high T₃, low T₄ and rT₃, slightly elevated TSH and severe psychomotor developmental delay, no verbal communication, mental retardation, generalized dystonia combined with spasticity and poor coordination. As for most X-linked diseases, female carriers have only mild thyroid test abnormalities and no neurologic manifestations. Identification of neurologically asymptomatic females allows the provision of prenatal diagnosis and genetic counseling for this serious condition.

Defects in TH metabolism

Iodothyronine deiodinases (Ds) are selenoproteins that regulate intracellular TH (Fig.2). This unique class of proteins requires selenocysteine (Sec) for enzymatic activity. Sec is incorporated into the nascent protein chain through recoding of an in frame UGA stop codon. Several factors are required for Sec insertion, cis-acting sequences present in the mRNA of a selenoprotein (UGA codon and Sec insertion sequence, SECIS) and trans-acting factors [elongation factor eEF^{Sec}, tRNA^{Sec} and SECIS-binding protein (SBP2)].

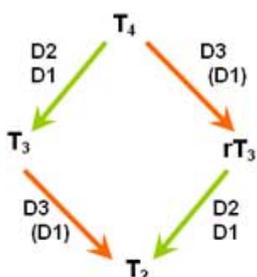


Figure 2. Diagram of TH metabolism.

D1 and D2 are the principal enzymes that convert T₄ to T₃ and rT₃ to 3,3'-diiodothyronine (T₂), while D3 and to a lesser degree D1 convert T₄ to rT₃ and T₃ to T₂ (27). D activities are modulated by the availability of substrate and environmental factors such as food intake and illness. While acquired changes in D activities are common, until recently inherited defects have not been identified in humans.

We identified two families with abnormal thyroid function tests (28) suggestive of abnormal TH metabolism. In a Bedouin Saudi family, two brothers and a sister had high serum T₄ (total and free), high rT₃, low T₃ and slightly increased TSH and transient growth retardation. The parents and other 4 siblings had normal tests. In an Irish family one child born from non-consanguineous parents had a similar phenotype. Linkage analysis and sequencing excluded abnormalities in all 3 DIO genes, yet in vivo TSH-suppression tests suggested a defect in T₄ to T₃ conversion in the affected

children. In vitro tests of patients' fibroblasts showed significantly reduced baseline and cAMP-stimulated D2 activity, despite a normal increase in DIO2 mRNA expression, suggesting a defect in generating or maintaining an active D2 enzyme. The selenocysteine present in the active center of D2 is required for proper enzymatic activity (27), and D2 is subject to ubiquitination (29, 30). Systematic linkage analysis with candidate genes excluded all known factors in these pathways except for SBP2.

At the SBP2 locus the affected Bedouin children shared homozygous haplotypes. A homozygous mutation R540Q was identified in these children, and the parents and the four unaffected siblings were heterozygous carriers of this mutation. The child of Irish origin harbored compound-heterozygous mutations in SBP2 not present in controls, a nonsense mutation K438X and an intronic mutation IVS8ds+29 G->A creating an alternative donor splice site and abnormal transcripts incorporating parts of intron 8.

All four affected children from both families had reduced levels of other selenoproteins. For example glutathione peroxidase (GPx) activity in serum and in fibroblasts was decreased, as were the levels of serum selenoprotein P (SePP) and total serum selenium. The global effect of SBP2 deficiency on the synthesis of selenoproteins has been documented and represents an interesting example of epistatic effect resulting in deficiency of selenoproteins. Although the reduction in GPx and SePP is not trivial, thyroid abnormalities resulting from decreased D2 activity and likely also D1 and D3, appear to dominate the clinical phenotype. Among the known selenoproteins, the UGA codon of DIO2 gene is most distant from the SECIS element and the half-life of the protein is less than 45 min. These factors and the hierarchy among selenoproteins might aggravate a deficit in Sec incorporation producing this

specific thyroid phenotype.

It is believed that SBP2 is the major determinant of Sec incorporation as its in vitro addition increases selenoprotein synthesis by 20-fold, whereas its immunodepletion eliminates Sec incorporation (31). The relatively mild phenotype manifested in the patients described above is due to partial loss of SBP2 function. In the Saudi family, the missense mutation is likely to function as a hypomorphic allele. In the affected child of the Irish family the intronic mutation results in partial alternative splicing with preservation of about 25% normal transcripts. Insight into consequences of SBP2 gene defect is novel and represents the first report of mutations in a component of the machinery leading to the synthesis of selenoproteins, and the first instance of inherited deiodinase deficiency in humans.

Concluding overview

Two novel mechanisms of reduced sensitivity to TH (Fig.3, lines B and C respectively) have been uncovered. These forms of reduced sensitivity to TH also present different modes of inheritance due to gene location and to protein function. RTH due to TR mutations is inherited as an autosomal dominant trait with the exception of the autosomal recessive inheritance of a deleted TR in the index family. The dominant inheritance requires that the mutant TR interfere with the function of the normal TR (dominant negative effect). MCT8 defect has an X-linked inheritance, and SBP2 defect has autosomal recessive inheritance. For the rest of the families with non-TR RTH of unknown cause, the mode of inheritance is less clear though dominant is apparent in some. Other defects at putative steps in TH action are still to be identified and non-Mendelian modes of inheritance and defects with low penetrance should be considered.

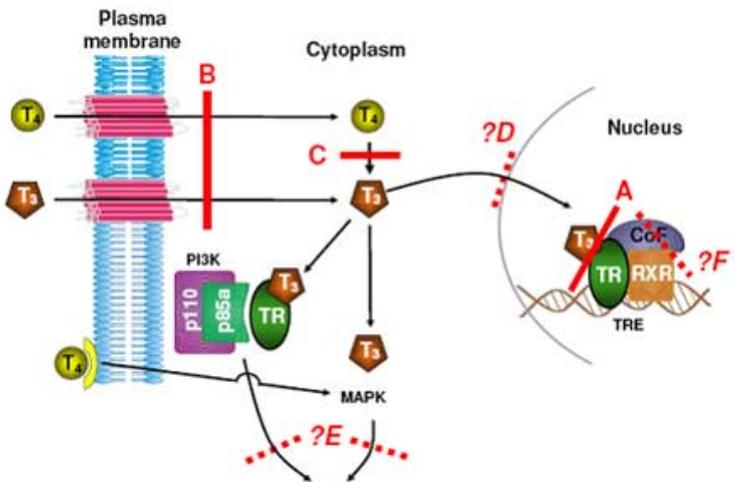


Figure 3. Defects in thyroid hormone action. Shown in red are reported (straight lines A, B, C) and putative (dotted line, D, E, F) defects.

Summarizing the characteristics of the 3 forms of reduced sensitivity to TH (Table 1), it is apparent that the two novel forms have distinctive patterns of TH concentrations in serum compared to those characteristic of TR mutations. Subjects with MCT8 defect have a more complex phenotype in terms of thyroid and neurological manifestations.

	TRβ defect	MCT8 defect	SBP2 defect
T4	↑	NI or ↓	↑
T3	NI or ↑	↑	NI or ↓
rT3	↑	↓	↑
TSH	NI or ↑	NI or ↑	NI or ↑

Table 1. Summary of thyroid tests abnormalities in the known syndromes of reduced sensitivity to TH. [NI = normal, ↑ = increased, ↓ = decreased, NI or ↑(↓) = indicates values often straddling the upper (lower) limit of normal).

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Autonomic innervation of the thyroid gland and its functional implications.

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Most endocrine glands are governed by both neuro-endocrine and neural factors. As for the thyroid gland, it is well established that the trophic hormone TSH, secreted by the anterior pituitary, is the principal endocrine regulator of thyroid function. The serum concentration of TSH is regulated within the context of the hypothalamus-pituitary-thyroid (HPT) axis, which has received a great deal of attention in the literature over the past decades. In addition to this neuro-endocrine regulation, it has been known for many years that the thyroid gland is also richly innervated by both sympathetic and parasympathetic nerve fibers. In this short review, we will focus on neuro-anatomical aspects and functional implications of autonomic innervation of the thyroid gland highlighting recent advances in this research area.

Neuro-anatomy

As early as in the 1930s, nerve-endings surrounding blood vessels and follicles in the thyroid were reported, suggesting a role in thyroid function (1). More recently, it appeared that several ganglia contribute to the innervation of the thyroid gland. Tracing studies using True Blue as a retrograde tracer indicated that the thyroid ganglion and superior cervical ganglion (SCG) contribute most to the nerve supply of the thyroid, with more moderate contributions from the jugular nodose and cervical dorsal root ganglia (2). By combining surgical resection of the SCG and immunohistochemistry, the SCG was identified as the major sympathetic ganglion projecting to the thyroid (3).

As for parasympathetic innervation of the thyroid, the picture is less clear. Acetylcholinesterase (AChE)-positive fibers have been identified in the rat thyroid. However, the assumption that these represent postganglionic parasympathetic fibers is uncertain, as also adrenergic and sensory neurons may contain AChE. The thyroid is thought to receive its parasympathetic innervation via vagal branches originating in the brain stem, namely the superior laryngeal nerve (thyroid nerve), and -although to a much lesser extent- the recurrent nerve, which anastomoses with the thyroid nerve (2). The thyroid nerve projects to the thyroid ganglion which contains mostly vasoactive intestinal peptide (VIP) and neuropeptide Y (NPY) expressing neurons, innervating the thyroid gland.

Until recently, no data were present as to the central nuclei within the central nervous system (CNS) contributing to the autonomic innervation of the thyroid. In order to further investigate this, we performed viral tracing studies using pseudorabies virus (PRV) as a retrograde, transsynaptic tracer. PRV was injected into the thyroid gland of rats and by applying different times of survival, successive stages of infection were visualized using immunocytochemistry. Following the virus as it traveled towards the CNS via neural pathways enabled us to visualize polysynaptic autonomic pathways from the (pre-autonomic) hypothalamus and brain stem to the thyroid gland (4). After two days of survival, infection of sympathetic motoneurons occurred in the intermediolateral nucleus (IML), while parasympathetic motoneurons were labeled in the dorsal vagal complex (DMV). This confirmed the existence of both sympathetic and parasympathetic thyroid innervation. Furthermore, at three days of survival, infected neurons in the hypothalamic paraventricular nucleus (PVN) became apparent. These findings provided evidence for the existence of multisynaptic neuronal pathways between the hypothalamic PVN and the thyroid, probably via both branches of the autonomic nervous system. A schematic representation of these pathways is presented in Figure 1.

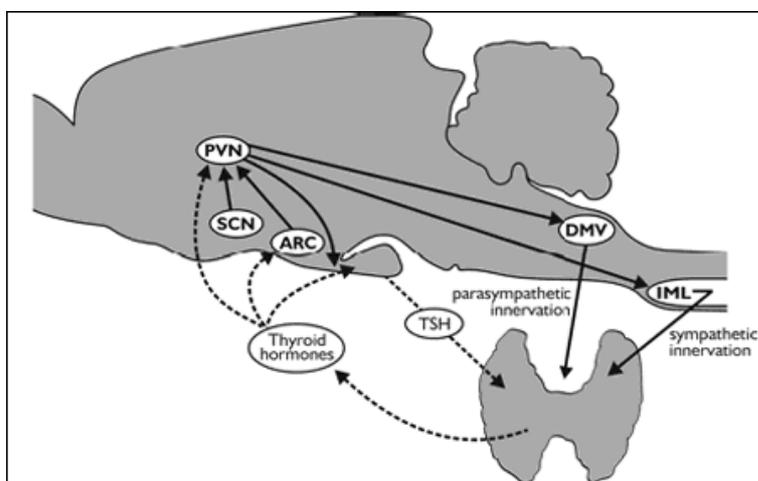


Fig 1. Schematic representation of endocrine and neural connections between the central nervous system and the thyroid gland. The hypothalamic suprachiasmatic nucleus (SCN) represents the biological clock, projecting to the paraventricular nucleus (PVN). The PVN harbours TRH expressing neurons that project to the median eminence, where TRH is released into the portal venous system. In the anterior pituitary, TRH stimulates the release of TSH, which acts on the thyroid gland to stimulate the synthesis and release of thyroid hormones. Negative feedback action by these hormones occurs both at the level of the anterior pituitary and the PVN. The hypothalamic arcuate nucleus is an additional site with

abundant expression of thyroid hormone receptors. In addition to this well established neuro-endocrine axis, the PVN projects to the dorsal motor nucleus of the vagus nerve (DMV) in the brain stem, where parasympathetic motor neurons innervating the thyroid gland are located. An additional autonomic projection from the PVN targets the intermediolateral column (IML) in the spinal cord, which contains sympathetic motor neurons projecting to the thyroid gland.

Functional implications

The potential of sympathetic input to the thyroid as to altering thyroid function has been shown in older studies. For example, unilateral electrical stimulation of the postganglionic cervical sympathetic trunk induced ipsilateral colloid droplet formation and a marked increase in plasma radiolabeled iodine in mice pre-treated with thyroxine in order to suppress TSH (5). More recently, electrical stimulation of the cervical sympathetic trunk was shown to induce NE release into the thyroid vein as well as a sharp decrease in thyroid blood flow in rats, suggesting indirect neural control of thyroid function by modulating thyroid blood flow (6). A functional role for parasympathetic innervation of the thyroid in modulating thyroid blood flow was shown in experimental thyrotoxicosis. In this condition, electrical stimulation of the thyroid nerve resulted in an increase in thyroid blood flow. This effect was partly blocked by atropine pre-treatment (7).

In addition to classical autonomic neurotransmitters such as noradrenaline and acetylcholine, neurons innervating the thyroid contain a variety of neuropeptides including NPY and VIP. These substances often appear to coexist and may be co-released by the same neuron (3). For example, NPY is co-expressed by noradrenergic neurons projecting to the thyroid from the SCG, and presumably parasympathetic fibers from the thyroid ganglion containing VIP may express NPY as well. VIP was shown to induce accumulation of cAMP in human thyroid cells in vitro and this was unaffected both by anti-adrenergic and by anticholinergic substances. In addition, VIP stimulates release of thyroxine (T4) from human thyroid slices (8). When administered exogenously in rats, VIP increases thyroid iodine uptake (9), blood flow (10) and thyroid hormone secretion (11).

Both NPY and VIP are potent vaso-active substances. In isolated rabbit blood vessels, NPY induces vasoconstriction and enhances noradrenaline-induced vasoconstriction (12). Also when administered exogenously in vivo, NPY -like noradrenaline- decreases thyroid vascular conductance while potentiating the vasoconstricting effect of noradrenaline in rats (13), suggesting a possible indirect control of thyroid function by modulating thyroid blood flow.

It is questionable if NPY and VIP are important players in vivo with respect to nervous signaling to the thyroid gland. In physiological experiments in rats, NA (but not NPY) appeared to be the primary mediator of the acute thyroid blood flow response to sympathetic nerve stimulation (6,13). In addition, decreased concentrations of triiodothyronine (T3) and T4 in thyroid venous plasma induced by iodine deficiency were not paralleled by changes in thyroidal or neural (ganglial) VIP or NPY protein or mRNA content (6). Also blocking VIPergic signaling by VIP antibodies had no effect on thyroid function or thyroid blood flow in rats (14). Therefore, the importance of these neuropeptides for neural regulation of the thyroid gland is probably less than the classical neurotransmitters.

Altered neural regulation should perhaps be considered in situations where a discrepancy exists between serum thyroid hormone concentrations on the one hand and serum TSH on the other. For example, when studying the role of the suprachiasmatic nucleus (SCN), i.e., the endogenous biological clock, in daily rhythmicity of plasma TSH and thyroid hormones, it appeared that SCN ablation had only minor effects on daily plasma TSH levels. However, the daily fluctuations in plasma levels of thyroid hormones, in particular T4, were clearly abolished (4) (Figure 2).

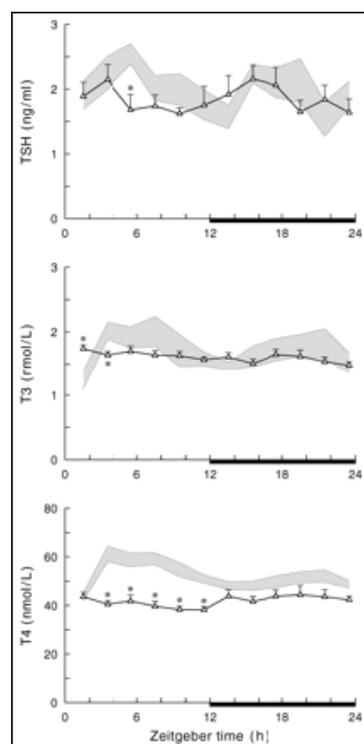


Figure 2. 24-hour rhythms in plasma concentrations of T3, T4 and TSH in SCN-lesioned male rats (Δ). The shaded area indicates the mean \pm SEM for control (SCN-intact) animals. Asterisks indicate time points that differ significantly between SCN-lesioned and SCN-intact animals. Copyright © 2000 by The Endocrine Society

This observation led us to speculate on alternative mechanisms by which the SCN could influence thyroid hormone secretion. Next to control of TSH release via its input to thyrotropin-releasing hormone (TRH) expressing neurons in the PVN (the neuroendocrine pathway), the SCN may influence thyroid function by its input to pre-autonomic neurons in the PVN that

project to the thyroid via multisynaptic autonomic pathways, as revealed earlier by our tracing studies.

Interestingly, double-labelling experiments using confocal laser scanning microscopy showed TRH immunoreactivity in some PRV-infected pre-autonomic neurons in the PVN after inoculation of PRV into the thyroid gland. Moreover, numerous TRH-immunoreactive fibers were seen in close approximation to a large proportion of PRV-infected preganglionic neurons in the IML, suggesting an extensive TRH input to sympathetic motor neurons in the spinal cord. This also holds, although to a lesser extent, for the DMV area in the brain stem harbouring parasympathetic preganglionic neurons. (Figure 3)(4).

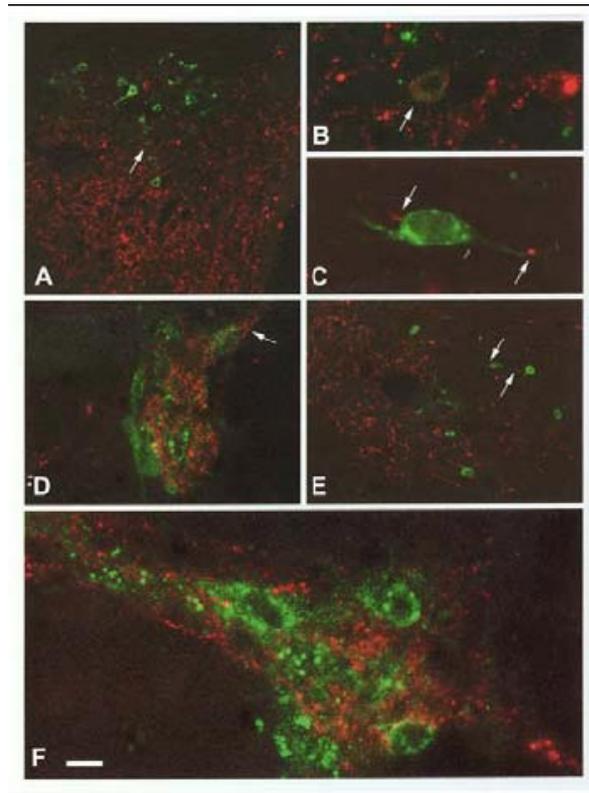


Figure 3 Composite two-color confocal laser scanning microscopical images of the same optical sections in the hypothalamus and spinal cord. Visualisation of TRH immunoreactive perikaryal profiles and axonal endings in red and PRV-FITC staining in green.

A, PRV (green) and TRH (red) staining in the paraventricular nucleus of the hypothalamus; B, a TRH-containing PVN neuron that is also infected by PRV (i.e. colocalization); (E) PRV and TRH (red) staining in the parasympathetic preganglionic nucleus of the solitary tract and DMV area; (C) Shows a PRV-infected DMV neuron receiving two TRH-immunoreactive contacts; (D,F) PRV and TRH (red) staining in the sympathetic preganglionic IML. Scale bar, 12 μ m in F.

The origin of these TRH fibers is uncertain at present. The colocalization of PRV and TRH in the hypothalamic PVN indicates the possibility of a TRH containing projection to the IML and DMV. However, it seems more likely that the TRH containing IML/DMV projections originate from the raphe nucleus and ventral medulla, where a population of TRH containing neurons has been shown to project to these autonomic motornuclei (15,16). Nevertheless, the colocalization of TRH and PRV in PVN neurons after PRV inoculation in the thyroid gland gives rise to the notion of a second population of TRH containing neurons in the PVN projecting to the thyroid via multisynaptic autonomic pathways, in addition to the well-known TRH containing neurons projecting from the PVN to the median eminence.

Theoretically, there are several mechanisms by which the autonomic input to the thyroid may interfere with T3 and T4 plasma concentrations. It could influence thyroid hormone release directly, it could alter thyroid responsiveness to TSH and it could interfere with the deiodination of the pro-hormone T4 to the biologically active hormone T3. In our earlier SCN lesioning experiments, there was a clear lowering effect on mean plasma concentrations of T4, while plasma TSH and T3 were unaffected (4) (Figure 2), suggesting impaired thyroid sensitivity to TSH in SCN-lesioned animals in keeping with the second possibility. The idea of autonomic nervous system involvement in the process of deiodination is supported by our recent experiments on type 2 deiodinase activity in the rat pineal gland, which also receives sympathetic input via the SCG. We showed that the large diurnal variation of type 2 deiodinase activity was abolished by lesioning of the SCN (17).

In several endocrine organs, including the adrenal cortex and the testis, a role for autonomic innervation in modulating the responsiveness to the corresponding pituitary hormones has been demonstrated (18,19). The notion of CNS control of thyroid responsiveness to TSH is supported by studies in mice showing that exogenous noradrenaline inhibits the thyroid response to TSH (20). Increased sympathetic tone of thyroid innervation has also been proposed as a partial explanation of discordant serum T3 and TSH in the framework of non-thyroidal illness (NTI) (21). Finally, recent studies in rats confirmed a functional role for sympathetic thyroid innervation by showing that unilateral SCG lesions induced a reduction in thyroid weight, and in TSH-stimulated radioiodine uptake (22).

Conclusion

Pre-autonomic neurons in the hypothalamic PVN, partly expressing TRH, project to the thyroid via sympathetic as well as parasympathetic pathways. There is growing evidence

suggesting a role for the autonomic nervous system in modulating thyroid function, and possibly thyroid size. Thus, in addition to the well-established neuro-endocrine HPT axis, neural control of the thyroid gland may prove to be an important modulator of thyroid function in health and disease.

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Importance of genetics for interindividual thyroid function variation in healthy subjects

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Introduction (Thyroid related phenotypes)

Despite intensive research, the mechanisms by which overt thyroid diseases develop are incompletely understood (1-3). Twin studies point toward a strong genetic influence in the aetiology of autoimmune thyroid disease (4-6) as well as simple goitre (7). Thus, in autoimmune thyroid disease, genetic susceptibility in combination with external factors (i.e. dietary iodine, cigarette smoking and infections) is believed to initiate the autoimmune response to thyroid antigens (1). Likewise, simple goitre seems to develop on the basis of genetic susceptibility interacting with environmental triggers, the two most important being inadequate iodine intake and cigarette smoking (3).

Health and disease are often defined by a continuum of biochemical and physiological measures (8). Despite distinct reference intervals, the definition of normality is not straightforward. There is a continuum from being disease-free and healthy without symptoms to having overt thyroid disease. Asymptomatic thyroid enlargement or presence of thyroid antibodies in euthyroid subjects illustrate this point. Another example is subclinical or mild thyroid dysfunction, which is defined by a condition with serum TSH levels outside the reference range and levels of free T4 and free T3 within the reference range (9). Studying, in healthy individuals, the regulation of biochemical and physiological measures related to the thyroid might be essential in understanding the pathways that eventually lead to thyroid disease. When individuals have been exposed to a disease status for a period, the physiology changes, and the original aetiological components may become invisible. Moreover, according to the endophenotypic approach, it could be useful to decompose a complex phenotype into a set of variables that might represent more basic processes (10,11). The understanding of the aetiology of overt thyroid disease may be enhanced by investigating underlying quantitative biochemical and physiological measures related to the thyroid.

Several thyroid-related measures/phenotypes exist, and different aspects of the thyroid homeostasis can be visualized and analysed. Investigations of parameters such as thyroid function, thyroid size, thyroid morphology and presence of thyroid autoantibodies are crucial in the diagnosis of thyroid disease. These phenotypes illustrate the thyroid homeostasis from different angles and each represents a small piece of a puzzle. Consequently, the serum TSH, free T4 and free T3 levels represent measures of the thyroid function set-point – reflected from different angles. The regulation of thyroid function is of special relevance, because the thyroid-specific genes may be partly involved in the genetic influence affecting most overt thyroid diseases.

Analyzing the causes of variation

Studies of the biological variation in thyroid function tests have shown that the intra-individual variation in serum T4, serum T3 and TSH levels, in healthy subjects, is narrow compared to the interindividual variation and the laboratory reference ranges (12-18). The distribution of serum T4 values in one individual is approximately half the width of the population-based reference range (15,16). This is compatible with a unique thyroid function set-point in each individual.

The sources of the interindividual differences in thyroid function are many. Ultimately, all those effects arise from genetic and environmental sources. Only a few family and twin studies have characterized the relative importance of the factors associated with thyroid function in healthy individuals. In general, previous studies suggest a low genetic influence on TSH and free T4 levels, the heritability estimates ranging from 0.32-0.44 (19-21). In the study by Samollow et al. (21), the heritability for TSH was only 0.32 – with an apparent difference between males and females. Excluding individuals with elevated serum TSH values (TSH>4.5 mIU/liter), the heritability estimate for serum TSH dropped to 0.20 and the gender difference disappeared, emphasizing the importance of an exact phenotype definition. In contrast, the heritability estimate for serum free T3 levels has been established to be higher - 0.67. Serum TSH, T3 and T4 depend on each other, and it therefore seems unlikely that the genetic influence is much different across the phenotypes that reflect the thyroid function set-point. It is important to keep in mind that heritability estimates are population specific. But actually, these studies are hampered by problems such as inadequate sample sizes (19,20,22), without specification of confidence intervals for the main results (20), crude statistical methods (20,22), and problems with phenotype- and zygosity definitions (22) making it difficult to interpret the results.

In our view, these limitations have been overcome in our recent twin studies. Based on well-defined phenotypes in a large study population of twins, we have found that intraclass correlations for serum TSH, free T4 and T3 concentrations were consistently higher for MZ than for DZ twin pairs indicating a strong genetic influence (23). Using structural equation model fitting (24,25) we have established that about 65% of the variation in serum TSH level is explained by genetic influences (23). Almost identical heritability estimates were found for serum free T4 (65%) and free T3 (64%) levels (23).

Reflections regarding the molecular basis of the estimated genetic influence

The interpretation of the above heritability estimates is not straightforward. The understanding of these results needs to be linked to the molecular knowledge of the regulation of thyroid function. The thyroid hormone homeostasis is achieved through the close interconnection of multiple, redundant mechanisms. Serum levels of TSH, free T4, and free T3 form part of a delicate feedback mechanism, and the variables are tightly interconnected. The circulating serum concentrations represent the product of balanced rates of secretion and metabolism of each of the variables along the hypothalamic-pituitary-thyroid-axis. Thus, the estimate of genetic effects includes genetic influences from many different levels and stages, and it is necessary to consider and describe the complete system to understand the estimated genetic influence.

A gene is a candidate gene if its product is known to be part of a relevant biochemical pathway to the phenotype in question. Many of the potential genes involved in the regulation of thyroid homeostasis are known from molecular studies (26-37). Moreover, the work identifying the specific genetic markers within these thyroid hormone pathway genes, associated with the circulating thyroid hormone phenotypes, has already been initiated. In the studies by Peeters et al. (38,39) distinct single nucleotide polymorphisms (SNP's) in three of the major thyroid hormone pathway genes (deiodinase type 1, deiodinase type 2 and TSHR) have been found to be significantly associated with plasma TSH and thyroid hormone levels in healthy individuals. On the other hand, the relative role of these distinct genes and specific polymorphisms in the biochemical pathways is unknown, so far. Trying to quantify the importance of these distinct genes and specific genetic markers is essential, because it would be an important step in trying to understand how the genes actually work together. With the advent of sophisticated statistical tools and molecular genetics it is possible to decompose the genetic variance into contributions from individual genetic loci, quantitative trait loci (QTL) (40). The ability to detect a QTL is a complex function of the effect size of the QTL itself (i.e. the proportion of the total phenotypic variance attributable to the QTL), the study design, sample size, and the characteristics of the genetic data.

The control of gene expression

Genetic factors do not act independently. The current molecular knowledge clearly indicates that the genes are modulating the effect of each other. The complex networks of interacting pathways and regulatory feedback mechanisms coordinate and regulate multiple functions, and they are likely to be the norm rather than the exception. The understanding of these genetic regulatory pathways is, however, in its infancy (41,42). In addition, the genetic background acts together with the exposures of external environmental factors, which modify the regulation of thyroid function.

A variety of mechanisms are involved. The interactions between genotype and the environment may change over time and gene expression may be context dependent. Besides, it is possible that the effects of environmental exposures accumulate throughout life. But most deny, or choose to ignore these issues, when designing, carrying out, and reporting genetic studies of complex traits and diseases. It is crucial to consider genes and environmental risk factors together. Most likely, the effects of environmental factors, such as iodine intake, influence the expression of distinct polymorphisms. It is necessary to consider genetic and environmental risk factors together to obtain information about the environmental control of gene expression, and how the effects of distinct environmental factors influence the expression of specific polymorphisms. Such investigations could be done by incorporating information of measured genetic markers located in thyroid related target genes into specific models, and quantify the proportion of the total phenotypic variance in serum levels of TSH, free T4 and free T3 attributable to specific polymorphisms. Information on specific covariates (factors such as age, gender, iodine intake, and cigarette smoking) could be added into the models. The continuous rapid development of genetic epidemiological approaches is promising and has permitted more sophisticated analyses. As an example, it is possible to extend the classical twin design by incorporating gene-environment-interaction effects as well as the effects of gene-gene interactions (epistasis) (43,44). A huge challenge is epigenetic changes, such as DNA-methylation, which enable cells to respond to environmental signals without having to alter the DNA itself (45).

A multivariate framework

The studies of thyroid hormone homeostasis have focused on the genetic-environmental analysis of one phenotype at a time, a univariate (one variable) approach. Clearly this is an oversimplification. A multivariate framework serves to distinguish whether a certain phenotype has variation that is shared or common, with other phenotypes in the model, or whether variation exists that is not being shared – i.e. unique. Both common and specific variation is valuable information. It is possible to investigate the genetic overlap between different phenotypes using multivariate models (46). When genes have influence on more than one trait it is called pleiotropy (47) As an example, it would be possible to address whether a set of “common” genetic and environmental factors influence serum levels of TSH as well as free T4 and/or free T3, or whether there is a set of “specific” genetic and environmental factors that are unique to e.g. serum TSH regulation. The major advantage compared to separate univariate analyses is greater statistical power.

A hierarchical model for thyroid homeostasis

When trying to describe and understand the complexity of a biological system, it might be useful to apply a model which reflects the system. This can be illustrated by applying a hierarchical model. In such a model the genes are assigned to the basement level. Biochemical and physiological traits are located at intermediate levels and the clinical signs - the clinical endpoints - are located at the top. Studying a trait at a lower level as compared to the clinical endpoint located at a more gross phenotypic level may contain some advantages. There are likely to be fewer genes involved in intermediate phenotypes, and the effect of environmental exposure may be less important.

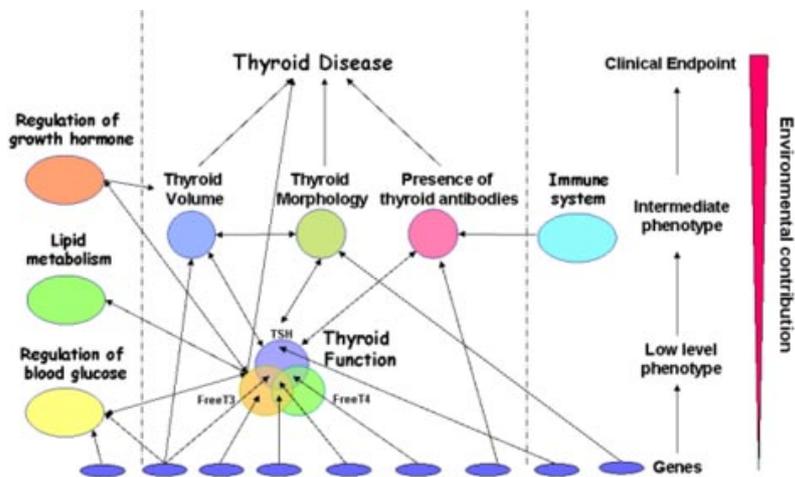


Figure 1

A simplified schematic diagram of the biological complexity assuming a hierarchical organisation of thyroid related phenotypes. The inverted triangle on the far right side of the figure represents the likely diminishing effect of environmental factors at lower and lower levels of a biochemical and physiological hierarchy.

According to a hierarchical model the regulation of thyroid function could be regarded as a low or intermediate level phenotype (Figure 1). Several of the genes involved in regulation of thyroid function are known, and it is possible that this system is close to the effect of the genes. Nevertheless, it is important to bear in mind that the regulation of thyroid function is connected and modulated by several other thyroid phenotypes/variables such as thyroid volume, thyroid morphology, and presence of thyroid antibodies. In addition, these systems are connected to other subsystems as well. Variation in thyroid hormone levels could be intimately connected with variation in health-related characteristics such as regulation of blood glucose, growth hormone levels, lipid metabolism, the immune system, and many more. It would be useful trying to connect these phenotypes, although such connections are complicated by a plethora of possibilities.

Whether or not this theoretical thinking is true, a hierarchical model does visualize the complexity, and it makes one realize that mechanisms in one subsystem affects other subsystems. This is important to take into consideration when applying simple aetiological models to biological systems.

Conclusion

Studying the regulation of biochemical and physiological measures related to the thyroid, in healthy individuals, might be essential in understanding the pathways that eventually lead to thyroid disease. It has been established that the thyroid function set-point (reflected by serum TSH, free T4 and free T3 levels) is under tight genetic control. However, the detailed understanding of this genetic influence is still an illusive goal. Research which combines molecular and physiological studies with clinical, epidemiological and statistical knowledge is necessary in order to increase our understanding of the regulation of thyroid function.

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THERMOGENESIS AND THYROID HORMONE

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ABSTRACT

Thyroid hormone (TH) plays a critical role in cells and whole body physiology, regulating the expression of developmental programs as well as specific cell functions. In homeothermic species, TH acquires another function, which is to regulate thermogenesis, playing a critical role in temperature homeostasis and thereby influencing the rate of metabolism and energy expenditure. I here review some basic concepts and progress made recently on the regulation of thermogenesis by TH. TH augments obligatory thermogenesis (heat produced as result of processes inherent to life) in homeothermic species basically by increasing ATP consumption, hence forcing the cell to produce more, with the attendant heat liberation, as well as by reducing the efficiency of ATP synthesis for the sake of producing heat. TH utilizes several mechanisms, such as increasing the calcium exchange between cytosol and sarcoplasmic reticulum and stimulating glycerol-3-phosphate shuttle in skeletal muscle as well as lowering the proton motive force in mitochondria. It is so far questionable whether UCP3 could mediate this latter effect. In addition, TH is essential for facultative thermogenesis (additional heat produced on demand in cold environments) for which it interacts synergistically with the SNS. The effects on facultative and obligatory thermogenesis are coordinated to ensure temperature homeostasis and avoiding hyperthermia in hyperthyroid states.

INTRODUCTION

The thyroid gland is present in all vertebrates, and its hormones, thyroxine (T_4) and triiodothyronine (T_3), collectively called here thyroid hormone (TH), play an essential role controlling the developmental programs and specific functions in various organs of the body. Only in homeothermic species TH seems to control thermogenesis [reviewed in (1)].

Whether one looks at whole body or separate organs, homeotherms have a faster metabolism than poikilothermic species (2;3). This means that the energy investment to sustain life is higher in homeotherms in order to produce more heat, that is, the homeothermic machine is thermodynamically less efficient for the sake of temperature homeostasis. Homeotherms indeed need more ATP to sustain life and produce ATP with more dissipation of energy as heat. Skeletal muscle, where it is easier to measure "work", is thermodynamically less efficient in homeothermic species (4). It appears that TH is responsible for a large fraction of the difference between poikilothermic and homeothermic species. Physiologically, that is in the transition from the hypothyroid to the euthyroid status, TH recapitulates the differences between the cold-blooded and warm-blooded species. Thus, for any amount of mechanical work, TH increases heat production by muscle (5), as it also increases the energy cost of gluconeogenesis and ureogenesis in hepatocytes [(6) and references therein]. This article reviews the possible mechanisms that may mediate the thermogenic effect of TH.

TH-INDUCED INCREASE IN ATP TURNOVER

Cells attempt to keep a critical level of ATP as an immediate source of energy to support activities inherent to life. Mitochondrial respiration is tightly regulated by the amount of ATP available. Increases in the ADP/ATP ratio as a consequence of increased ATP utilization cause proportional increments of mitochondrial respiration. As in any process of energy capture from exergonic reactions, ATP synthesis captures only a fraction of the free energy of substrates, the difference being dissipated as heat. Thus, changes in ATP turnover are associated with proportional changes in heat production. The stimulation of numerous processes requiring energy i.e. ATP, is one of the mechanisms whereby TH increases heat production.

The maintenance of sodium and potassium gradients across cell membranes by Na/K ATPase consumes a substantial fraction of cell ATP. Indeed, the requirement of ATP needed to maintain these gradients explains a significant fraction of the difference in energy expenditure (oxygen consumption, QO_2) between poikilothermic and homeothermic species (2). Initially, there was excitement about this enzyme being a major thermogenic mechanism for thyroid hormone (7). Even though this enzyme activity could explain about 20% of resting metabolic rate in mammals, about 2/3 of this is accounted for by brain and kidney, and in these tissues the enzyme is not responsive to TH (8), so that the fraction that is TH-dependent is small. It is possible that the energy (ATP) required to maintain these gradients is more important in the hyperthyroid state, but not physiologically because the difference in Na/K ATPase activity between the hypothyroid and the euthyroid state is small (9).

Another postulated mechanism of increased ATP consumption is the stimulation of Ca^{2+} transfer from cytosol to sarcoplasmic reticulum (8). By stimulating the activity of the sarcoplasmic-endoplasmic reticulum Ca^{2+} -dependent ATPase (SERCA) in skeletal muscle [reviewed in (10)], TH increases the sarcoplasmic calcium pool, the release of calcium during contraction and ultimately ATP demands to return the calcium back into the sarcoplasmic reticulum. TH administration to hypothyroid rats increases the SERCA activity and the amount of sarcoplasmic reticulum, which is associated with a substantial increase in the amount of energy spent in Ca^{2+} pumping (11). Slow twitch muscle is more responsive to T_3 than fast muscle, largely owing to the predominant stimulatory effect TH on SERCA1 gene expression, the baseline expression of which is low in this type of muscle (10;12;13). In addition to increasing calcium fluxes during activity, it is possible that TH enhances the "leak" of calcium at rest by stimulating the number and activity of ryanodine receptors (14;15), thus enhancing ATP expenditure also in the resting condition. Interestingly, for any level of mechanical work (tension) in both fast-twitch and slow-twitch muscle heat production is

significantly greater in the euthyroid than in the hypothyroid state (5). Moreover, extrapolating the heat vs. tension curves to zero tension i.e. rest, the difference between the two thyroid states is maintained (5), providing independent support to the idea that resting muscle heat production is also greater in euthyroidism than in hypothyroidism. Since skeletal muscle normally contributes about 20-30% of resting metabolic rate [(16) and references therein], Ca²⁺ transport in muscle could explain as much as 10% of the effect of TH on resting thermogenesis. Lastly, it is important to recall that slow-twitch muscles are relatively more abundant in larger mammals, such as humans, who in addition have less brown fat, so that these mechanisms are quantitatively more important in larger species. The presence of type II iodothyronine 5'-deiodinase (D2) in human but not in rodent skeletal muscle (17) may also be an indication that muscle TH-dependent thermogenesis is more important in humans.

Yet another mechanism whereby TH can stimulate the expenditure of ATP is by accelerating metabolic cycles, such as lipolysis-lipogenesis, glycolysis-gluconeogenesis. Increased consumption of substrates is coupled to synthesis, and TH stimulates the expression of key enzyme genes such as gluconeogenic and lipogenic enzymes. However, in animals fed ad libitum, synthetic process do not seem to account for a major fraction of ATP consumption. Lipogenesis can account for no more than 10% of difference in energy expenditure between hypo- and hyperthyroid rats (18). There are possibly multiple other mechanisms whereby thyroid hormone may increase ATP consumption, such as increased non-exercise physical activity, increased heart activity, etc. Although there are no rigorous measurements of ATP turnover in whole body in different thyroid states, the TH-induced increase in ATP demands probably does not account for more than 50% of differences in thermogenesis between the euthyroid and the hypothyroid state.

TH-INDUCED RESPIRATION UNCOUPLED OF ATP SYNTHESIS

Brown adipose tissue (BAT) was until recently the only site where there was controlled reduction in coupling between oxidation and ATP synthesis, which is mediated by a 32,000 Mr protein, thermogenin or uncoupling protein (UCP), now called uncoupling protein-1 (UCP1), in view of the cloning of several new homologues [see (19;20) for recent reviews]. The idea that TH could uncouple phosphorylation received attention in the 1950's but was then abandoned in the late 1960's following the demonstration that the earliest effect of TH was to increase nuclear RNA synthesis (21). In recent years, work by Brand's and by Berry's groups have provided evidence that a good fraction of the increase in QO₂ following the administration of TH cannot be explained by increased ATP synthesis [see (6;22) and references therein]. Brand's group demonstrated the presence of proton leak across the mitochondrial membrane that could account for 20-40% of basal liver respiration (23). This was later shown to occur in other tissues, notably in muscle and to be regulated by TH (22). Supporting the idea expressed above regarding the contribution of ATP turnover stimulation to TH thermogenesis, the analysis of hepatocytes indicates that the increase in QO₂ in the transition from the hypothyroid to the euthyroid state is mostly due to increased proton leak and non-mitochondrial sources, with little being due to increased ATP synthesis, whereas this is as important as the proton leak in thyrotoxic hepatocytes (24).

The cloning of UCP1 homologues more ubiquitously expressed, most notably UCP2 and UCP3 raised the expectation that they would explain the proton leak and that they would be stimulated by TH, but so far the evidence is missing (20). UCP3 is clearly stimulated by T₃ (25), but transgenic UCP3-deficient mice do not seem to have a thermogenic deficiency and their body temperature and QO₂ respond normally to T₃ (26). Such negative findings should be interpreted cautiously, though, because studies in transgenic models of gene ablation have revealed redundancy of mechanisms and recruitment of compensatory mechanisms that can conceal the effect of the ablation of a given gene.

Another mechanism whereby TH could reduce the efficiency of ATP production is by favoring the use of the glycerol-3-phosphate (G3P) shuttle to dump cytoplasm-generated reducing equivalents in the mitochondria, as this shuttle produces only 2 instead of 3 ATPs per pair of electrons or per molecule of generated water. The NADH-G3P shuttle constitutes a rapid way to generate ATP aerobically out of oxidative processes occurring in the cytoplasm (27), and the rate-limiting enzyme of the shuttle, the FAD-linked mitochondrial glycerol phosphate dehydrogenase (EC 1.1.99.5, mGPD), has been known for long to be stimulated by TH in several tissues (28) in proportion to the stimulation by TH of QO₂ (29). Furthermore, TH does not stimulate mGPD activity either in homeothermic tissues where it does not stimulate QO₂, such as the brain (28;29), or in poikilothermic species (30). We have carefully studied a transgenic mouse lacking mGPD and found the genotype associated with reduced obligatory thermogenesis partially compensated by increased BAT activity and serum TH levels (31). The tissue most affected by the lack of mGPD was skeletal muscle, as revealed by an increase in G3P and lactate/pyruvate ratio (32). While these studies show that mGPD is involved in TH-stimulated thermogenesis, they also indicate it is not essential because hypothyroid mGPD^{-/-} mice increase normally their QO₂ when given T₃ over the physiological replacement (31). Interestingly, male mGPD^{-/-} mice have an increase in UCP3 (31;32).

FACULTATIVE THERMOGENESIS AND TH

While the well-known cold intolerance of hypothyroid animals and humans may be explained by the reduced obligatory thermogenesis, studies in rats suggest that it is largely due to failing facultative thermogenesis caused by the lack of T₃ in BAT. Prompted by the presence of type II iodothyronine 5'-deiodinase (D2) in BAT and its activation by the sympathetic nervous system (33) as well as by hypothyroxinemia (34), we treated hypothyroid rats acutely with replacement doses of T₄ and found that within 48 h they restored their cold tolerance and UCP1 response to cold, whereas such brief and small regimen did not affect the thyroid status of the liver nor restored plasma levels of T₃ (35). Such effect of T₄ was abolished by the blockade of D2 with iopanoic acid, even though T₃ was concomitantly given in these studies to maintain plasma T₃ levels (35). It is unclear, though, how relevant these findings are to humans. BAT is in a quiescent state in adult humans and it is unlikely to be an important site of facultative thermogenesis. It is likely that in humans and other large mammals skeletal muscle play an important role. Interestingly, humans but not rodents express D2 in skeletal muscle (17).

TH is essential for the full response of the UCP1 gene to adrenergic stimulation. A complex TH-response element (TRE) is found in a critical upstream enhancer of the gene. This TRE acts synergistically with cAMP to maximize the transcription of the gene [reviewed in (36)]. In addition, TH is needed for integrity of the norepinephrine-signaling pathway both in BAT [see (37) and references therein] and in other tissues. We recently reported that mice lacking the TH receptor α (TR α 0/0) have an impaired thermal response of BAT to exogenous

norepinephrine, even though UCP1 and other relevant genes responded well to cold, revealing the existence of yet another T_3 -dependent step essential for the ultimate thermogenic response of BAT to adrenergic stimulation, in this case specifically requiring $TR\alpha$ for T_3 stimulation (38).

The multiple mechanisms whereby TH contributes to thermogenesis, temperature homeostasis and energy balance are schematically summarized in Figure 1.

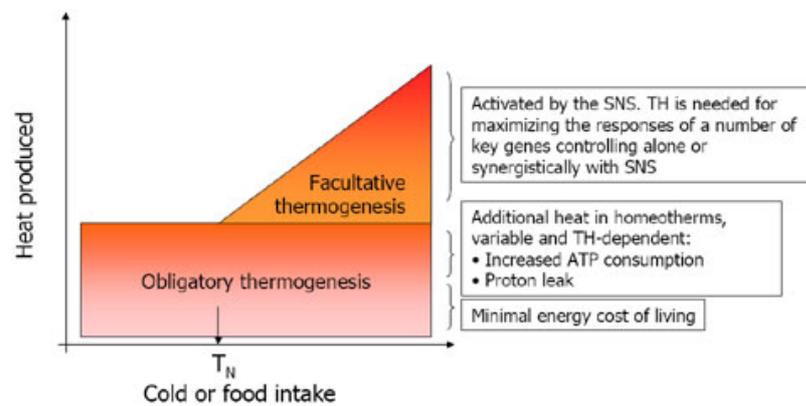


Figure 1. Heat production as a function of ambient temperature and food intake. Homeothermic species produce more heat than poikilothermic species. This excess heat produced is largely TH-dependent and it is quite variable in humans, probably reflecting selections pressures. In addition, homeothermic species produce heat on demand, in a facultative or adaptive manner (facultative thermogenesis). T_N represents the point of equilibrium between obligatory thermogenesis and ambient temperature or food intake. Below and over this point, adaptive responses are necessary. Facultative thermogenesis, one of these responses, is stimulated both by cold and excess food intake and the magnitude of its response is also TH-dependent.

CONCLUDING REMARKS

The stimulation of thermogenesis is a new role acquired by TH with the advent of homeothermy. The thermogenic mechanisms stimulated by TH antecede homeothermy but they evolved into being TH-dependent in warm-blooded species. Part of the stimulation of obligatory thermogenesis by TH results from TH increasing ATP utilization, forcing the cells to produce more ATP with the attendant production of heat. A significant fraction, possibly 50% or more, is due to stimulation of respiration without ATP production, that is, pure heat production, but the biochemical mechanisms mediating this effect have not been defined, but are likely related to a reduction in the proton motive force in the mitochondria in the form of a proton leak. Lastly TH is essential for facultative thermogenesis interacting with the sympathetic nervous system in a synergistic manner, well exemplified in rodents BAT, but less understood in humans and larger mammals.

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Aging and the thyroid

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Introduction

The thyroid undergoes slight "physiological" changes with aging, either as a result of its participation in the senescence process or as an effect of other systems' changes. Thus, thyroid alterations in the elderly may be either the expression of aging evolution by itself or the result of the senescence process. Moreover, some thyroid diseases are more frequently encountered in the elderly and others, which start as subtle dysfunctions in younger people, may appear as clinically overt disorders in older individuals. Hypothyroidism from atrophic chronic autoimmune thyroiditis is known to be much more common in the elderly. Non-toxic goiter, usually starting as a diffuse thyroid enlargement in younger people, frequently acquires nodularity and autonomous function with age and eventually, but not necessarily, evolves in toxic nodular goiter. In their evolution from normal to abnormal thyroid function, both chronic autoimmune thyroiditis and goiter often show a phase of subclinical thyroid dysfunction (subclinical hypothyroidism and subclinical hyperthyroidism, respectively). Frequently, the interpretation of thyroid function tests is also difficult in aging individuals, because true "physiological" age-associated changes cannot be easily distinguished from the alterations secondary to subclinical thyroid disease, to acute or chronic nonthyroidal illness and / or drugs often taken by elderly patients. The definition of "physiological" age-associated thyroid changes with aging and the correct understanding of these modifications is not merely speculative, since it greatly helps in differentiating "physiological" alterations from subclinical thyroid disease and in resolving the question of treatment of uncertain clinical situations. Moreover, the effects of thyroid dysfunctions may be to a variable extent different in the elderly. The consequences of hyperthyroidism on cardiovascular function and of hypothyroidism on atherosclerosis, that are more marked in older than in younger individuals, are two examples of this situation.

We will present some topics on thyroid function and diseases in the elderly, with particular accent on the latest and more controversial issues.

Physiological changes of thyroid with aging

The results of studies on thyroid function in the elderly have been long controversial because of the inaccurate selection of study samples (1). Indeed, it is now apparent that subclinical thyroid disease and nonthyroidal illness are common in the elderly. Thus an accurate selection of population samples, which must include only healthy subjects, is crucial to define the normal thyroid function in the elderly. In a population of healthy elders, carefully selected to exclude subjects with nonthyroidal illness, we showed that TSH decreased, FT4 remained steady, FT3 decreased and rT3 increased with aging (2). These changes of TSH and thyroid hormones are consistent with the presence in the elderly of a partial central hypothyroidism, associated with an impaired activity of type I deiodinase (5'D-I). A similar reduction of TSH levels had been previously reported in carefully selected healthy old subjects (3) and in a small proportion of unselected ambulatory patients, unrelated to subclinical hyperthyroidism (4). However, in the Whickham survey, after people with circulating thyroid autoantibodies and a family or personal history of thyroid disease had been excluded, the levels of TSH in aging people resulted unchanged (5). Moreover, a recent survey on a large population in the USA (6) reported that in the reference population, which included people without circulating thyroid autoantibodies and no other risk factors for thyroid function, TSH increased with aging. These dissimilar results may be explained, to some extent, with a diverse iodine intake and a different prevalence of subclinical thyroid disease in the populations evaluated in these studies. In conclusion, a number of complex, albeit minor, changes in functional parameters occur with aging: the question of whether these changes are beneficial or detrimental to the aging process per se remains to be established.

Aging and thyroid autoimmunity

An age-dependent increase of the prevalence of anti-thyroperoxidase (TPOAb) and anti-thyroglobulin autoantibodies (TgAb), particularly in females over 60 years of age has been long documented (reviewed in 1: 6). Nevertheless, with the exception of atrophic lymphocytic thyroiditis, the prevalence of clinically overt autoimmune thyroid disease is not increased in the elderly. We investigated the prevalence of TPOAb and TgAb in very old subjects (7). The prevalence of thyroid autoantibodies resulted significantly greater in subjects aged 70-85 compared with people aged less than 50. By contrast, the prevalence in centenarians was not different from subjects aged less than 50. Therefore, the age-dependent increase in the prevalence of thyroid autoantibodies in the elderly is not seen after the ninth decade of life. In another study, we reported that the prevalence of thyroid autoantibodies was higher in unselected or hospitalized elderly patients and lower in centenarians and in "younger" healthy elderly as compared with an apparently normal population (8). Therefore, the appearance of thyroid autoantibodies might be related to age-associated disease, rather than to the aging process per se and healthy older subjects might represent a selected population with an unusually efficient immune system (1). Moreover, it is well established that thyroid autoimmunity is often associated with other autoimmune diseases.

On these grounds, it is appealing to speculate a link between circulating thyroid autoantibodies and other diseases. Indeed, some authors have proposed a correlation between thyroid autoimmunity and some age-associated diseases, in which (auto)immune mechanisms are believed to be involved (reviewed in 9). The increased prevalence of circulating thyroid autoantibodies observed in the elderly might reflect an activation of the (auto)immune process involved in the atherosclerotic process. However, the slight increase of coronary heart disease associated with positive serum thyroid autoantibodies, reported by some epidemiological studies (reviewed in 1: 10; 11; 12). In 1966 Brain et al. described a patient that was on levo-thyroxine treatment for hypothyroidism, due to Hashimoto's disease, who developed several episodes of cerebral disorders (13). After this first report, few papers describing an association of thyroid autoantibodies with encephalopathy ("Hashimoto's encephalopathy") have been published (reviewed in 14). However, chronic lymphocytic thyroiditis is seldom associated with serious neurological manifestations, with the exception of myxedema coma, and the reports on "Hashimoto's encephalopathy" are very few compared with the large prevalence of thyroid autoimmunity in general population. A recent paper, describing an elevated numbers of perfusion defects in

euthyroid patients with autoimmune thyroiditis, recalls the hypothesis that cerebral vasculitis might be implicated in this situation ⁽¹⁵⁾. Therefore, it is conceivable that not Hashimoto's thyroiditis by itself but some correlated autoimmune disorder might be responsible for Hashimoto's encephalopathy. However, a definitive role for thyroid autoantibodies in neurological disorders has not been proved yet.

Hypothyroidism and hyperthyroidism in the elderly

Definition of overt and subclinical hypothyroidism and hyperthyroidism

Overt hypothyroidism is characterized by low levels of thyroid hormones and increased levels of TSH, whereas overt hyperthyroidism is characterized by high levels of thyroid hormones and a low TSH. The term subclinical thyroid disease currently include subclinical hypothyroidism and subclinical hyperthyroidism, which are defined as states with serum TSH levels either above or below the reference range, respectively, and normal thyroid hormone levels in the absence of clinical symptoms and signs. The definition of subclinical thyroid disease is widely recognized as somewhat arbitrary and uncertainties in the definition and the terminology of subclinical hyperthyroidism and subclinical hypothyroidism should also be considered ⁽¹⁶⁾.

Prevalence of hypothyroidism and hyperthyroidism in the elderly

The prevalence of hypothyroidism and hyperthyroidism varies widely according to gender, age and the environment. As reported in iodine sufficient areas, hypothyroidism occurs in 4 to 9.5% of the general population, hyperthyroidism in 0.4 to 3.2% ^(5; 6; 17; 18). Both subclinical hypothyroidism and subclinical hyperthyroidism are 2-4 fold more common than the corresponding overt conditions and are more prevalent in women and in the elderly. Hypothyroidism is more common in iodine sufficient areas, hyperthyroidism in iodine deficient areas ⁽¹⁹⁾. In the latter, nodular goiter with hyperthyroidism or various degrees of thyroid autonomy, including complete TSH suppression, is relatively frequent ^(20; 21; 22; 23). The prevalence of subclinical hyperthyroidism resulted 15% in elderly subjects (>75 years) living in Pescopagano, an iodine deficient area ⁽²¹⁾, and 6.5% in people over 85 years living in the United States, an iodine sufficient area ⁽⁶⁾. In the Pescopagano survey the prevalence of subclinical hyperthyroidism resulted equal in the two sexes ⁽²¹⁾, whereas in the United States was more frequent in women ⁽⁶⁾. The prevalence of hypothyroidism increases with aging up to 17-20% of females and 3-16% of men over 75 years of age in iodine sufficient areas ^(5; 17).

Etiology of hypothyroidism

Several conditions, transient or permanent, may be associated with an elevated serum TSH level and normal or low serum thyroid hormone concentrations (subclinical or overt hypothyroidism, respectively). Half of cases of permanent hypothyroidism of the elderly are due to chronic autoimmune thyroiditis (Hashimoto's thyroiditis), a condition that is more common in women. Indeed, a higher prevalence of thyroiditis in patients over age 60 than in younger women has been reported by an autopsic study ⁽²⁴⁾. Moreover, in the Whickham survey the incidence of antithyroglobulin and antimicrosomial autoantibodies was 7% and 9% in females over 75 years of age compared with 2% and 5% in total population, respectively ⁽⁵⁾. A permanently elevated TSH may be also caused by a previous treatment of hyperthyroidism, by congenital hypothyroidism (including inactivating mutations of the TSH receptor) and by external radiotherapy. Circulating bioinactive TSH and heterophilic antibodies against mouse protein can induce falsely elevated levels of TSH. A transient increase of TSH levels is typical of the recovery phase from postpartum thyroiditis and subacute thyroiditis, of nonthyroidal illness and of drug (lithium, amiodarone) (reviewed in ²⁵) treatment.

Clinical presentation and diagnosis of hypothyroidism

The clinical appearance of hypothyroidism may be subtle ⁽²⁶⁾, particularly in elderly patients, in whom the classical symptoms of overt hypothyroidism may be confused with normal aging. Furthermore, clinical presentation of hypothyroidism in the elderly is quite different than in younger individuals (reviewed in ²⁷). "Subclinical" hypothyroidism, particularly in presence of TSH levels >10 mIU/L, has been associated with neuromuscular symptoms ⁽²⁸⁾, increased cholesterol levels and other lipid abnormalities ^(17; 29; 30), cardiac alterations ^(31; 32; 33; 34) and vascular impairment ^(35; 36). Subclinical hypothyroidism has recently been reported to be an independent risk factor for atherosclerosis and myocardial infarction in postmenopausal women ⁽¹¹⁾ and in men ⁽¹²⁾. However, in another paper mortality was unchanged in presence of subclinical hypothyroidism ⁽³⁷⁾. The validity of most of the above mentioned studies has been questioned by a recent review ⁽¹⁸⁾. In our view, identify the etiology of subclinical hypothyroidism is mandatory, to differentiate chronic autoimmune thyroiditis, previous treatment of thyroid disease and other causes of permanent thyroid damage from rare case of isolated hyperthyrotropinemia caused by a TSH increase due to partially inactivating mutations of the TSH-receptor or other causes ⁽¹⁶⁾.

Treatment of hypothyroidism

Treatment of overt hypothyroidism in elderly patients should be started and monitored carefully in order to maintain TSH and FT4 within their normal ranges. The initial dose of levothyroxine should be very low (even 12.5 µg/day) and increased every 4-6 weeks, in order to reach the replacement dose in 3-4 months. We have recently shown that individual L-T4 requirements are dependent on lean body mass ⁽³⁸⁾. The replacement dose of L-T4 in the elderly is usually lower than 1.6 µg/kg/day, the dose usually employed in younger patients. This reduction appears dependent on a relative decrease in lean body mass with aging. Replacement treatment must be carefully monitored in elder patients, with TSH measurement every three months, in order to avoid overtreatment. In some patients a lesser than substitutive dose of levo-thyroxine is required to avoid ischemic heart symptoms.

While need of treatment of clinical hypothyroidism is unanimously accepted, that of subclinical hypothyroidism is under discussion. A major matter of debate concerns the role of the "evidence-based medicine" approach in managing subclinical hypothyroidism. Recently, a consensus panel recommended against treatment of patients with subclinical hypothyroidism with TSH level 4.5-10 mIU/L, because there were no large randomized control trials to support benefit ⁽¹⁸⁾. This and other panel's suggestions have later been criticized by a Consensus Statement from the American Association of Clinical Endocrinologists, the American Thyroid Association and the Endocrine Society that stated that these recommendations were based on "the lack of evidence for benefit rather than on evidence for a lack of benefit" and that there is no evidence that treatment of subclinical hypothyroidism is harmful ^(39;40). We share the latter view, and recommend that a practical approach should be used, taking into account that, even if not definitively, many reports have shown that treatment may normalize some abnormalities induced by subclinical hypothyroidism. Indeed, a direct correlation between TSH values and total and LDL cholesterol levels has been demonstrated ^(17;41). The optimal ranges for total and LDL cholesterol are still uncertain and the new concept is that "lower is better" ⁽⁴²⁾. In this setting an aggressive control of LDL cholesterol levels is required, particularly in presence of coronary heart disease (CHD) or CHD risk equivalents ⁽⁴³⁾, a common occurrence in the elderly. Basically, in presence of an increased cholesterol level, determination of TSH value is already a widely accepted practice ⁽⁴³⁾. Moreover, effectiveness of treatment of subclinical hypothyroidism in normalizing total and/or

LDL cholesterol levels and other lipid abnormalities, particularly in patients with greater basal TSH and cholesterol levels, have been reported in many (44; 45; 46; 47), though not all studies (48;49). Therefore, when subclinical hypothyroidism is associated with elevated cholesterol values, clinical judgment would suggest that treatment with levo-thyroxine would be beneficial for CHD, despite the fact that no definitive data are available. Similar considerations can be drawn for the other abnormalities reported in subclinical hypothyroidism, with papers showing that treatment has beneficial effect on non-specific symptoms (28; 46), cardiovascular dysfunctions (28;33; 46;50) and vascular impairments(35; 36). A different conclusion has been reached by other authors (49). The bulk of available data are sufficient to support treatment of subclinical hypothyroidism, whereas management of isolated hyperthyrotropinemia is still uncertain (16;51; 40;52).

Etiology of hyperthyroidism

In the elderly, the most common causes of permanent hyperthyroidism are Graves' disease in iodine sufficient areas, nodular goiter and functioning thyroid adenoma in iodine deficient areas, respectively (20; 21). A transient thyrotoxicosis may occur in silent and subacute thyroiditis and during treatment with drugs including amiodarone (particularly in iodine deficient areas) (reviewed in 25), levo-thyroxine and interferon. With the exception of amiodarone-induced thyrotoxicosis, which may represent a major therapeutic challenge (25), the other transient drug-induced thyrotoxicosis are self-limited and remit with drugs withdrawal.

Clinical presentation and diagnosis of hyperthyroidism

The hyperkinetic symptoms, typical of clinical hyperthyroidism in younger patients, are generally less evident or absent in older patients, that usually exhibit the so called "apathetic" hyperthyroidism (reviewed in 53). Atrial fibrillation is more common in elderly hyperthyroid patients than in younger patients (54). Age, male gender, ischemic heart disease, congestive heart failure, aortic and/or mitralic valve disease have been identified as risk factors for atrial fibrillation in patients with hyperthyroidism (55). Angina pectoris may occasionally occur in hyperthyroid patients with normal coronary arteries. Hyperthyroidism in the elderly is associated with greater bone density reduction and fracture risk (reviewed in 56). In clinically euthyroid patients over 60 years a TSH value <0.1 mIU/L was associated with a relative risk of atrial fibrillation of 3.1 (57) and, more recently, a TSH level <0.5 mIU/L in people aged 60 years or older, not taking levo-thyroxine, was related to increased mortality (37). Surprisingly, in the latter study no difference was noted between patients with low but detectable TSH and patients with undetectable TSH. Further studies are needed to confirm these data. Moreover, subclinical thyrotoxicosis due to suppressive levo-thyroxine treatment has been associated with cardiac abnormalities (58; 59). A decreased bone density has been reported in post-menopausal women taking levo-thyroxine suppressive treatment (60; 61; 62), whereas we did not find any significant effect on bone mass and bone metabolism in men in whom levo-thyroxine had been carefully titrated in order to use the minimal dose able to suppress TSH (63). Since studies investigating the effects of subclinical hyperthyroidism often included patient on suppressive levo-thyroxine treatment, the opportunity to extend these conclusions to spontaneous subclinical hyperthyroidism has been questioned (64).

FT4 is elevated in most but not all elderly hyperthyroid patients. The concomitance of T3-thyrotoxicosis, that is more common in the elderly, confirm the diagnosis. However, FT3 may be normal in many elderly patients and if the normal decrease of T3 with aging is not considered, an elevated FT3 may be mistakenly considered as normal. Furthermore, nonthyroidal illness and drugs (propranolol and iodinate contrast material) may lower T3 levels.

TSH levels are suppressed in hyperthyroidism, with the exception of central hyperthyroidism. As reported above, diagnosis of subclinical hyperthyroidism may be cumbersome and the presence of an isolated low TSH value, particularly if only partially suppressed, may not necessary indicate subclinical hyperthyroidism. Indeed, a low serum TSH value (<0.1 mIU/L) alone showed a low predictive value for hyperthyroidism in person older than 60 years, while a concomitant elevated T4 raised its predictive value (4). Administration of drugs that reduce TSH levels, as dopamine and glucocorticoid, must be ruled out. Positive circulating anti-thyroid autoantibodies and a goiter at palpation or ultrasound examination are useful tools to confirm the presence of thyroid abnormalities and to distinguish between Graves' disease and nodular goiter.

Treatment of hyperthyroidism

Although clinically hyperthyroid elderly patients may be maintained on anti-thyroid drugs, definitive treatment with 131-I or surgery is advisable. 131-I is the treatment of choice, especially when concomitant cardiovascular diseases are present, while surgery may be considered in presence of obstructive symptoms caused by large goiters, nevertheless radioiodine may be successfully used in large compressive goiters (76 reviewed in 65). Administration of beta blockers may prevent symptoms of thyrotoxicosis following 131-I treatment. 60% of patients with atrial fibrillation reverted to sinus rhythm within four months after restoration of euthyroidism (66). A long duration of hyperthyroidism and old age are negative determinants for reversion to sinus rhythm.

At the moment, most authors advice treatment of elderly patients with subclinical hyperthyroidism and a clearly suppressed TSH level (<0.1 mIU/L) and follow up for patients with TSH levels between 0.1 and 0.4 mIU/L (16; 18; 40). Beta-blockers have been proposed to reduce the cardiac effects of levo-thyroxine suppressive treatment (67).

Screening for subclinical hypothyroidism and subclinical hyperthyroidism

As a consequence of the uncertainty about effectiveness of treatment of subclinical thyroid disease, and particularly of subclinical hypothyroidism, the need of screening for thyroid disease is under discussion as well. Benefits of screening have been considered inadequate by some authors, who rather advocated aggressive case-finding in subjects at risk (18; 68). In our view, the relatively high frequency of subclinical thyroid disease in the elderly population, the vague clinical presentation of hypothyroidism in this age group and the need for treatment of many patients are strong arguments favoring the screening for thyroid disease at this age. This is in keeping with the recommendations of the American Thyroid Association and the American Association of Clinical Endocrinologists (40; 69).

Non toxic goiter in the elderly

With age, goiter size increases, thyroid nodularity develops and TSH decreases. The largest goiters are observed in the oldest age groups, living in iodine deficient areas. At this age goiters are commonly multinodular and are frequently associated with a suppressed TSH, expression of thyroid autonomy, that can progress to overt thyrotoxicosis. The prevalence of diffuse and nodular goiter in young adults participating in the Pescopagano (an iodine deficient area) survey was 30% in young adult and increased up to 75% in the age group 55-65 years, with nodular goiter accounting for about one third of the total (21). Goiter is more common in women than in men (70). As reported above, in the Pescopagano survey, prevalence

of thyroid functional autonomy was 15% in subjects over 75 years, with multinodular goiter accounting for the majority of cases. Large goiters may cause obstructive symptoms. Follow-up is the choice when symptoms are absent, whereas treatment is warranted in goiters presenting with hyperthyroidism (and thyroid autonomy) or compressive symptoms. Since thyroid autonomy is very common in the elderly, suppressive levo-thyroxine treatment is not recommended at this age. ¹³¹I is the standard treatment for thyroid autonomy and hyperthyroidism, whereas surgery see above is advised for large non-toxic goiters causing significant compressive symptoms. ¹³¹I therapy has been proposed in order to reduce thyroid volume in non-toxic goiters, with satisfactory results. In multinodular goiters, the nodules are structurally and functionally heterogeneous, and are separated by varying amounts of extracellular matrix. In addition, large nodular goiters often present with cysts, hemorrhage, fibrosis and calcifications. Shrinkage of goiter in the 6-12 months after ¹³¹I treatment has been described in several studies, but there was a large variation in the radioiodine activity administered and the degree of shrinkage. Certainly, the reduction of goiter size is related to the dose of ¹³¹I administered per gram of thyroid tissue and very large nodular goiter, with irregular distribution of radioiodine uptake due to cold nodules and areas of involution, may require bigger doses of ¹³¹I.

Recently, administration of recombinant human TSH (rhTSH) before ¹³¹I has been proposed in non-toxic and autonomously functioning goiter. In the earlier studies, rhTSH increased ¹³¹I uptake in nontoxic nodular goiter (⁷¹), particularly in cold areas (⁷²). More recent reports have indicated that pretreatment with rhTSH may increase the efficacy of ¹³¹I therapy, supporting the concept that it may reduce the required activity of radioiodine (⁷³; ⁷⁴). Acute side effects have been reported to be absent in one study (⁷³), and relatively common in another (⁷⁴). Since these studies altogether included a relatively small number of subjects with different thyroid size and function (non toxic, autonomous, toxic), definitive conclusions can not be drawn. In particular, the amount of rhTSH to be administered, the timing of its administration and the dose of ¹³¹I have not been established yet. Thus, appropriate treatment protocols should be defined in order to avoid severe acute adverse effects caused by radiation-induced thyroiditis and oesophagitis after rhTSH pretreatment (⁷⁵).

Conclusions

The aging process is associated with a number of thyroid function changes. The question of whether and to what extent these changes are expression of the aging process per se or of an age-associated thyroidal and nonthyroidal illness is a matter of debate. Studies on healthy elderly subjects suggest that several age-related changes in thyroid function are indeed independent of nonthyroidal illness. Human aging is often associated with an increased prevalence of thyroid autoantibodies, that may be not the consequence of the aging process by itself, but rather an expression of age-associated disease. Thyroid diseases in older patients differ from those observed in younger patients in their prevalence and clinical expression and their treatment often deserves special attention because of the increased risk of complications. For all these reasons clinical evaluation of thyroid status is, to some extent, different in elderly patients and deserves a particular consideration.

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Redifferentiation therapy of thyroid cancer

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Introduction

The classical molecular genetic view of cancer states that carcinogenesis is a multistep process whereby cells accumulate mutations in essential genes that give them growth advantages and finally develop into malignantly transformed cancer cells with the ability to invade neighboring tissues and blood vessels to give rise to metastases [1]. Recently, this view is being complemented by conceding a major role for interactions between tumors and their stromal environment [2] as well as for so-called epigenetic changes, such as histone modification and DNA methylation, that influence gene activity without altering gene sequence [3]. In the course of these events, cells abandon the expression of genes encoding tissue-specific proteins and consequently lose the ability to perform their proper physiological functions, i.e., they undergo de-differentiation. Concerning thyroid cells, this includes, probably due to a deficit in thyroid-specific transcription factors [4-6], the loss of thyroperoxidase (TPO), thyrotropin receptor (TSHr), thyroglobulin (Tg) and, as a comparably early event, of the sodium iodide symporter (NIS) [7] which then also has therapeutic consequences in terms of the feasibility of radioiodide therapy. Also, this process may induce slowly growing thyrocytes, showing only minimal probability to undergo division during their lifetime, to give rise to one of the most aggressive and rapidly proliferating cancers in humans, an anaplastic thyroid carcinoma (ATC) [8].

A recent alternative to cytotoxic drug treatment of cancers is re-differentiation therapy. In case of the thyroid gland this option is tested especially for those cases that do no longer respond to established treatment protocols [9]. Here, it is the aim to reactivate at least some of the features of differentiated cells, e.g. to control rapid tumor growth by stimulating appropriate cell cycle regulation or an adequate level of apoptosis, or to reinduce typical functions, such as radioiodide uptake, being exploitable for treatment. This must again involve changes in gene expression and may be achieved by various types of drugs targeting different, although mutually interdependent, mechanisms of the control of gene activity: 1) by providing transcriptional signals, 2) by influencing the accessibility of genes for the transcriptional machinery or 3) by altering the long-term activation or silencing of genes. As it is of course impossible to revert chromosomal rearrangements, deletions or mutations of essential tumor suppressor or oncogenes, it is the epigenetic level that has become a main focus of re-differentiation therapy. Concerning thyroid carcinomas, all these principles have been applied, either experimentally in vitro and in vivo or in clinical trials, and will be discussed in the following sections.

1) Modulating gene expression using nuclear receptor ligands

Retinoic acid

A class of substances widely utilized for chemoprevention and therapy of hematological and solid tumors are the vitamin A-derived retinoic acids (RA) and some of their synthetic derivatives [10]. RA are key regulators of morphogenesis, proliferation, and differentiation during development and are involved in vision, spermatogenesis and the maintenance of healthy, functional tissues, such as the lung, in the adult. Various effects of RA in cancer cells have been described. They have been shown to affect signaling pathways regulating growth, differentiation and apoptosis via proteins such as AP-1, MAPK, PI3 kinase, Akt, cyclins, cyclin-dependent kinases and their inhibitors, Bcl proteins and caspases [11].

RA exert their effects via nuclear receptors acting as ligand dependent transcription factors. There are two subfamilies of RA receptors, retinoic acid receptors (RAR) which bind all-trans RA and 9-cis RA and retinoid X receptors (RXR) which bind only 9-cis RA. They usually function as RAR-RXR heterodimers and interact with RA response elements in the regulatory region of RA-responsive genes. In the absence of ligand, they recruit so-called co-repressor complexes. These contain histone deacetylase (HDAC) activities which remove acetyl residues from the tails of histones H3 and H4 and thereby induce a compact and closed conformation of the chromatin. In the presence of ligand, nuclear receptors recruit co-activator complexes. These include histone acetyl transferase (HAT) activities adding acetyl residues to histones. This causes an opening of the chromatin making DNA accessible for the transcriptional machinery which then initiates transcription [12]. Necessarily, this is a global process affecting many RA-responsive genes simultaneously. However, as RA-regulated genes are involved in cell cycle regulation and apoptosis, a growth-limiting effect may be the consequence. Moreover, as it is the case in the thyroid gland, therapeutically "useful" genes may be induced (see below).

From that it is evident that cells must have an adequate supply of RARs and RXRs as a prerequisite for RA signal transduction. Several publications report that RARs and RXRs are present in thyroid carcinomas. However, their expression levels may vary between tumor cell lines as well as tumors and control thyroid tissues [13-17]. There may also be a redistribution of the normal nuclear localization of the receptor to the cytoplasm as demonstrated by immunocytochemistry of thyroid carcinoma specimen [17]. Tang et al. [16] reported that loss of mRNA expression for one or more RAR or RXR correlated with increased proliferation and altered histological features of PTC tumors. There may even be an association between the responsiveness of thyroid carcinoma cells to RA and the loss of certain receptor subtypes, namely RXR γ or RAR β [18-20] as only those lines expressing these isoforms exhibit growth reduction after RA treatment. Thus, the RAR/RXR repertoire of thyroid tumors may determine, if they will respond to RA re-differentiation therapy.

Nevertheless, a couple of experimental studies demonstrated that RA treatment can indeed alter the expression of certain differentiation-related markers in thyroid carcinoma cells. These are, among others, Tg [21], ICAM-1 [22], and type I 5'-deiodinase (5'DI) [23,24] which are up-regulated and CD97 [25] and urokinase which are down-regulated [26]. Growth reduction in vitro or in a xenotransplantation model was also observed [19,25]. Most interesting, NIS mRNA expression and activity are increased by RA [27,28], which is due to a RA response element in the human NIS promoter [29]. This is therapeutically promising, as it opens the possibility to treat patients exhibiting non-accumulating tumors or metastases with

radioiodide after applying RA. Interestingly, this RA responsiveness is also exhibited by NIS aberrantly overexpressed in the mammary cancer cell line MCF-7 so that NIS is discussed also for radioiodide therapy of breast cancer (reviewed in [30]). Furthermore, a combination between NIS gene therapy and RA differentiation therapy has been considered for the treatment of prostate cancer [31]. 5'DI is a differentiation marker in the thyroid, as 5'DI expression and enzyme activity are high in the healthy gland but progressively lost in thyroid carcinoma in correlation with increasing malignancy. Stimulation of 5'DI mRNA and protein expression as well as enzyme activity was observed in follicular, but not in anaplastic thyroid carcinoma cell lines [23] and, furthermore, in a fine needle aspiration biopsy obtained from a thyroid carcinoma patient treated with RA [32]. A RA responsive element is also present in the 5'DI promoter [33,34].

Several studies have been conducted to elucidate RA effects on thyroid carcinoma in patients. In a multi-center pilot study [35-37], 75 patients with advanced thyroid cancer and without the therapeutic options of operation or radioiodide therapy were treated with 13-cis RA at a dosage of 1.5 mg/kg body weight daily over 5 weeks. Of the 50 patients evaluated, 13 showed a clear increase in radioiodine uptake, and eight a mild increase. Tg levels (a tumor marker for thyroid cancer progression) were unchanged or decreased in 20 patients. Tumor size was assessable in 37 patients; regression was observed in 6, and there was no change in 22. Enhanced glucose uptake into tumors results from anaerobic metabolism and is indicative of rapid malignant growth. Glucose uptake was measured by ¹⁸F-FDG PET, and there was an increase after RA in 1, a decrease in 6 and no change in 25 of 32 patients. In total, a response, classified as a reduction of tumor size and/or Tg levels with or without concomitant increase in radioiodide uptake, was observed in 10 of 50 patients; a stabilization of these two parameters was observed in another 9 patients. Response to retinoid therapy did not always correlate with increased radioiodine uptake, so other direct antiproliferative effects had to be assumed. Several other studies with smaller numbers of patients report on similar [38-42] or lower efficiencies [43] of RA with respect to the parameters mentioned. However, therapeutically relevant doses of radioiodide were accumulated by some of the lesions with previously insufficient or absent radioiodide uptake after RA treatment [35,38]. In one study, two of 25 patients were completely free of symptoms after a follow-up of two years [42]. A summary of the various studies is presented in Table 1.

 Table1 as PDF

To further elucidate the potential of this therapeutic modality, a multi-center, randomized, prospective phase II/III trial ("MSSR study") [44] has now started to recruit patients. 120 participants will be enrolled in two arms, one receiving 13-cis RA at 1.5 mg/kg body weight daily for six weeks alone, the other arm ablative ¹³¹I -radioiodide therapy in addition. Monitoring will include whole body scan, determination of Tg serum levels, ¹⁸F-FDG PET and determination of tumor size.

RA is the only compound discussed for re-differentiation of thyroid cancer for which already therapeutic experience is available from several clinical trials. A couple of other compounds are being evaluated in vitro and in animal models, and so far, some first studies in patients have been initiated. The respective data are reviewed in the following sections.

PPAR γ ligands

PPAR γ is, like RAR and RXR, a member of the nuclear receptor family. This receptor which functions as a heterodimer with RXR is, above all, known for its role in lipid metabolism and adipocyte differentiation. Its ligands are, on the one hand, natural compounds such as polyunsaturated fatty acids and eicosanoids, on the other hand, synthetic drugs such as glitazones or thiazolidinediones [45]. In the context of thyroid carcinoma, PPAR γ came into focus after the description of a chromosomal translocation, t(2;3)(q13;p25), in follicular thyroid carcinomas and later also in follicular thyroid adenomas [46-50]. The product, a fusion protein of thyroid transcription factor PAX8 to peroxisome proliferator-activated receptor &gamma (PPAR γ), is a dominant-negative inhibitor of PPAR γ signal transduction, accelerates cell proliferation, reduces apoptosis and permits anchorage independent and growth without contact inhibition [46,51], indicating that PPAR γ is involved in tumor suppression as well as epithelial differentiation in the thyroid gland. Consistent with these observations, restoration of PPAR γ expression in PPAR γ -negative NPA cells derived from a papillary thyroid carcinoma (PTC) decreased cell growth, and PPAR γ agonists induced further inhibition, both associated with increased p27(kip1) protein levels and apoptotic cell death [52]. A dose-dependent growth-inhibitory effect was also observed in the PPAR γ -positive ATC cell lines OCUT-1 and ACT-1 after treatment with the PPAR γ ligands troglitazone and 15-deoxy-delta 12,14-prostaglandin J2. Cells were arrested in G1 via a p53-independent, but p21- and p27-dependent cytostatic pathway [53].

1 α ,25-dihydroxyvitamin D₃ (VitD3)

VitD3 also activates a nuclear receptor that belongs to the same family as RAR and PPAR γ and also forms heterodimers with RXR. VitD3 plays a role in Ca²⁺ and phosphate metabolism, in the differentiation of bone and skin and has shown anti-tumor effects in several preclinical and clinical studies [54-56]. VitD3 as well as its synthetic analogs, 22-oxa-1,25 (OH)₂D₃ (OCT) and EB1089 exhibited a dose-dependent, growth-inhibitory effect on human PTC-derived NPA cells in vitro [57,58]. VitD3 and EB1089 were also shown to induce a G1-phase arrest accompanied by an increase in the expression of the cyclin-dependent kinase inhibitor, p27(kip1) due to reduced degradation. Dackiw et al. [59] used a model of 4- to 5-week-old female SCID mice xenografted with WRO cells which are derived from a human follicular thyroid carcinoma (FTC). Mice were treated i.p. three times a week with 0.75 μ g/kg VitD3 or vehicle and killed after 21 d. Tumors from vehicle-treated animals demonstrated morphological features of epithelial malignancies with characteristics of insular carcinoma and multiple metastases to the lungs, whereas tumors from VitD3-treated animals demonstrated signs of differentiation with restoration of Tg staining, associated with a marked accumulation of p27(kip1) immunoreactivity in the nuclear compartment.

2) Modulating gene expression via HDAC inhibitors:

As described above, a consequence of nuclear receptor-dependent transcriptional regulation is an alteration in chromatin modification by interaction with histone modifying enzymes. Instead of this indirect mechanism, histone modifying proteins may be targeted directly, e.g., by the use of HDAC inhibitors. This is a quite heterogeneous class of substances, including, e.g., hydroxamic acids (Trichostatin A (TSA), suberoylanilide hydroxamic acid), carboxylic acids (valproic (VPA) and butyric acid), benzamides (MS-275, N-acetylaldinaline), and fungal metabolites (depsipeptide (FR901228, FK228), apicidin). The rationale for their use against cancer is the empirical observation that HDAC activity is generally increased in cancer cells,

resulting in altered gene transcription. Furthermore, there is evidence that genes for HDACs are disrupted in certain malignancies or that there is an aberrant recruitment of HDACs by oncogenic transcription factors. In normal cells, the pattern of histone acetylation together with that of other histone modifications define the so-called "histone-code" which is involved in discriminating domains of active from those of inactive chromatin. The effects of the application of HDAC inhibitors on gene expression are, of course, more global, as the result is not the regulation of very specific responsive promoters, but a more general relief of gene silencing. Nevertheless, a significant up- or down-regulation is displayed by no more than 4 – 10 % of genes in cultured cells. Many of these genes are involved in growth arrest, apoptosis, angiogenesis and differentiation, e.g., the one coding for cyclin-dependent kinase inhibitor p21^{WAF}, which is up-regulated by all known HDAC inhibitors. A couple of publications describe anti-cancer properties of HDAC inhibitors in several cancer cell lines and animal models, and several phase I/II clinical studies indicate that they are well-tolerated drugs [60,61].

Valproic acid (VPA)

VPA has been used for years as an antiepileptic drug before its properties as a HDAC inhibitor were discovered [62], and both its pharmacological properties and the spectrum of its side effects are well characterized. Pharmacologic concentrations determined in patients taking VPA for anticonvulsant therapy reach 0.7 mM. Catalano et al. [63] demonstrated effects of VPA in the poorly differentiated PTC cell lines NPA and BHT-101. At concentrations ≥ 1 mM, they observed a decrease in cell viability, an increase of apoptosis via caspase 9, and an induction of cell cycle arrest at G1. The latter was accompanied by up-regulation of p21^{WAF} and down-regulation of cyclin A mRNAs and proteins. Furthermore, NIS mRNA expression is increased in NPA cells at VPA concentrations ≥ 1 mM and in the ATC cell line ARO at concentrations ≥ 1.5 mM. This was accompanied by an induction of NIS protein, which was localized in the plasma membrane of NPA but intracellularly in ARO cells. Iodide uptake was only increased in NPA cells [64].

Depsipeptide (FR901228, FK228):

Furuya et al. [65] described the powerful effects of depsipeptide on BHP18–21v (derived from a PTC) and ARO cells. At concentrations from 1 to 10 ng/ml, NIS, TPO and Tg mRNA and protein expression were stimulated. Iodide uptake was increased from 3 ng/ml on in both cell lines. Furthermore, in the treated cells, accumulated iodide was organified into a protein species of more than 210 kDa molecular weight, probably by depsipeptide-induced TPO, as this effect could be blocked by the TPO inhibitor methimazol at a concentration of 300 μ M. Depsipeptide also increased accumulation, retention and organification of iodide in tumors that had grown from xenotransplanted BHP18-21v cells on nude mice. In these tumors depsipeptide could also induce the expression of NIS, Tg and TPO mRNAs, whereas TSH-R mRNA was not stimulated. Depsipeptide also increased dose-dependently the expression of TTF-1, but not of PAX8, mRNA in BHP-18-21v and ARO cells.

Kitazono et al. [66] performed all their experiments using FTC-133, FTC-236, Kat-4 and SW-1736 cells at a depsipeptide concentration of 1 ng/ml, which is only minimally cytotoxic as determined by MTT assay. After 48 and/or 72 h, they observed increased histone acetylation by Western blot and immunocytochemistry, elevated iodide uptake after 72 h and enhanced activity of a Tg promoter/luciferase reporter construct in all cell lines. Depsipeptide also restored expression and function of p53 in SW-1736 cells which are "pseudo-null" for this protein [67].

It may be added that the concentrations required to see effects in thyroid carcinoma cell lines can easily be achieved in patients, as in phase I studies concentrations up to 500 ng/ml have been tolerated without significant toxicity [66].

Trichostatin A

A reduced cell viability at TSA concentrations up to 250 ng/ml was described for TPC-1, FTC-133 and XTC-1 cells, deriving from a PTC, an FTC and a Huerthle cell carcinoma (HTC), with cytotoxicity occurring from 1000 ng/ml on. NIS mRNA was induced by 50 and 100 ng/ml TSA and basal pendrin gene expression was reduced by these two concentrations [68]. In cell lines derived from primary ATC, sodium butyrate and TSA repressed proliferation independent of the p53 status through the induction of apoptosis and cell cycle arrest in G1 or G2/M. Apoptosis was associated with the appearance of the cleaved form of the caspase substrate, poly-(ADP-ribose) polymerase (PARP), while a reduced expression of cyclins A and B, an increased expression of the cyclin-dependent kinase inhibitors, p21^{WAF1} and p27(kip1), a reduced phosphorylation of the retinoblastoma protein and a reduction in cdk2- and cdk1-associated kinase activities accompanied cell cycle arrest. However, in ATC cells overexpressing cyclin E, drug treatment failed to replicate these events [69].

3) Modulating gene expression by inhibition of DNA methyl transferases

DNA methylation is a covalent modification of CpG dinucleotides in genomic DNA which plays a role in the regulation of gene activity, namely transcriptional repression, and results, e.g., in long-term silencing of genes by X chromosome inactivation or genomic imprinting. Two mechanisms have been described: One is the direct inhibition of transcription factor binding to a methylated response element. The other is based on proteins such as MBD 1-3 and MECP2 that specifically bind to methylated CpG dinucleotides, interact with histone methylases or HDACs and thereby contribute to the generation of inaccessible chromatin [70]. Abnormal patterns of DNA methylation are observed in human cancers [71], among them those of the thyroid gland [72], and include general genomic hypomethylation in combination with regional hypermethylation. Hypermethylation-triggered silencing affects genes involved in almost all of the important steps of cancerogenesis: cell-cycle regulation, DNA repair, drug resistance and detoxification, apoptosis, differentiation, angiogenesis, and metastasis, e.g., the genes coding for CRABP1, CDKN2/p16INK4A, RASSF1, LKB1, MT1G, maspin, E-cadherin, TSH receptor, NIS, pendrin and 5'DI in thyroid cancer and/or carcinoma cell lines [73-85]. Recently, Hoque et al. [86] showed hypermethylation for Rassf1A, TSHR, RAR-beta2, DAPK, CDH1, TIMP3, and TGF-beta, and, furthermore, a trend toward multiple hypermethylation in thyroid cancer tissues. Hypermethylation of 2 or more markers was detectable in 25% of hyperplasias, 38% of adenomas, 48% of thyroid cancers, and 100% of cell lines, and a subset of these markers were epigenetically modified in concert, probably reflecting an organ-specific regulation process. E.g., a positive correlation was found between the BRAF mutation and RAR-beta2, and a negative correlation was found between BRAF and Rassf1A.

Interference with DNA methylation to release gene silencing and thereby activate dormant tumor suppressor genes for experimental and clinical purposes makes use of derivatives of cytidine derivatives, namely 5-aza-cytidine or 5-aza-2'-deoxycytidine (decitabine). They get incorporated into DNA during replication (or, as for 5-aza-cytidine, also into RNA) and then inhibit the action of DNA methyltransferases. Several clinical phase-I/II trials have been performed for solid tumors, however, the highest potential for these substances has so far been

demonstrated in the case of hematological malignancies [87].

As for thyroid cancer, Venkataraman et al. [4] analyzed a collection of human thyroid carcinomas with different pathologies and reported variable methylation patterns of the hNIS promoter and first exon which, however, did not correlate with NIS mRNA expression or clinical iodide uptake. When seven human thyroid carcinoma cell lines lacking NIS mRNA were treated with 5-azacytidine or sodium butyrate, NIS mRNA expression was re-induced in four and iodide transport in two cell lines. This was associated with demethylation of NIS DNA in the untranslated region within the first exon and with restoration of the expression of TTF-1.

An association has been demonstrated between de-differentiation of thyroid carcinomas and E-cadherin expression in ATC, and E-cadherin expression is an independent prognostic factor for highly differentiated thyroid tumors. Hypermethylation of CpG islands in the proximal promoter region of the E-cadherin gene could be demonstrated in thyroid tumors and in E-cadherin-negative cell lines derived from thyroid carcinomas [88]. On the other hand, treatment with 5-aza-2'-deoxycytidine restored E-cadherin expression in 5 thyroid carcinoma cell lines [89].

Results from our group indicate that methylation of a GC-rich region close to a thyroid hormone responsive element in the 5'DI promoter may be responsible for silencing of the 5'DI gene in human thyroid FTC cell lines FTC-133, FTC-238 and HTh74. Treatment of these cell lines with 5-aza-2'-deoxycytidine drastically increases both 5'DI mRNA and enzyme activity [85]. Interestingly, this increase was also seen in the ATC cell line HTh74, where 5'DI was not stimulated by RA [23].

4) Miscellaneous

Phenylacetate

Phenylacetate and phenylbutyrate are reported to inhibit growth and to modulate differentiation in a variety of cancer cell lines as well as in patients at concentrations exhibiting minimal toxicity. Inhibition of HDACs and activation of PPAR γ are discussed as possible mechanisms [90,91]. In five FTC cell lines, cell cycle arrest in the G0-1 phase occurred at a dose of 2.5-10 mmol/L phenylacetate. Increased radioiodide uptake (in 2 out of 5 cell lines) and decreased secretion of vascular endothelial growth factor were also observed [92]. Furthermore, combination of phenylacetate and RA had synergistic effects: In FTC-133 cells, RA (2.5 μ mol/L) inhibited growth by 16% and PA (10 mmol/L) by 35% versus controls, whereas the combination inhibited growth by 60% at 5 days [93].

Resveratrol

Resveratrol, trans-3,5,4'-trihydroxystilbene, is a fruit constituent of various plants, including grapes, berries and peanuts. Besides cardioprotective effects, resveratrol exhibits anticancer properties in a wide variety of tumor cells. The growth-inhibitory effects of resveratrol are mediated through cell-cycle arrest and activation of caspases; suppression of angiogenesis has also been described. In vivo, resveratrol shows both chemopreventive and therapeutic effects against cancer and seems to be pharmacologically quite safe [94]. In two PTC and two FTC cell lines, treatment with 1-10 μ M resveratrol induced apoptosis by increasing p53 expression and serine phosphorylation via a Ras-MAPK kinase-MAPK signal transduction pathway [95].

3-Hydroxymethyl-3-methylglutaryl coenzyme A (HMG-CoA) inhibitors: Statins

Statins have been approved for the treatment of lipid disorders. However, studies in cell culture, with experimental animals and first trials in patients also indicated antitumor properties such as induction of growth arrest and apoptosis as well as inhibition of metastasis and of angiogenesis [96]. In thyroid carcinoma cell lines, lovastatin induced apoptosis, and several different mechanisms have been discussed, such as inhibition of geranylgeranylation of Rho proteins, lamin B proteolysis and cytochrome C release from mitochondria [97].

Concluding remarks

From the data reviewed above it is clear that there is a broad selection of drugs inducing (partial) re-differentiation and exerting anti-tumor effects on experimental models for thyroid cancer, which now have to be tested in patients. To date, there are five clinical trials evaluating re-differentiation therapy for thyroid cancer listed by the NIH [98]: a phase I and a phase II study on desipeptide, a phase II study on 5-aza-2'-deoxycytidine, and a phase I study on rosiglitazone; a phase I on azacytidine is no longer recruiting patients (Table 2).

 Table 2 as PDF

A multi-center phase II/III trial on RA has just been started ("MSSR study")[44]. Data published on RA show that a certain number of patients seems to experience benefit from this kind of therapy, however, most patients did not respond. Whereas studies initially reported response rates of about 20 % [35,39], they may be lower after longer follow-up intervals [42]. For an extensive discussion, see references [34-42]. Other re-differentiating agents might be more efficient, especially as PPAR γ ligands, HDAC inhibitors or DNA methylation inhibitors show positive effects in ATC cell lines where RA was inactive [52,64,85]. The most promising feature of these drugs, however, might be their use in combination. As they target several different aspects of the same process, i.e., control of gene activity, their combination may be synergistic. Thus, HDAC inhibitors could open up chromatin and give nuclear RA or VitD3 receptors access to gene regulatory sequences. Similar considerations hold true for a combination of HDAC and DNA methylase inhibitors due to the interaction of methylated CpG-binding proteins with HDACs. Combination of drugs may also help to reduce doses of single compounds to circumvent possible dangerous side effects that are elicited by methyl transferase inhibitors [99]. Conforming with these considerations, the HDAC inhibitor m-carboxycinnamic acid bis-hydroxamide (CBHA) at 50, 100, and 200 mg/kg/day inhibited growth of SMS-KCN-69 neuroblastoma tumor xenografts on immunodeficient mice in a dose-dependent fashion, with 200 mg/kg CBHA resulting in a complete suppression of tumor growth. The efficacy of 50 and 100 mg/kg CBHA was enhanced by the addition of 2.5 mg/kg all-trans RA, while this dose of all-trans RA was ineffective when administered alone [100]. In a mouse model for lung cancer, treatment with low doses of 5-aza-2'-deoxycytidine (0.5 mg/kg) decreased the incidence of neoplasms by 30%. When the methyl transferase inhibitor 5-aza-2'-deoxycytidine was combined with the HDAC inhibitor phenylbutyrate (300 mg/kg), lung tumor development was significantly reduced by >50%, while no effect was seen with

phenylbutyrate alone [101].

Thyroid cancer in general is an easily treatable disease with a good prognosis. However, in a considerable number of cases, de-differentiation of the tumors makes classical therapy obsolete and, therefore, other approaches are required. Starting from the first attempts using RA, the field of re-differentiation therapy for thyroid cancer has developed and now offers promising new prospects in vitro and in experimental animals. Hopefully, it will be possible, after they have shown their potential in the ongoing clinical trials, to transform them into new therapeutic alternatives for otherwise untreatable thyroid cancer patients.

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TSH-Receptor structure and mechanism of activation

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In a previous review (1) on the structure and mechanism of activation of the thyroid stimulating hormone receptor (TSHR), published in 2001, we summarized the evidence in relation to structure and function of the TSHR. Since 2001 several new and important insights in TSHR structure and signal generation have been reported. In this review we will summarize these recent key findings concerning the TSHR research of the last years.

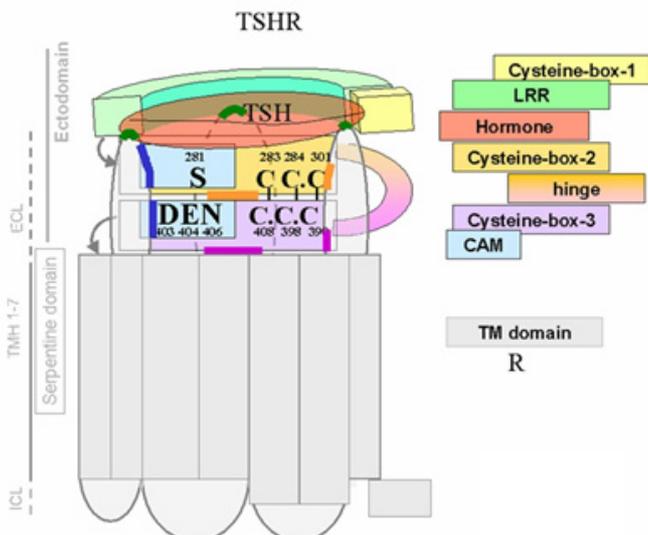
The thyroid stimulating hormone (TSH) as a member of the glycoprotein hormone family is secreted by the pituitary gland and controls thyroid function and proliferation via interaction with the membrane associated TSHR. Binding of TSH results in activation of the G protein-coupled TSHR and in stimulation of second messenger pathways, like cyclic adenosine monophosphate (cAMP), inositolphosphates (IPs) and diacylglycerol (DAG). Intracellular accumulation of these second messengers leads to modification of different physiological properties of thyroid cells.

The TSHR belongs together with the choriogonadotropin/leutinizing hormone receptor (CG/LHR), follicle stimulating hormone receptor (FSHR) and leucin rich repeat containing glycoprotein receptors (LGRs) to the subfamily of glycoprotein hormone receptors (GPHR) (2-4). The TSHR, LHR and FSHR are characterized by a large N-terminal extracellular domain (ECD), a seven transmembrane spanning helix motif (TM1-7) connected by three extra- and three intracellular loops (ECL1-3; ICL1-3) and a C-terminal intracellular domain.

Despite functional characterization of numerous in vivo TSHR mutants less is known about the precise mechanisms of receptor activation. The understanding is hindered by the lack of information about the three-dimensional structure of the TSHR. One reason is the small number of functionally characterized amino acid exchanges for the large extracellular domain and the extracellular loops of the TSHR. The major part of naturally occurring mutations is located in the TMs (5, www.uni-leipzig.de/~innere/tsh/). The occurrence of in vivo activating mutations revealed first insights into intra- and intermolecular structure-function relationships of the TSHR and led to new approaches for the elucidation of the mechanism of TSHR activation by site-directed mutagenesis and molecular modelling.

Extracellular Domain and Extracellular Loops

The large N-terminal extracellular domain consists of five molecular sections: (i) an N-terminal Cysteine-box-1 (C-b1) (hTSHR: 1-54), (ii) a 230 aa spanning leucin rich repeat motif (LRR) (hTSHR: 55-279), (iii) the central Cysteine-box-2 (C-b2) (hTSHR: 280-314), (iv) a TSHR specific 50 aa insertion (hTSHR: 317-366) and (v) the C-terminal Cysteine-box-3 (C-b3) (hTSHR: 370-410). (6, Figure).



LEGEND: TSHR: cartoon representing a molecular model (6) for the very tight packing of the ectodomain (LRR, Cysteine-box-2, Cysteine-box-3) being located very close to or even in between the extracellular loops of the serpentine domain. A new LRR template based on the Nogo receptor with much higher sequence similarity to the TSHR was introduced, whose 'scythe blade' shape also allows an interaction of the hormone parallel to the LRR structure. Furthermore, a new template for Cysteine-boxes -2 and -3 was identified based on a complex structure of the chemokine IL8 and a portion of the N-terminal tail of the IL8 receptor. These findings also support the hypothesis of a disulfide bridge between Cys398/Cys408 (C-b3) either to Cys283/Cys284 (C-b2) or in a reverse manner with Cys408/Cys398. (6, 20). Furthermore, the hydrophilic amino acids Asp403, Glu404 and Asn406 of C-b3, spatially located in proximity to Ser281, are likely to be involved in intramolecular signal transduction from the ECD towards the serpentine domain.

The largest structural characteristic of the extracellular domain is the leucine rich repeat (LRR) motif (2-4). The LRRs are not only a direct interaction partner for the ligand, but also have an essential role for receptor function. Mutagenesis of the N-terminal part of the TSHR and CG/LHR has shown that activity of the TSHR can be directly influenced by changes in the extracellular domain (7, 8). Based on the crystal structure of LRR containing proteins only the structural properties of the LRRs in the middle of the ECD sequence of the GPHRs were determined (9, 10) To evaluate an optimal structure template for the LRR Kleinau et al. (6) determined the LRR motif based on the Nogo receptor (11) as the best matching motif for the LRR for all three human GPHRs. To investigate the influence of the ECD for TSHR activation in more detail, Zhang et al. (12) deleted the ECD. This resulted in a strong constitutive activation of the receptor. A similar experiment described the loss of constitutive activity of the naturally occurring mutations Ile486Phe (ECL1) and Ile568Thr (ECL2) after deletion of the ECD, which implies that the ECD likely interacts with regions of the ECLs as a linked inverse agonist to maintain the inactive receptor conformation (13). In addition, these findings suggest that the structure of the ECD alternates from a tethered inverse agonist to an agonist in the process of receptor activation. Another experimental approach for the CG/LHR suggests that a structure within the ECD serves as an agonist for the ECLs as part of the transmembrane domain after agonist binding or mutational alterations (14). These findings underline the importance of the ECD and the ECLs in the process of TSHR activation and support a model in which the ectodomain acts as a silencer of the serpentine domain of the receptor. The current research is mainly focused on the identification of residues or epitopes in the ECD and ECLs, respectively, which are involved in the maintenance of the inactive conformation of the TSHR. Ser281 is the only position in the ECD, which is affected by constitutively activating in vivo mutations (15-18), and it was intensively characterized together with the surrounding residues (C-b2). The highly conserved Ser281 is situated in the hinge region between the LRR motif of the ECD and the TMs like the homologous positions Ser277 in the CG/LHR and S273 in the FSHR. Mutagenesis of amino acids Pro280, Cys283 and Cys284 in the vicinity of Ser281 also led to constitutive activation of the TSHR (19). In addition, recent studies on the corresponding position Ser277 in the CG/LHR (TSHR: Ser281) by substitution of all other 19 amino acids suggest that Ser277 is an integral part of a loop like epitope that may be involved in stability and signal generation of the CG/LHR (14, 20). In fact, 15 substitutions at position Ser277 in the CG/LHR led to constitutive activation of the receptor. Taken together, the C-b2 epitope 279YPSHCC284 can probably act as an intramolecular switch for receptor activation.

In another approach a systematic search with fragments of the ECD in the protein structure database (PDB) was performed (6). Based on sequence similarities two new structural templates were identified. First, a new LRR template based on the Nogo receptor with much higher sequence similarity to the TSHR was introduced, whose 'scythe blade' shape allows also an interaction of the hormone parallel to the LRR structure. Second, a new template for C-b2 and C-b3 was identified. This template showed homologous properties to the structure of the chemokine IL8 and to a portion of the N-terminal tail of the IL8 receptor. In this way sequence similarities of C-b2 to the chemokine IL8 and C-b3 to the IL8 receptor, respectively, were determined. The very tight packing of LRR, C-b2 and C-b3 with the extracellular loops also supports the hypothesis of disulfide bridges between Cys398/Cys408 (C-b3) or between one or both of these cysteins with Cys283/Cys284 (C-b2) and/or Cys408/Cys398. (6, 21). Furthermore, the hydrophilic amino acids Asp403, Glu404 and Asn406 of C-b3 were likely to be involved in intramolecular signal transduction from the ECD towards the serpentine domain (6). Substitution of these residues by amino acids with an opposite charge and the smallest nonpolar amino acid alanine, respectively, leads to constitutive activation of the TSHR in three of five mutants (Asp403Ala, Glu404Lys, Asn406Ala). Residues Asp403 and Asn406 are highly conserved in GPHRs and alanine substitutions at these positions lead to constitutive activation of the receptor. Interestingly, Glu404, a specific amino acid for the TSHR, showed no differences in basal cAMP accumulation when mutated to alanine, whereas Glu404Lys revealed a strong basal cAMP accumulation. The authors suggest a spatial proximity of the epitope Asp403-Asn406 (C-b3) to the Ser281 epitope (C-b2) based on combined data from homology models and functional data. It is important in this context that these two epitopes in the ECD are the only ones that have been reported to lead to constitutive activation of the TSHR by in vivo mutations (Ser281Thr/Ile/Asn) or mutagenesis (Asp403-Asn406). Based on sequence similarities between the three members of the GPHRs it has been suggested that mutations of the CG/LHR and FSHR at positions corresponding to Asp403-Asn406 in the TSHR also cause constitutive activity (Figure).

To provide a hypothesis for interactions between the ECD and ECLs (12, 13) more knowledge about the structural and functional properties of these regions is necessary. In contrast to the CG/LHR and FSHR, only very few functionally characterized in vivo and in vitro mutations in the extracellular loops of the TSHR are available. Most of them are in vivo mutations: Thr477Ile (ECL1), Ile486Phe, Met (ECL1), Ile568Thr (ECL2), Asn650Thr (ECL3), Val656Phe (ECL3) and del658-661 (ECL3) (5). Only position Asp474 in the TSHR has been intensively characterized by mutagenesis (22). The ECLs seem to be important for both, the interactions with the ECD and for signal transduction towards the serpentine domain. Agretti et al. (23) generated a receptor harboring the inactivating mutation Thr477Ile in the ECL1 and the activating mutation Pro639Ser in the 6th transmembrane segment (5, 24), resulting in a dominant effect of the activating Pro639Ser mutation. Interestingly, Thr477Ile was characterized by an impaired cell surface expression and Pro639Ser showed a cell surface expression comparable to the wt TSHR. However, the double mutant Thr477Ile/Pro639Ser was expressed on the cell surface like the single mutant Pro639Ser. This suggested that not only signal pathways but also structural properties between the ECL1 and TM6 were affected, which are necessary for correct folding and trafficking to the cell surface. In a second approach these authors combined two constitutively activating mutations (Ile486Phe in the ECL1 and Pro639Ser in the ECL2), which led to an increased basal cAMP accumulation. This observation was also reported by Kosugi et al. (25) and Angelova et al. (26) for the CG/LHR. Much more is known about the ECLs of the CG/LHR and FSHR, especially for the ECL3. An alanine scan of the ECL3 including residues Lys580-Lys590 of the FSHR (TSHR: An650-Lys660) identified several residues which are crucial for activation of the Gs and Gq signal pathway and for hormone binding (27, 28). Based on the highly conserved amino acid sequence of the ECL3 within the GPHRs it is likely that the ECL3 also plays an important role in signal generation and hormone binding for the CG/LHR and TSHR. Only for position Lys660 (unpublished data) in the TSHR and Lys583 in the CG/LHR (corresponding to Lys590 in the FSHR) functional data are available (29). Comparing the results regarding cell surface expression, cAMP and IP synthesis and hormone binding similar effects of this position could be determined within

these GPHRs. In addition to the ECL3 of the FSHR, Ryu et al. (30) characterized the ECL2 of the CG/LHR by an alanine scan. Also for the ECL2 several residues, which are involved in signal generation and hormone binding, were identified. Taken together, these data can be used for refining the three dimensional models of the receptors to determine residues which are interaction points between the ECD and the transmembrane domain.

Transmembrane Domain

The transmembrane domains of GPCR consist of seven helices connected by three extracellular and three intracellular loops. Based on sequence alignments of the G protein-coupled receptors a homology of more than 70% could be determined (2-4). New insights into structural relationships of the TMs were possible with the description of the x-ray crystal structure of the bovine rhodopsin with a high resolution (31). Due to the high homology of GPCRs within their TMs this structure model is used as a template for many members of the large superfamily of G protein-coupled receptors. In addition, this model allowed many groups to provide new important insights into the architecture and side chain orientations of the TMs. The TMs of the TSHR are characterized by a large number of constitutively activating *in vivo* mutations. Most of them are located in TM6 between residues 629 and 639 (5). Furthermore, naturally occurring mutations were identified in TM2, TM3, TM5 and TM7. The high number of *in vivo* mutations underlines the importance of the TMs with regard to stabilization of the inactive receptor conformation. In particular, interactions between TM5-TM6 and TM6-TM7 seem to be involved in maintaining the native receptor conformation. Neumann et al. (32) have identified a hydrogen bond between Asp633 (TM6) and Asn674 (TM7) by a combined approach of mutagenesis and modeling guided by naturally occurring mutations. This finding was supported by Kosugi et al. (33) and Lin et al. (34) which have characterized the homologous position Asp578 in LHR (corresponding to Asp633 in TSHR) by mutagenesis. A breakdown of this interaction between TM6 and TM7 leads to a change in receptor conformation and finally to a constitutive activation of the receptor. A recent report demonstrated that residue Met389 in the LHR (corresponding to Met453 in TSHR) is essential to maintain the inactive receptor conformation by interactions with the highly conserved residues Ile460 in TM3 (Ile515 in TSHR), Met571 in TM6 (Met626 in TSHR) and Tyr623 in TM7 (Tyr678 in TSHR) (35). Due to the fact that mutation of the homologous residue Met453 in the TSHR also causes constitutive activity, it is likely that a similar network of interactions exists in the TSHR (36-39).

Intracellular Loops

A common feature of all GPCRs is their interaction with G-proteins and subsequently the activation of downstream signaling cascades. Although many studies have focused on the molecular mechanisms of G-protein activation, conserved structure motives participating in the processes of G-protein recognition and selectivity have not been identified yet (40, 41). However, studies on several GPCRs revealed that residues, which are involved in G-protein coupling and selectivity, are primarily localized at the transmembrane/cytoplasmic borders between TM3/ICL2, TM5/ICL3 and ICL3/TM6 (42). Similarly, ICLs 2 and 3 of the TSHR were found to be important for recognition and selective activation of G_{α_s} and G_{α_q} (1, 43). Initially, studies on chimeric TSHRs containing homologous sequences of the α_1 - and β_2 adrenergic receptors revealed the impact of the ICL2 for cAMP- and IP signaling (44). Kosugi et al. demonstrated the importance of the middle part of the ICL2 for G_s activation and identified amino acids 528-532 as important for G_q mediated IP production. Mutagenesis studies of the LH and FSH receptor also provided evidence for an important functional role of several amino acid residues within ICL2 (45, 46). However, only single selected residues in ICL2 were substituted in these studies. To further investigate the influence of this domain on TSHR signaling, deletion studies and alanine-scanning mutagenesis were carried out (43). Deletions of four to five residues and their corresponding multiple alanine substitutions were introduced into ICL2. Residues I523-D530, comprising mainly the N-terminal half of ICL2, appeared to be critical for G_s - and G_q -mediated signaling. A single alanine substitution screening within ICL2 revealed hydrophobic residue M527 in particular and to lesser extents F525, R528, L529 and D530 as residues that selectively abolished or strongly impaired G_q -activation. Further, double mutants between residues in ICL2 and 3 suggested interactions between these loops in the vicinity of Phe525 and Thr607, indicating a conformational cooperation between ICLs 2 and 3 during G_q -activation by TSHR.

So far, constitutively activating mutations have only been identified in the ICL3 of the TSHR, but not in the ICL1 and 2 or the C-terminus (47). Both constitutively activating point mutations in ICL 3 Asp619Gly and Ala623Ile are localized in the C-terminal part of the loop and at the ICL3/TM6 junction. Further, the activating deletion mutation del613-621 also includes the C-terminus of ICL3. Mutagenesis studies on hybrid angiotensin II AT₁ and AT₂ receptors showed, that the N-terminal part of ICL3 is primarily responsible for G_q recognition. Further, for the AT1 receptor it was shown that residues in the C-terminus of ICL3 also contribute to G_q activation. Investigation of chimeric TSHR containing homologous sequences of the α_1 - and β_2 -adrenergic receptors revealed the importance of N- and C-terminal amino acids in ICL3 for G_q mediated signaling (48). In this work Kosugi et al. showed that substitution of residues in the N- and C-termini of ICL3 abolished G_q mediated IP formation, whereas mutation of amino acids in the middle portion had no effect on TSHR signaling. In contrast, substitution of amino acids 617-620 resulted in increased basal activity regarding to the G_s – cAMP pathway. Based on the constitutively active *in vivo* deletion del613-621, different deletion- and alanine substitution mutants were characterized (49). Thereby, it has been shown that not the loss of a specific group of amino acids residues is decisive for the constitutive activation of the TSHR. However, shortening of ICL3 leads to a relative movement of TM6 towards the cytoplasm enabling critical transmembrane portions to interact with G_{α_s} . This assumption is supported by recent work of Janz et al. (50) demonstrating interactions of transducin with hydrophobic residues in TM6 following the activation of Rhodopsin. Moreover, the conserved Asp619 at the ICL3/TM6 junction was found to be necessary for maintaining the inactive conformation of TSHR and of CG/LHR as well (49). It has been suggested that Asp619 is involved in a helix-capping structure, indicating that the inactive receptor state is stabilized by interactions between residues within the helix of ICL3/TM6 junction and adjacent structures. In addition to constitutively activating and inactivating *in vivo* mutations, several polymorphisms were identified in the TSH receptor gene. The most frequently found polymorphisms are Ile606Met (ICL3) and Ala703Gly, Gln703Glu and Asp727Glu in the C-terminal tail. Several studies produced controversial results regarding the association between these polymorphisms and thyroid diseases. Gabriel et al. (51) found Asp727Glu to be an important factor in the pathogenesis of toxic multinodular goiter, whereas Ile606Met, Ala703Gly and Gln703Glu had no effect. In addition, Asp727Glu was also postulated to be involved in the development of Graves' Disease (52). In contrast, other studies found no correlation between Asp727Glu or other polymorphisms and the appearance of thyroid disorders (53, 54, 55). In the context of the wild type TSHR, the polymorphism Asp727Glu does not seem to be functionally important. Interestingly, both basal and TSH stimulated cAMP levels of the constitutively activating mutant Ala593Asn could be significantly reduced by generation of the Ala593Asn/Asp727Glu double mutant (56). This finding suggests possible unknown properties of TSH receptor polymorphisms which should be further investigated.

Conclusions

The identification of activating and inactivating mutations has revealed first insights into TSHR activation and intramolecular interactions. The combination of site-directed mutagenesis and molecular modelling represents a valuable tool for understanding of structure-function relationships and will lead to a continuous refining of the 3D structure of the TSHR. This improved TSHR model will have the potential for rational design of new therapeutical compounds, the development of TSHR agonists, antagonists or superagonists and can help to understand the molecular pathogenesis of thyroid diseases.

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THE THYROID IN NON-THYROIDAL ILLNESS**Elaine M. Kaptein, MD, FRCP(C), FACP**

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INTRODUCTION/TERMINOLOGY

In the absence of hypothalamic, pituitary or thyroid diseases, systemic illnesses have multiple effects on thyroid hormone metabolism and on serum thyroid hormone concentrations (1-3). These changes are primarily related to the severity (2) (Figure), and perhaps to the chronicity of illnesses (4), rather than to the specific disease states. Changes related to chronicity may be secondary to worsening malnutrition and/or catabolism which result in progressive declines in thyroid binding protein concentrations.

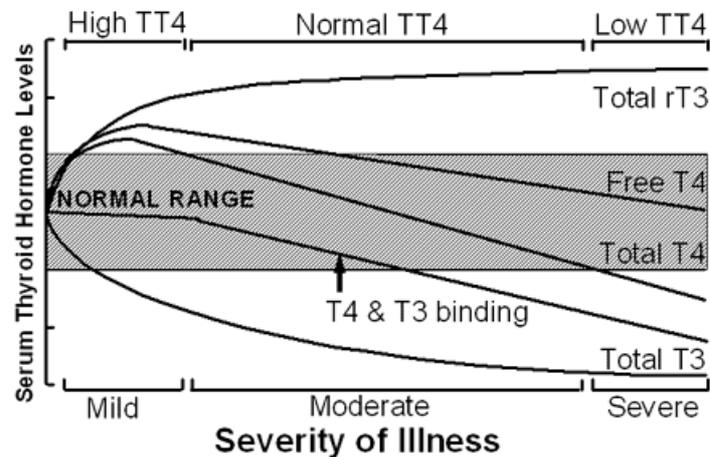


Figure : Relationship of serum thyroid hormone levels to the severity of nonthyroidal illnesses. Adapted from (3).

Most frequently, serum levels of total T3 are reduced leading to the term "the low T3 syndrome" or "the low T3 state of nonthyroidal illness". Less frequently, serum total and free T4 values and TSH concentrations are increased or decreased. Some have termed patients with low total T4 and T3 levels as "the low T3-low T4 state" of nonthyroidal illness. Many authors believe that these patients are euthyroid, hence the label "euthyroid sick syndrome", although this area remains controversial (5, 6). The term "nonthyroidal illness syndrome" (NTIS) encompasses the transient changes in serum thyroid hormone levels as well as the alterations in thyroid hormone metabolism induced by systemic illnesses in patients without concurrent hypothalamic, pituitary or thyroid diseases, and does not imply thyroid hormone status.

GOALS

The primary goals in clinical practice are to 1) understand the pathophysiology of changes in thyroid hormone metabolism and serum thyroid hormone alterations in NTIS and the potential clinical significance of these alterations, 2) differentiate changes in serum thyroid hormone levels due to NTIS from those due to concurrent hyper- or hypothyroidism, and 3) recognize effects of NTIS on serum thyroid hormone levels in patients with concurrent hyperthyroidism or hypothyroidism. In addition, a number of assumptions have been made regarding the thyroid hormone status of patients with NTIS and the potential beneficial or detrimental effects of thyroid hormone replacement therapy in these patients. These issues will be summarized.

EPIDEMIOLOGY OF NTIS

Serum total T3 concentrations are frequently reduced even with mild nonthyroidal disorders such as caloric deprivation, with the highest frequency and lowest values occurring in patients with the most severe illnesses (1, 2). Serum total T3 values return to normal only after complete recovery of the nonthyroidal illness and of the nutritional deficiency.

In the general population, altered free T4 index values are as frequently due to NTIS as to hyper- or hypothyroidism (1). Free T4 index values were transiently increased in 0.2 to 0.9% due to NTIS and in 0.3 to 0.5% due to hyperthyroidism. Transiently increased free T4 estimates are usually associated with mild nonthyroidal illnesses and acute psychiatric disorders (1). Free T4 index values were transiently reduced in 0.2 to 1.1% due to NTIS and in 0.6 to 1.1% of patients due to primary hypothyroidism (1). Transiently reduced free T4 estimates (indexes and immunoassays) occur most commonly with moderate to severe nonthyroidal illnesses, with the lowest values occurring in critically ill patients with the highest mortality rates (1). In NTIS patients with altered free T4 estimates, serum TSH values are usually within the reference range of values for euthyroid subjects facilitating differentiation from primary hypothyroidism or hyperthyroidism (1).

In hospitalized patients, serum TSH levels may be increased or decreased due to NTIS or to thyroid disease (1). Serum TSH values below 0.1 mIU/L were due to hyperthyroidism in 24% with the remainder transiently reduced due to NTIS (1). TSH values below 0.01 mIU/L were due to hyperthyroidism in 73%, and to NTIS in the remainder, while only 8% with TSH values between 0.01 and 0.1 mIU/L were due to hyperthyroidism (1). Only 14% of TSH values between 6.8 and 20 mIU/L were due to primary hypothyroidism, whereas 50% of TSH values above 20 mIU/L were due to primary hypothyroidism and 50% were transiently elevated due to NTIS (1). NTIS patients with transiently elevated or reduced serum TSH levels typically had free T4 index values in the reference range for euthyroid subjects (1). Consequently, differentiating hyper- and hypothyroidism from NTIS in sick patients frequently requires assessment of a serum free T4 estimate as well as a TSH value, and may require reassessment after recovery.

ARE NTIS PATIENTS EUTHYROID OR HYPOTHYROID ?

Controversy continues as to whether patients with nonthyroidal illnesses, in the absence of concurrent hypothalamic, pituitary or thyroid disease, are hypothyroid or euthyroid (1, 2, 5-9), as summarized in Table 1. The major problems in assessing the hypothalamic-pituitary-thyroid axis in NTIS are that immunoactive TSH assays may not reflect bioactive TSH levels, and free T4 and free T3 values measured in sera from sick patients are highly method dependent (3). Systemic illnesses reduce thyroid hormone binding to serum binding proteins, causing disparities among non-dialysis free T4 estimates, which are dependent on T4 binding to serum proteins, and direct dialysis free T4 measurements, which are not (3, 10). Consequently, clinicians must be aware of the performance of the assay methods used for assessing patients with NTIS. Further, the thyroid hormone status of the tissues in patients with NTIS has not been accurately determined.

TABLE 1: THYROID HORMONE STATUS IN NTIS

Evidence **supporting** euthyroidism in NTIS

- 1) Serum TSH and free T₄, by ultrafiltration of minimally diluted sera (11) or direct dialysis, are normal in the majority of NTIS patients (1, 3).
- 2) T₄ production/degradation rates in critically ill NTIS patients may be lower than in normal subjects but are **similar** to those of healthy euthyroid subjects with low serum T₄ binding (2). Low T₄ binding in serum may result in underestimation of total T₄ concentrations (12).
- 3) Reverse T₃ production from T₄ in critically ill patients with low total T₄ levels are similar to those of NTIS patients with normal total T₄ levels (2).
- 4) Free T₃ concentrations in NTIS are highly method dependent and are **normal** in critically ill NTIS patients by ultrafiltration of minimally diluted sera (3, 11)
- 5) Hepatic T₃ nuclear receptor proteins are increased in chronically diseased human livers (13).
- 6) Thyroid hormone-regulated mRNAs encoding TBG, SHBG, CBG, and transthyretin are present in normal quantities in chronically diseased human livers (14).
- 7) In critically ill NTIS patients, free cortisol by direct equilibrium dialysis or cortisol index values are appropriately elevated (15, 16) despite reduced total cortisol levels (4) indicating a functional hypothalamic-pituitary-adrenocortical axis.

Evidence **not supporting** euthyroidism in NTIS

- 1) TRH gene expression may be reduced in NTIS (6, 17).
- 2) Nocturnal TSH surge and pulsatile secretion of TSH, GH and PRL are decreased, and TSH response to exogenous TRH is blunted in NTIS (1, 3).
- 3) T₄ uptake into rapidly equilibrating tissues like liver may be reduced in critically ill patients with NTIS (2).
- 4) Total T₃ content of cerebral cortex, hypothalamus, anterior pituitary, liver, kidney, and lung are reduced in NTIS as is hepatic content of total T₄; total hormone content may reflect primarily **bound** hormone (18).
- 5) T₃ production rates are decreased in all NTIS patients (2), however, total T₃ levels, and, therefore, T₃ production rates, may be underestimated if free T₃ concentrations are normal (11).

HYPOTHALAMIC-PITUITARY FUNCTION IN NTIS

Controversy exists as to whether the fall in total T₄ concentrations during acute NTIS are primarily due to central suppression indicating hypothyroidism, to reduced serum T₄ binding to TBG due to protease cleavage, indicating euthyroidism, or a combination of these factors (2, 6, 19). Wadwekar and Kabadi (20) addressed this question in a study of six euthyroid men with primary hypothyroidism maintained on L-T₄ replacement therapy during an acute nonthyroidal illness. Prior to the illness, total T₄, total T₃ and TSH levels were in the reference range for euthyroid subjects. All of these values decreased during the acute illnesses, returning to pre-illness values with recovery (20). These findings are more consistent with acutely impaired serum T₄ binding than with central hypothyroidism. Since oral L-T₄ replacement was continued during hospitalization, TSH suppression could not reduce thyroidal T₄ production. Rather, rapid reduction of T₄ binding to TBG during acute illness (19, 21) could release T₄ from TBG, reduce total T₄ levels, transiently increase free T₄ and suppress TSH levels, as well as increasing serum T₄ clearance rates and reducing serum T₄ half-life values (2, 19). During recovery, normalization of T₄ binding to TBG could increase total T₄, and transiently reduce free T₄ levels resulting in increased TSH values, until a steady state was re-established.

IS THYROID HORMONE OR SECRETOGOGUE THERAPY BENEFICIAL IN NTIS ?

Controversy continues regarding thyroid hormone therapy in NTIS (5, 7-9). Many studies utilized pharmacological rather than physiological doses of L-T₄ or L-T₃. Normal T₄ production rates range from 80 to 100 ug per day for a 70 kg person, while normal T₃ production rates range from 25 to 32 ug per day (22, 23). Absorption of L-T₄ and L-T₃ after oral administration varies from 50-80%. Oral L-T₄ replacement doses range from 112 to 126 ug per day while oral L-T₃ doses range from 42 to 56 ug per day (22, 23).

Two weeks of L-T₄ therapy in physiological doses (1.5 ug/kg intravenously per day, 105 ug per day if 70 kg) given to intensive care unit patients with low total T₄ concentrations did not hasten recovery or improve survival compared to placebo treated patients (7). However, pharmacological doses of L-T₄ (300 ug per day intravenously) given to patients with acute renal failure for 48 hours in a prospective, randomized, double-controlled, double-blinded trial, increased mortality more than 3 fold (7).

L-T₃ therapy in physiological doses (30 ug/d orally) administered during fasting did not affect protein metabolism (24, 25), while a higher physiological dose (40 ug/d orally) increased protein catabolism (26). One week of L-T₃ 0.8 ug/kg orally (56 ug/d if 70 kg) given to uremic patients adversely affected nitrogen balance and increased amino acid turnover (27). Pharmacological doses of L-T₃ (60-150 ug/d orally) increased protein catabolism during fasting (24, 28).

L-T₄ (150 ug per day intravenously) plus L-T₃ (0.6 ug/kg per day intravenously, 42 ug for 70 kg) administered to critically ill NTIS resulted in TSH suppression and higher serum total T₃ concentrations than in untreated patients; outcome data were not provided (29).

Pharmacological doses of L-T₃ have been administered to patients with burns without affect, and after coronary artery bypass surgery or heart transplantation with improvement in cardiac function, decreases in pressor requirements, and shortened time to recovery, without an affect on overall mortality (7, 8). In an uncontrolled study, pharmacological doses of L-T₃ therapy for congestive heart failure increased cardiac output and reduced systemic vascular resistance (8). Pharmacological doses of L-T₃ in brain-dead heart transplant donors showed no significant differences in hemodynamic status or myocardial function (7).

TRH in combination with growth hormone secretogues given to NTIS patients with prolonged critical illnesses augmented the pulsatility of TSH release and increased total T₄ and total T₃ level but no clinical benefit was demonstrated (30).

CONCLUSIONS:

- 1) There is no compelling evidence to support overt T₄ or T₃ deficiency in NTIS.
- 2) Data do not indicate that physiological replacement doses of either L-T₄ or L-T₃ improve outcome in NTIS patients and such therapy may be detrimental.
- 3) If thyroid hormone therapy is proposed in NTIS patients without concurrent hypothalamic, pituitary or thyroid disease, large prospective, randomized, double-blinded, controlled studies should be performed in a homogeneous clinical setting with well defined and clinically relevant end-points.
- 4) Although pharmacological doses of L-T₃ may have beneficial effects in certain circumstances, these effects are unrelated to thyroid hormone deficiency in NTIS.

- 5) Thyroid hormone replacement therapy in NTIS patients should be restricted to treating hypothyroidism due to documented hypothalamic, pituitary, or thyroid disease.
- 6) The clinician should focus on differentiating changes in serum thyroid hormone levels due to NTIS from those due to concurrent hypothyroidism or hyperthyroidism, and on treating patients with concurrent hypothyroidism or hyperthyroidism and NTIS appropriately.

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ENDOCYTIC RECEPTORS FOR THYROGLOBULIN IN THYROID EPITHELIAL CELLS

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Interactions between thyroglobulin (Tg) and endocytic receptors on the apical membrane of thyroid epithelial cells (TEC) result in Tg uptake and delivery to post-endocytic pathways. Several receptors have been postulated or demonstrated to mediate Tg endocytosis and some are involved in thyroid homeostasis. The known Tg endocytic receptors are here reviewed, preceded by a brief introduction on intrathyroidal metabolism of Tg and mechanisms of Tg uptake.

Intrathyroidal metabolism of Tg

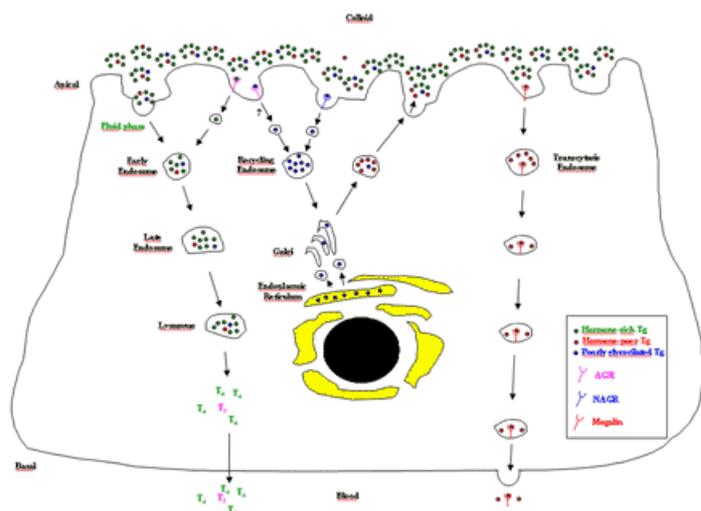
Tg is secreted by TEC into the thyroid follicular lumen, where it is involved in thyroid hormone formation by iodination and coupling of its tyrosine residues.(1). Then, Tg is either stored to form the colloid or is processed further for hormone release. For the latter, Tg is endocytosed by TEC, followed by lysosomal degradation, even though hormones can also be released within the colloid through the action of extracellular proteases (1). Although most of the internalized Tg undergoes lysosomal degradation, some follows other intracellular routes. After uptake, poorly glycosylated Tg molecules undergo a further passage through the trans-Golgi network, where glycosylation is completed, and are then recycled back into the follicular lumen (1-2). In addition, Tg can undergo transepithelial transport from the colloid to the bloodstream (transcytosis), thereby reaching the circulation (1-2). It is unknown how Tg crosses the capillary wall from the basolateral interstitium following transcytosis.

Mechanisms of Tg uptake by TEC: role of nonspecific and receptor-mediated endocytosis

In rodents, TEC can internalize Tg by macropinocytosis, but in most species, including humans, uptake of Tg occurs mainly by micropinocytosis (1-2). The majority of investigators agree that fluid phase micropinocytosis is the major route of uptake leading to hormone release, because of the very high concentrations of Tg within the colloid (1-2). Thus, fluid phase uptake is a constitutive process that occurs by passive gradient diffusion of substances that are highly concentrated in extracellular fluids and it ends invariably in lysosomes (1-2). Because endocytic receptors take up substances that are present in very low concentrations in extracellular fluids, it is unlikely that they mediate uptake of large amounts of Tg (1-2). Receptors may contribute to hormone release under special circumstances, for example iodine deficiency, but under physiological conditions they are more likely to sort Tg molecules to post-endocytic pathways that do not lead to hormone release, namely recycling or transcytosis (2). The contribution of the various means of endocytosis depends on the biochemical features of Tg, especially hormone content and degree of glycosylation (Fig. 1). Ultimately, both fluid phase endocytosis and receptor mediated endocytosis are aimed at the same goal, namely to render hormone release effective, which is achieved not only by targeting of Tg to lysosomes, but also by favoring complete glycosylation of immature Tg molecules through their recycling, or by eliminating hormone-poor Tg molecules from the colloid by transcytosis. The latter process is probably especially important when hormone-poor Tg molecules are present in excess within the colloid.

Tg endocytic receptors

Several molecules expressed on the membrane of TEC have been proposed to function as Tg endocytic receptors. Two of them, the asialoglycoprotein receptor (AGR) and megalin, are well characterized receptors. The characteristics of another receptor that binds to exposed N-acetylglucosamine residues of Tg [N-acetylglucosamine receptor (NAGR)] are unknown. As detailed below, the three identified Tg receptors probably deliver Tg to different, post-endocytic pathways (Fig.).



Legend: Fig. Schematic representation of the known Tg endocytic pathways and of the receptors involved.

It is likely that other receptors for Tg also exist (2), but their nature and roles are not established.

The thyroid AGR

The existence of a thyroid AGR similar to the liver receptor was postulated because removal of sialic acid units from Tg increases its binding to thyroid membranes (3). Indeed, AGR is expressed by TEC and expression of AGR in thyroid PC C13 cells is TSH-dependent (4-6), suggesting a thyroid-specific function of the receptor. By immunohistochemistry, AGR can be found on the apical membrane of TEC in rat thyroid sections (5), directly facing the follicular lumen, therefore in the ideal position to mediate Tg endocytosis.

Asialo-Tg binds to AGR in solid phase assays and it also binds to the native receptor in PC C13 cells (4-6). In addition, Tg uptake by PC C13 cells can be in part reduced by an antibody against AGR (6), suggesting that, at least in cultured cells, AGR is involved in Tg endocytosis.

AGR binds especially to Tg molecules with a low degree of glycosylation, a feature that makes it an ideal candidate to be involved in Tg recycling. In addition, early studies showed that binding of asialo-Tg to thyroid membranes occurs optimally at low pH (3), which should prevent dissociation of Tg from the receptor due to the acidic pH of endosomes, as it sometimes occurs in ligand recycling or transcytosis. Nevertheless, degradation of Tg in PC C13 cells is reduced by an antibody against AGR (6), suggesting that, at least in part, AGR delivers Tg to lysosomes, which presumably should result in hormone release. It is possible that, also in view of its TSH dependence, AGR may facilitate maximal hormone release under special circumstances, for example iodine deficiency. However, a dual role with AGR mediating to some extent also Tg recycling cannot be completely excluded (Fig.). Unfortunately, no *in vivo* data are available, and therefore the actual impact of AGR on thyroid function remains to be elucidated.

NAGR

Asialo-agalacto-Tg, obtained by digestion of asialo-Tg with galactosidase, bears exposed N-acetylglucosamine residues and it binds to thyroid membranes to a greater extent than undigested Tg (7). In addition, asialo-agalacto-bovine serum albumin (BSA) binds to thyroid membranes with high affinity (saturation point 13 nM), and binding can be inhibited by unlabeled native Tg and to an even greater extent by asialo-Tg and asialo-agalacto-Tg (8). These findings suggest the existence of a NAGR capable of interacting with Tg, which may be responsible for recycling of poorly glycosylated Tg molecules (Fig.). In support of this, asialo-agalacto-BSA is released undegraded following endocytosis by cultured TEC, and ovomucoid, a glycoprotein with exposed N-acetylglucosamine residues, accumulates in the Golgi following endocytosis by cultured TEC (9). Although NAGR is very likely to mediate recycling, the exact identity of the receptor is unknown, as previous attempts to identify it failed. In addition, because studies *in vivo* are not available, the impact of NAGR on thyroid function remains to be established.

Megalyn

Megalyn is a member of the LDL receptor family expressed by a restricted group of absorptive cells, including TEC, where it can be found on the apical surface (2), thus in the ideal position to mediate Tg endocytosis. Megalyn expression in TEC is up-regulated by TSH (2, 10), suggesting a thyroid-specific function.

Tg binds to purified megalyn with high affinity (Kd ~9-11 nM), both in solid phase assays and to the native receptor in FRTL-5 cells (10). In the latter, megalyn competitors reduce Tg uptake by ~50% (10), suggesting the receptor is involved in Tg endocytosis.

In most instances, megalyn-mediated uptake of ligands results in their delivery to lysosomes (10). However, certain ligands undergo a different intracellular fate, namely transcytosis, which is the case for Tg, representing one of the mechanisms by which Tg enters the bloodstream. Thus, transport of Tg across FRTL-5 cell layers is reduced by megalyn competitors (2, 10), and portions of the megalyn ectodomain (secretory components) remain complexed with transcytosed Tg (11). Furthermore, in conditions associated with increased megalyn expression, due to TSH (hypothyroid rats) or TSH-like (patients with Graves' disease) stimulation, a relatively high proportion of serum Tg is complexed with megalyn secretory components (11) and serum Tg levels in megalyn KO mice are reduced (12).

The molecular mechanisms responsible for targeting of the Tg-megalyn complex to transcytosis are known only in part. Although binding of Tg to megalyn is optimal at low pH, transcytosis is only minimally affected by increasing intracellular pH, suggesting that pH resistance is not a major factor (13). The calcium-calmodulin pathway and phosphoinositide 3-kinase (PI3-K) affect Tg transcytosis. Calmodulin antagonists reduce transcytosis and increase T3 release, indicating calmodulin favors transcytosis prior to Tg sorting (10). In contrast, a PI3-K inhibitor increases Tg transcytosis but does not affect T3 release, suggesting that PI3-K exerts an inhibitory effect at a post-sorting stage (14).

A major role in determining targeting of Tg to transcytosis is related to the ability of Tg to bind to cell surface heparinoids. As other megalyn ligands, Tg is a heparin-binding protein and heparin and megalyn binding sites are functionally related (10). Occupation of a major heparin-binding sequence of rat Tg (2489-2503) abolishes Tg binding to megalyn. In addition, transcytosis of rat Tg in FRTL-5 cells is reduced by enzymatic removal of cell surface heparan sulfate proteoglycans (HSPGs) as well as by an antibody against Tg2489-2503 (12). In this regard, optimal exposure of Tg2489-2503 is crucial, and a greater exposure of Tg2489-2503 in hormone-poor rat Tg is responsible for its preferential transcytosis compared with hormone-rich Tg (12). The role of Tg binding to heparinoids appears to be more important in rodents than in humans, because Tg2489-2583 is identical in mice and rats, but differs in humans by 6 residues, resulting in a weaker Tg binding to heparin (15).

As mentioned above, transcytosis is preferential for hormone-poor Tg molecules. Thus, Tg transport across FRTL-5 cells is greater for hormone-poor than for hormone-rich Tg (12). In addition, megalyn-mediated transcytosis *in vivo*, estimated by the proportion of serum Tg complexed with megalyn secretory components, is enhanced by inhibition of hormone formation within Tg due to thionamide treatment (12). This selective mode of transcytosis renders hormone release more effective, by preventing hormone-poor Tg to enter the lysosomal pathway, thereby avoiding competition with hormone-rich Tg, as well as wasteful transcytosis of hormone-rich Tg. Thus, megalyn KO mice are hypothyroid (Lisi et al., manuscript in preparation). Whether megalyn deficiency exists in humans and it causes thyroid dysfunction remains to be established.

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Clinical Use of TSH Suppression: Why, When, and How?

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Introduction

Thyroid stimulating hormone (TSH)-suppressive therapy has been variously defined in different clinical contexts and studies. In general, exogenous thyroid hormone, usually L-thyroxine (L-T4), is used to induce subclinical thyrotoxicosis. The rationale for this treatment is that pharmacologic doses of L-T4 lead to decreased pituitary release of TSH, which, in turn, may inhibit the growth and proliferation of TSH-responsive thyroid cells. Before sensitive TSH assays were available, thyrotropin releasing hormone (TRH) suppression tests were used as an index of TSH suppression (1). TRH is no longer available in the United States, and serum TSH values are now used for the titration of TSH suppressive L-T4 doses. There is some disagreement about the optimal therapeutic degree of TSH suppression, but in general the goal is the maintenance of a serum TSH value below the normal range with a normal or minimally elevated serum free T4 value.

Effectiveness of TSH Suppression

For Treatment of Benign Thyroid Disease

TSH-suppressive L-T4 therapy has been commonly used since the 1960s for the treatment of benign nodular thyroid disease. The goal of such therapy is to reduce nodule size or prevent further nodule growth in order to delay any need for surgical treatment for compressive symptoms or cosmetic reasons. Although there are regional variations in treatment preferences, approximately half of endocrinologists recently surveyed in Europe and North America indicated that they would use TSH-suppressive therapy in the management of typical cases of benign nodular thyroid disease (2, 3, 4). However, numerous clinical studies of the efficacy of TSH suppressive therapy for nodular thyroid disease have had mixed results, and this practice has become increasingly controversial. Many of the published studies assessing the effectiveness of TSH suppressive therapy for decreasing nodule size or limiting benign nodule growth have been very short-term, had small sample sizes, have been non-randomized, have utilized imprecise outcome measures, or have lacked appropriate controls (5). Some studies have not documented appropriate TSH suppression. Difficulties in interpreting conflicting data may also stem in part from the fact that thyroid nodules have heterogeneous etiologies and that there may be substantial variability in the growth rate of individual thyroid nodules and their response to TSH suppression (6, 7). Finally, the fine needle aspiration biopsies used in most studies to rule out malignancy may themselves significantly alter nodule size (8).

The most long-term prospective randomized trial of TSH-suppressive therapy for nodular thyroid disease to date found that treated subjects had only a borderline decrease in thyroid size after 5 years of follow-up ($p = 0.051$), but concluded that TSH suppressive therapy prevented the thyroid growth and appearance of new nodules seen in the control group (9). In a recent trial, 27% of subjects on TSH suppressive therapy had a =50% decrease in dominant thyroid nodule size compared to 17% of controls ($p = 0.04$); these investigators also noted a decrease in the number of non-dominant nodules detectable by ultrasound in treated patients after 18 months (10).

Several recent meta-analyses have attempted to synthesize the results of clinical trials evaluating the effectiveness of TSH-suppressive therapy on benign solitary thyroid nodule growth. One meta-analysis of seven prospective trials found that TSH-suppressive therapy was associated with decreases in nodule size by ultrasound measurement in 17% of subjects (11). Another meta-analysis of five randomized trials found that subjects taking TSH suppressive therapy were 1.9 to 2.5 times more likely to achieve a 50% reduction in nodule size compared to controls (12). A third meta-analysis of six randomized clinical trials using ultrasonographic measurements found that the size of nodules decreased by more than 50% in subjects on TSH suppressive therapy, but these results did not reach statistical significance (13). A fourth meta-analysis of nine randomized trials similarly concluded that there was a nonsignificant trend toward decrease in nodule size by at least 50% in the L-T4 treated group (14).

The degree of TSH suppression employed has varied from study to study. A recent randomized crossover study compared the effects of high-level (serum TSH =0.01 mU/L) and low-level (TSH 0.4 – 0.6 mU/L) TSH suppression and concluded that both were equally effective at decreasing nodule size (15). The study also noted that thyroid nodules increased to their pre-treatment size in placebo-treated patients who had previously been treated with TSH-suppressive therapy. One important drawback to TSH suppression is that nodule growth typically recurs after discontinuation of L-T4 therapy (16).

There are several alternatives to TSH suppressive therapy for benign nodular thyroid disease.

Total or partial thyroidectomy is one option. In patients who undergo partial thyroidectomy for thyroid nodules, randomized trials have not shown that the use of TSH suppressive therapy reduces nodule recurrence rates (17, 18, 19), and use of TSH suppressive therapy post-operatively is not cost-effective (20). Since hypothyroidism usually occurs after surgery, replacement doses of L-T4 are given to maintain the serum TSH in the low-normal or mid-normal range. However, patients with radiation-associated benign thyroid nodules may be an exception, as post-operative nodule recurrence rates were lower in L-T4 treated patients than in controls in a nonrandomized study of 511 patients who had received local head and neck irradiation in childhood (21). Another alternative to TSH suppressive therapy in patients with benign nodular thyroid disease is watchful waiting; as long as patients are not bothered by compressive symptoms this does not entail any evident risks (22). Finally, radioactive iodine therapy may be employed; a recent study suggests that this may be more effective and have fewer side effects than TSH-suppressive therapy (23).

For Treatment of Thyroid Carcinoma

Although TSH-suppressive therapy is widely used following thyroidectomy in patients with differentiated thyroid carcinoma and is considered standard of care, its use has never been rigorously evaluated in clinical trials. Given the clear rationale for and relatively low risks of TSH-suppressive therapy, such a trial would probably not be considered ethical. Available information regarding the efficacy of TSH suppression in preventing thyroid carcinoma progression or recurrence comes from observational studies. A recent meta-analysis of 10 observational studies concluded that TSH-suppressive therapy for differentiated thyroid cancer patients resulted in improved clinical outcomes (24).

Different dosing regimens have been proposed for TSH suppression in thyroid cancer patients. Some authors have advocated the use of just enough L-T4 to suppress the serum TSH value to just below the normal reference range (25). Others feel that more aggressive TSH suppression is warranted, at least in high-risk patients. A 1996 study of differentiated thyroid carcinoma patients compared 15 subjects whose serum TSH values were consistently ≈ 1 mU/L following thyroidectomy to a group of 18 subjects whose serum TSH values were consistently <0.05 mU/L (26). There were no differences in age, sex, or initial tumor grade between the two groups. The patients with consistently suppressed serum TSH values had significantly longer disease-free survival times than the patients without TSH suppression. Another group evaluated the effects of TSH suppression on thyroid cancer in subjects from a U.S. thyroid cancer registry (27). They found no effect of the degree of TSH suppression on disease progression in low-risk patients, but a trend toward a protective effect of more aggressive TSH suppression in high-risk patients, defined as those with tumor stages III or IV. In light of this finding, it seems reasonable to adjust the degree of TSH suppression according to tumor grade as well as to other prognostic factors. For example, thyroglobulin antibody-negative patients who have undetectable stimulated (by rhTSH or L-T4 withdrawal) serum thyroglobulin levels following thyroidectomy and radioactive iodine ablation are at low risk for tumor recurrence and may require only slightly low to low serum TSH values rather than complete TSH suppression.

Adverse Effects of TSH Suppression

Cardiovascular Effects

An increased risk for atrial fibrillation has been described in patients with low serum TSH values (28). This finding was recently confirmed in a cohort of subjects not taking thyroid hormone in whom the prevalence of atrial fibrillation in patients with serum TSH values <0.4 mU/L (with normal free T3 and free T4 values) was 12.7%, compared with 2.3% in euthyroid subjects (29).

Some (30, 31, 32, 33), but not all (34), echocardiographic case control studies have noted impaired diastolic function characterized by delayed relaxation in patients on TSH-suppressive therapy. Some of these studies have reported increased left ventricular mass, particularly increased posterior wall and interventricular septum thickness (32, 33), but this finding has not been universal (30, 31). Cardiac changes in patients taking TSH-suppressive therapy appear to be associated with decreased exercise capacity. When Mercurio et al. (32) reduced subjects' L-T4 dose to the minimal amount required to maintain the serum TSH concentration at 0.1 mU/L or lower, echocardiographic and ergometric parameters normalized. These studies are limited by small sample sizes, and some are further limited by the use of control subjects unmatched for body mass index and usual physical activity.

A recent study compared measurements of plasma coagulation factors in 14 thyroid cancer patients on TSH-suppressive therapy to samples obtained while the patients were hypothyroid for cancer treatment. The investigators concluded that TSH-suppressive therapy may be pro-thrombotic (35).

An important question is whether the relatively subtle cardiovascular changes seen in patients taking TSH-suppressive L-T4 doses have an effect on survival. A community-based British study analyzed thyroid status in a cohort of 1191 patients aged 60 or older and found increases in all-cause mortality at 2, 3, 4, and 5 years of follow-up for subjects with subclinical thyrotoxicosis (serum TSH values <0.5 mU/L) at baseline (36). This difference was mainly due to increases in cardiovascular mortality. No difference in mortality was seen at 10 years. Outcome ascertainment in this study was based on death certificate information, which may not have been complete. In addition, results of this study were not age-adjusted, and the group of subjects with low serum TSH values was slightly older than other groups at baseline, which may have accounted for some of the difference seen.

Skeletal Effects

Results of studies describing the effects of TSH suppression on bone have been inconsistent, in part because of differing methodologies and small sample sizes. One meta-analysis pooled data from 13 studies of bone mineral density in women on long-term TSH-suppressive therapy compared to euthyroid control subjects (37). Among premenopausal women, bone density did not differ between the L-T4 treated group and controls. However, bone density was significantly lower in treated postmenopausal women than in controls. These results were confirmed by another meta-analysis, which combined data from 41 studies examining the effects of suppressive L-T4 therapy on bone density (38). In this analysis, TSH-suppressive therapy was associated with significant bone loss at all skeletal sites in postmenopausal women, but not in premenopausal women. To date there is no strong evidence that TSH suppression causes decreases in bone density in men (39, 40).

In postmenopausal women, administration of estrogen may prevent L-T4 induced bone loss in women with suppressed serum TSH values (41). Treatment with intravenous pamidronate has been shown to increase bone density in patients receiving TSH-suppressive therapy (42); it is likely that oral bisphosphonates would also be effective although this has not been studied to date.

Thyrotoxic Symptoms

Depending on their degree of thyrotoxicosis, patients receiving TSH suppressive L-T4 doses may complain of symptoms such as anxiety, heat intolerance, tremors, sweaty skin, insomnia, forgetfulness, or mood disorders.

In one study, 24 young men were treated with 300 mcg L-T4 or placebo for 3-week periods in a double-blind crossover design (43). The L-T4 treated men required a greater effort to complete a visual search task, demonstrating effects of TSH-suppressive therapy on central information processing. However, the L-T4 doses in this study were higher than those typically used clinically for TSH suppression.

Conclusions

There are many controversies surrounding the use of TSH suppressive therapy. We believe that TSH-lowering doses of L-T4 are certainly warranted for the post-thyroidectomy treatment of differentiated thyroid cancer, and that evidence suggests that TSH suppression should be more aggressive in high-risk cancer patients than in patients with lower-risk tumors (based on tumor grade and other prognostic factors). Treatment of benign nodular thyroid disease is less straightforward, with the preponderance of evidence suggesting that TSH suppression is effective at decreasing the size or reducing the growth of at least a subset of benign thyroid nodules. However, this limited benefit must be weighed against the risks of long-term TSH-suppressive therapy, including the development of thyrotoxic symptoms, decreased bone density in postmenopausal women, and increased risk for atrial fibrillation. Patients with

longstanding nodular goiter may develop functional thyroid autonomy; in those patients L-T4 therapy may cause iatrogenic overt thyrotoxicosis. Based on current evidence, we believe that TSH-suppressive therapy is not warranted for most patients with benign thyroid disease. If TSH-suppressive therapy is used for benign nodular thyroid disease, risks should be minimized by using the minimal dose of L-T4 required to decrease serum TSH values to the low but detectable range, and the TSH-suppressive therapy should be discontinued after 6 to 12 months if there is no clear therapeutic response.

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THYROID INCIDENTALOMA

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Definition

The problem of the thyroid incidentaloma is the problem of the clinical significance of an impalpable small thyroid nodule (usually less than 10 mm diameter and not more than 15 mm) discovered incidentally during investigation for another disease process or during screening tests on a well subject. With minimal simplification, the point at issue is the risk of the nodule being papillary thyroid microcarcinoma and the significance of this diagnosis i.e. the potential morbidity and mortality. The term incidentaloma is a clinical one best used for the as yet undiagnosed nodule and if a malignancy is subsequently identified the term then to be used is microcarcinoma. It is impossible to avoid a degree of arbitrariness in definition, both in the criteria of size and impalpability. A nodule of 15 mm or even more in the postero-medial aspect of the gland may be impalpable whereas one of less than 10 mm in the isthmus might be readily apparent clinically. Nevertheless such categorization allows formal studies and is useful in management decisions.

Demographics

Palpable thyroid nodules have prevalence in iodine-replete populations of about 5% and an incidence of about 1 per 1000 per year (1). Ultrasonographically detectable thyroid nodules are even more common at 17-67% (2) but clinically important thyroid cancer is uncommon (20-80/million/year) and deaths from thyroid cancer are rare (5-8/million/year). Nevertheless it is clear that even impalpable thyroid nodules can be malignant and that malignancy in the multinodular thyroid has the same prevalence as in the solitary thyroid nodule (3).

It has been known for many years that there is a high prevalence of occult thyroid cancer detected at autopsy (almost entirely papillary carcinoma,) ranging from 2.7-28.4% in various surveys (4) with a median prevalence of about 6% (5) and it has been thought to pursue an indolent course (6, 7, 8). Recent publications reaffirm the generally indolent course and good prognosis of small papillary carcinomas under 10 mm (9) or under 15 mm (10).

The previously largely academic issue of occult thyroid carcinoma has now moved into the clinical domain as the problem of the thyroid incidentaloma, now commonly detected since the advent of widespread use of ultrasonography, although incidentalomas are now also being found by positron emission tomographic scanning (PET scanning) (11) which is (as yet) in much more restricted use. Kang et al (12) found a 2.4% prevalence of focal thyroid incidentalomas by PET scanning in a cancer screening program and a 1.6% prevalence in a combined group including subjects being evaluated for metastases from a non-thyroidal malignancy, indicating a high prevalence of incidentaloma although lower than that of both ultrasonographically-detected nodules and occult microcarcinoma.

TABLE 1: THYROID NODULE AND CANCER STATISTICS

Prevalence of palpable nodules	5%
Incidence of new palpable nodules (1/1000/year)	1000/ million/ year
Incidence of manifest carcinoma	50/ million/ year
Calculated cancer prevalence per nodule	5%
Observed cancer prevalence per nodule	5%
Death rate from thyroid cancer	5/million/ year
Prevalence of impalpable nodules on ultrasonography	25%
Cancer prevalence in impalpable nodules (upper)	25%
Estimated occult cancer in population	5%
Prevalence of occult microcarcinoma at autopsy	5%

These figures are approximate and have been rounded off for mnemonic value. In particular it should be noted the prevalence of nodules on ultrasonography increases with age.

The clinical problem

If an impalpable thyroid nodule is identified on ultrasonography performed for an unrelated reason the main questions that arise are:

Should we investigate further?

If we biopsy the nodule what does the cytological diagnosis of malignancy mean biologically? i.e. is the prognosis the same as for clinically apparent cancer and should our management protocols be the same?

Furthermore, if occult thyroid cancer is common should we actively seek it by population screening or case-finding in high-risk groups?

Although ultrasonography has created the problem, it at least offers a method of evaluation by allowing guided fine needle aspiration biopsy [USGFNA] (13) and ultrasonographic imaging characteristics have the potential to assist in defining the risk of malignancy.

Review of recent relevant studies

These studies have better defined the extent of the problem, in particular the prevalence of carcinoma and its likely anatomical extent, the ultrasonographic criteria of malignancy, and have provided some data assisting in prognostication and formulation of a management plan.

Leenhardt et al (14) in 450 impalpable nodules, found that malignancy was present in 21% of those operated [20/94] or in 4.4% of the total nodules, with a rate of 33% [8/24] in nodules under 10mm and 17% [12/70] in nodules 10mm or more. Solid hypoechoic nodules were significantly more likely to be malignant but neither blurred nodule margins nor calcification were significantly associated with malignancy.

Papini et al (15) found that of 402 impalpable nodules in which adequate cytology was obtained, carcinoma was present in 9.1% of nodules with a diameter more than 10 mm and in 7.0% of those with a diameter less than 10mm diameter. Extrathyroidal extension was present in 35.5% and regional lymph node extension in 19.4%. Ultrasonographic features of solid hypoechoic nodules, irregular nodule margins, intranodular vascular spots, and microcalcification were associated with malignancy.

Nam-Goong et al (16) performed USGFNA on 267 patients with 317 impalpable nodules ranging from 2-15 mm in a retrospective analysis of all patients referred to them over a two-year period. The histologically proven malignancy prevalence was 12%. Of particular importance is the finding that one or both of extrathyroidal extension of malignancy and regional lymph node metastases was present in 69% of the occult thyroid cancers. Extrathyroidal extension was present in 44%, regional lymph node metastases in 50%, and 39% of cancers were multifocal. No distant metastases were found after thyroidectomy and whole body [131I] scanning. There was no difference in the malignancy rate between thyroids with single or multiple nodules, or between nodules less than 10 mm diameter or 10-15 mm diameter. Ultrasonographic characteristics significantly associated with the subsequent confirmed diagnosis of malignancy were solidity, hypoechogenicity, and punctate calcification. Ill-defined nodule margins and increased intranodular vascularity on colour-flow Doppler imaging had an observed but non-significant association with malignancy.

Kang et al (17) in a retrospective analysis of 198 incidentalomas less than 15 mm diameter, in which 28.8% were malignant, identified ultrasonographic characteristics assisting in the diagnosis of malignancy. Malignancies were less likely to have a well-defined border, or to be cystic, and more likely to be solid, hypoechoic and to have calcification. Using these factors to derive an ultrasonographic index, a score of less than 2 had a diagnostic accuracy for a benign lesion of 85.5% and a score of more than 3 a diagnostic accuracy for malignancy of 89.9%. 12% had involved lymph nodes and 20% capsular invasion.

TABLE 2: ULTRASONOGRAPHIC CRITERIA OF THYROID MALIGNANCY

	Leenhardt	Papini	Nam-Goong	Kang
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Reference number	14	15	16	17
Solidity	YES	YES	YES	YES
Hypoechoogenicity	YES	YES	YES	YES
Ill-defined margins	NS	YES	NS	YES
Calcification	NS	YES	YES	YES
Intranodular vascularity	ND	YES	NS	ND

YES: significant association with malignancy NS: no significant association found

ND; not done or not reported

High quality ultrasonography of the incidentaloma itself may thus aid in diagnosis but is not informative about biological behaviour. A potentially helpful prognostic ultrasonographic feature, however, has recently been reported by Ito et al (18) of a higher rate of local lymph node recurrence when pre-operative ultrasonography has identified lateral compartment lymph node metastases (6.0% vs. 1.1% when absent) whereas medial compartment lymph node metastases had no such adverse correlation. The ultrasonographic diagnosis of lateral compartment lymph node metastases was likely to be confirmed at operation (positive predictive value 81%) but was insensitive (38%) although this insensitivity had no adverse effect in follow-up (19).

The malignancy rate in incidentalomas discovered by PET scanning appears to be similar. A high rate of clinically relevant malignancies has been suggested from a series of eight patient referred with suspicion of various malignancies (20) in whom two medullary thyroid carcinomas and three papillary carcinomas were found at surgery. In a retrospective series of 1330 subjects Kang et al (12) found 21 focal incidentalomas of which 15 were diagnosed histologically or cytologically diagnoses, and 4 (26.7%) were papillary carcinomas. The mean standard uptake value (SUV) of the malignancies was higher than that of the benign lesions.

Pellegriti et al (10) in a retrospective study of 299 patients operated for papillary carcinomas less than 15mm found 32% to be multifocal, 20% extrathyroidal, and 30% to have lymph node metastases. Tumours incidental to multinodular goitre, Graves' disease, autonomous functioning nodules, and cysts (n=151) were less likely to be multifocal (25%), extrathyroidal (11%), or to have lymph node metastases (16%) but the rates of these adverse features are still of concern. Distant metastases were uncommon in the total group (8/299) but appeared to be even more uncommon in incidentalomas (1/151).

Discussion

These rates of invasive disease reported by Nam-Goong et al (16) Papini et al (15), Kang et al (17), and Pellegriti et al (10) are evidence against the view that such small thyroid cancers are mostly indolent and of little biological importance. It might seem axiomatic to conclude that invasive or metastatic carcinoma must have an adverse outcome unless it is managed

aggressively but for thyroid carcinoma this view must be considered against the high prevalence of thyroid nodules, the much lower incidence of clinically apparent thyroid carcinoma, and the even lower mortality rate of thyroid carcinoma. For instance, the reported thyroid carcinoma incidence rate in Korea is 64/million/year and the death rate is 5/million/year similar to that of the USA for 1995-1999 of an incidence rate of 66/million/year and death rate of 5/million/year. In contrast, were the prevalence of thyroid carcinoma suggested by Nam-Goong et al (16) to be applicable to thyroid nodules in general, over 4% of the population would harbour thyroid cancer and 3% would have invasive disease.

How can we reconcile these disparate observations? First, it is probable that widespread use of thyroid ultrasonography, either in a screening program or as case finding in a higher-risk segment of the population, would indeed lead to a rise in incidence rates. Although retrospective studies and studies composed of subjects referred for investigation may result in some distortion of the true incidence in the general population it still seems likely that study findings are accurately showing in a clinical context what the past autopsy data has shown in a pathological context. Thus the critical issue is the natural history and prognosis of microcarcinomas presenting as incidentalomas not their incidence. Mortality rate data are sturdier than incidence rate data and are not directly challenged by any of the current studies of prevalence and invasiveness. If 3-5% of the population have invasive thyroid carcinoma with a presumptive adverse prognosis then we would have clearly seen this reflected in mortality data given we know the high prevalence of occult thyroid carcinoma is not a new phenomenon. Thus the benign assessments of an earlier era (6, 7) cannot be discounted however much we feel concerned about the data on invasion and regional lymph node metastases. Indeed the study by Pellegriti et al (10) confirmed the generally indolent course of thyroid microcarcinoma, with distant metastases uncommon in the total group (8/299) and even more uncommon in incidentalomas (1/151), and showed no mortality over 3.8 years mean follow-up, admittedly a period too short for conclusive assessment. Of note in this study was that incidentalomas had less persistent or relapsing disease (6/115, 5.2%) compared to non-incidental tumours (7/67, 10.4%) supporting the view that the clinical presentation should be taken into account in management strategy.

Although we still lack a conclusive understanding of the clinical significance of the high rate of differentiated thyroid malignancy in impalpable nodules, and of the prognosis even if locally invasive, any tendency to an aggressive strategy should be tempered by the knowledge that screening programs for early cancers (lung, breast, prostate and neuroblastoma) have not demonstrated a mortality difference between screened and unscreened populations despite detecting more disease at an earlier stage and demonstrating longer survival in screened groups (21).

Despite the difficulty of acquiring this full understanding without randomised prospective trials of observation versus standard thyroid cancer care, with all the attendant practical and ethical problems, Ito et al (9) have provided a partial answer. In a prospective study of 732 patients with papillary microcarcinoma less than 10 mm of whom 162 chose observation [now extended to 211, (19)] after surgery was offered to those with various features considered unfavourable (high grade cytology, primary near the trachea, possible invasion of the recurrent laryngeal nerve, possible lateral lymph node invasion on ultrasonography) more than 70% of observed tumours did not grow in over 4 years mean observation and in those followed

for over 6 years stability or shrinkage in the size of the tumour was seen in 75%. Only 11% became greater than 10 mm, and only 1.2% came to surgery for metastases to lateral lymph nodes (although 60 of the extended observation group eventually underwent operation for a variety of reasons). There were no distant metastases or deaths in any of the 211 (151 unoperated). In the surgical group lymph node metastases were found in 51% and multiple foci in 43%, and it was considered that these preoperatively unidentified features would have been present in the observed group at a similar frequency, thus suggesting that many patients with lymph node metastases and multifocality nevertheless have an excellent prognosis.

Conclusions

Pearce and Braverman (22) interpret the data from Pellegriti et al (10) to support the previous recommendation to observe incidentalomas under 10mm and perform FNB only if more than 10mm (2), on the grounds that there is no evidence that more rigorous management improves survival and it would impose a high burden on health care providers. Certainly the opportunity cost of imposing such an investigative regimen might be prohibitive even in the health care systems of the wealthy nations of North America and Europe, and would be quite impracticable and of very low priority in countries with developing economies. It is suggested that in line with these previous recommendations (2, 22) the clinician can counsel observation of an incidentaloma of 10 mm diameter or less. It is suggested that adverse ultrasonographic characteristics so far identified relate to the possible diagnosis of malignancy per se and not to whether the course will prove to be indolent or aggressive, with the possible exception of lateral compartment lymph node enlargement (18), and thus should not lead to an alteration of a management strategy based on size alone. Growth of an incidentaloma or appearance of lateral lymph node compartment enlargement should lead to USGFNA provided the degree of imprecision of ultrasonographic reassessment is taken into account. The diagnosis of papillary carcinoma in such lesions should lead to total thyroidectomy (10, 22, 23). In general, non-incidental lesions should be investigated by USGFNA in view of the evident less indolent course (10). Conversely current evidence strongly indicates there is no basis for population screening despite the likely high occult papillary microcarcinoma prevalence. Nor, in non-irradiated patients, is there a basis for case finding of incidentalomas in specific patient groups. Finally, it will be evident to the clinician that some patients will select a more aggressive approach and others a less aggressive approach than the clinician has recommended depending on individual attitudes to the possible diagnosis of cancer, to proposed follow-up regimens, and to neck surgery. This is appropriate provided the discussion of the problem is balanced, with an accurate account of the uncertainties involved.

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Cardiovascular consequences of subclinical hyper- and hypothyroidism

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Introduction

Subclinical thyroid dysfunctions are defined by normal free-triiodothyronine (FT3) and free-thyroxine (FT4) concentrations in the presence of abnormal TSH, which is low-undetectable in subclinical hyperthyroidism (SH) and increased in subclinical hypothyroidism (Sh).

The clinical significance of subclinical thyroid dysfunction is much debated (1-3). The presence of tissue effects, symptoms and signs of mild thyroid hormone excess or deficiency and the management and treatment of these conditions are controversial issues. Similarly, the TSH cut-off point that determines the effects of subclinical thyroid dysfunction remains to be established.

The cardiovascular system, which is a major target of thyroid hormone, is sensitive to the effects of thyroid hormone excess or deficiency at the tissue level. Many symptoms and signs in patients with overt hypo- or hyperthyroidism are related to the reduced or increased action of thyroid hormone on cardiac function. Triiodothyronine (T3) affects the heart and vascular system through genomic and non-genomic mechanisms; it influences heart rate, systolic and diastolic function and systemic vascular resistance, and hence cardiac performance (4,5).

In human overt hyperthyroidism, the increase in left ventricular performance is predominantly sustained by the increased preload that results in enhanced left ventricular diastolic function (6,7). The reduced systemic vascular resistance, coupled with increased venous return and preload, enhances cardiac output (6,7). The decreased cardiac output in hypothyroid patients at rest depends largely on altered diastolic relaxation and hemodynamic loading (5,8). The reduced cardiac preload, combined with bradycardia and slightly depressed myocardial contractility, accounts for a subnormal cardiac output in overt hypothyroidism, whereas peripheral vascular resistance is remarkably increased (4,5,8). Moreover, cardiovascular alterations have been found in individuals with subclinical thyroid disease (9). This review covers the data about the progression of subclinical thyroid dysfunctions and cardiovascular risk. It also deals with the cardiovascular risk and the need for treatment as estimated from epidemiological data on cardiovascular morbidity and mortality.

Subclinical hyperthyroidism

Causes, prevalence and progression

The reported overall prevalence of SH ranges between 0.5 and 6.3%, the prevalence being higher in patients over 65 years. The prevalence can differ in relation to iodine intake and the TSH cut-off point used for diagnosis (10,11).

Subclinical hyperthyroidism may be caused by exogenous or endogenous factors. The exogenous form is usually related to TSH-suppressive therapy with L-thyroxine for the treatment of benign thyroid disease and differentiated thyroid carcinoma. The endogenous form is usually related to the same causes as overt thyrotoxicosis subsequent to autonomously functioning

thyroid adenomas, and multinodular goitre. TSH suppressive or unintentional over-replacement L-thyroxine therapy was the most common form of SH (20.7%) among subjects taking L-thyroxine in the Colorado study (11), whereas endogenous factors accounted for a minority of cases and prevailed in areas of iodine insufficiency (12). Endogenous SH is usually a slowly progressive disorder and may last several years before developing into overt thyrotoxicosis. The risk of SH progressing to overt hyperthyroidism varies between 2% and 7% per year in patients with undetectable TSH (12). Unfortunately, there are no data on the progression of SH in patients with TSH between 0.1-0.5 mU/L.

Cardiovascular risk

Exogenous and endogenous SH may lead to signs and symptoms of thyroid hormone excess thereby mimicking adrenergic overactivity and impairing quality of life (12, 13). Subclinical hyperthyroidism affects the cardiovascular system in various ways, and its increased cardiovascular risk is well documented in the elderly (14,15). The cardiovascular abnormalities are similar in stable endogenous and exogenous SH (12)

Table 1

Potential cardiac effects of subclinical hyperthyroidism

- **Increased heart rate**
- **Increased prevalence of atrial arrhythmias**
- **Increased systolic function at rest**
- **Increased left ventricular mass**
- **Impaired diastolic function**
- **Systolic dysfunction during effort**

The major cardiovascular findings in patients with SH coupled with undetectable TSH are a higher heart rate and a higher risk of supraventricular arrhythmias (15-17). The most consistent cardiac abnormality is a significant increase in left ventricular mass with unchanged or increased at-rest systolic function and, usually, impaired diastolic function (16,18-20). Moreover, reduced systolic performance on effort and decreased exercise tolerance has been reported in patients with SH who had a greater increase in left ventricular mass (21). Thyroid hormone-induced hypertrophy in SH is due primarily to the cardiac response induced by the increased cardiac workload. This is accordance with cardiac hypertrophy induced in rats by thyroid hormone excess (22). Moreover, the significant increase in left ventricular mass with a tendency towards LV concentric remodelling reported in patients with long-standing SH (16,18,19) may counteract the favourable effect acutely exerted by thyroid hormone on diastolic performance, and so lead to impaired ventricular relaxation and systolic dysfunction during effort (20,21). The altered passive elasticity of the ventricle (chamber stiffness) determined by the presence of myocardial hypertrophy is the major determinant of diastolic dysfunction in patients with SH.

The prognostic significance of these cardiovascular alterations in patients with SH remains to be clarified especially in young and middle-aged patients with low TSH. Unfortunately, there is no study of the effects on cardiovascular system of minimally suppressed TSH (i.e., TSH between 0.1-0.4 mU/L). However, the increase in heart rate and in left ventricular mass usually precedes the onset of more severe cardiovascular disease, and is an independent risk factor for increased cardiovascular morbidity and mortality in the general population (23).

The detrimental effects of SH are well documented in the elderly and atrial fibrillation represents an important cardiovascular risk. The Framingham study evaluated the risk of atrial fibrillation during the 10-year follow-up in 2007 people aged 60 years or older with endogenous or exogenous SH (15). The adjusted relative risk for atrial fibrillation was 3.1 times higher in the group with serum TSH \geq 0.1 mU/L compared with those with normal TSH concentrations ($>$ 0.4-5.0mU/L). The relative risk of atrial fibrillation was 1.6 times higher in the group with slightly low TSH concentrations (0.1-0.4 mU/L) ($p=0.04$) with an incidence of atrial fibrillation in 16/1000 patients person-years) ($p=0.11$) (15). Similarly, in a large retrospective study on hospitalized consecutive older subjects, the relative risk of atrial fibrillation was 5.2 ($p<0.01$) in patients affected by SH with TSH $<$ 0.4 mU/L. (17). The combination of subclinical hyperthyroidism and age may have deleterious effects on the heart (12,23). Furthermore, the possible onset of overt hyperthyroidism in hearts previously exposed to longstanding untreated SH may further increase the cardiovascular risk (9). This body of data is in agreement with the increased cardiovascular mortality reported in a community-based review of subjects aged 60 years or older with endogenous SH and TSH values $<$ 0.5 mU/L monitored for 10 years (14).

In patients with benign thyroid disease and in low-risk patients with differentiated thyroid cancer, cardiovascular parameters and quality of life can be improved by reducing L-T4 dosage to keep TSH at the lower limit of normal range (24). A cardioselective β -blocking drug, in addition to L-thyroxine, can be used in high-risk thyroid cancer patients to attenuate symptoms caused by mild thyroid hormone excess, and to reduce the risk of atrial arrhythmias and an increase in left ventricular mass (12). Methimazole administration and radioiodine therapy may restore euthyroidism and so improve the cardiovascular risk in patients with endogenous SH (25,26).

A panel of experts recently recommended that treatment of endogenous SH should be considered in case of TSH $<$ 0.1 mU/L especially when patients are older than 60 years, and if there are symptoms or risk of heart disease (3). The routine treatment of SH if TSH is between 0.1 and 0.4 mU/L is not recommended (3). In fact, despite increased mortality in the elderly, it remains to be established if a subnormal TSH concentration induces the same adverse effects as suppressed TSH on the heart of young and middle-aged patients. However, it is important to stress that the clinical manifestations of 'subclinical' hyperthyroidism may be related to an individual's sensitivity to thyroid hormone excess, which depends on the patient's thyroid function set-point (12), and may be triggered by individual predisposing conditions (12,27).

Subclinical hypothyroidism

Causes, prevalence and progression

Subclinical hypothyroidism reflects an early and mild form of thyroid failure. Most patients with Sh have chronic autoimmune thyroiditis and test positive for serum antithyroid peroxidase (anti-TPO) antibodies; in these patients, the risk of progression to overt disease is particularly increased. Poor compliance with L-T4 therapy or suboptimal treatment, may also result in Sh.

Medications (e.g., lithium, iodine, interferon, etc.), 131I therapy or thyroidectomy and external irradiation of the neck can also cause Sh. The epidemiologic data from three large population-based screening studies (the Whickham Survey, the Colorado Thyroid Disease Prevalence Study and the National Health and Nutrition Examination Survey III) (10,11,28) show that the prevalence of Sh is 4-10% and that this condition increases significantly with age, so that by the ninth decade of life the prevalence is 15%-20%.

A mild TSH increase (between 4-10 mU/L) is present in about 74% of Sh patients, and whether to treat Sh associated with this TSH range is hotly debated. The progression to overt hypothyroidism occurs at a rate of 2-5% per year and it is increased in patients with TSH >6 mU/L and positive thyroid antibodies.

The reasons for treating Sh are: to prevent progression to overt disease, to attenuate symptoms, and to correct lipid profile and cardiovascular abnormalities and so reduce the cardiovascular risk. However, the TSH cut-off at which to start replacement therapy with L-thyroxine is still debated (1-3).

Cardiovascular risk

The cardiovascular risk in patients with Sh results from changes in cardiovascular function and from accelerated atherosclerosis (8,9)

Table 2

Subclinical hypothyroidism and cardiovascular risk

Increased risk for functional cardiovascular changes

- Normal/depressed systolic function at rest
- Left ventricular diastolic dysfunction at rest and during exercise
- Impaired left ventricular systolic function on exercise
- Increased systemic vascular resistance
- Increased prevalence of diastolic heart failure in the elderly

Increased risk for atherosclerosis

- Increased prevalence of hypertension
- Endothelial dysfunction
- Atherogenic lipid profile
- Hypofibrinolytic and hypercoagulable state
- Elevated C-reactive protein levels

The most consistent cardiac abnormality in patients with Sh is impaired left ventricular diastolic function, which is characterized by slowed myocardial relaxation and impaired early ventricular filling (9,29). Impaired left ventricular relaxation was identified in patients with Sh by echocardiography and radionuclide ventriculography (30-33). All studies of young and

middle-aged patients with a mild and persistent TSH increase (4-10 mU/L) due to Hashimoto thyroiditis show that diastolic dysfunction of the left ventricle is a common finding in patients with persistent Sh (30-33). Diastolic function is impaired both at rest and during exercise (33). Slowed left ventricle relaxation is in accordance with the finding that thyroid hormone affects the calcium-regulating proteins SERCA and PLB thereby slowing down calcium re-uptake into the sarcoplasmic reticulum during diastole (29). Altered diastolic function can be reversed by L-thyroxine replacement therapy (8,9,29-33).

Conflicting results on systolic function are reported in patients with overt and Sh (8). Impaired left ventricular systolic function on effort was documented in patients with Sh by using radionuclide ventriculography, Doppler echocardiography and cardiopulmonary exercise testing (29, 34). The negative effect induced by Sh on systolic function at rest and during effort is reverted by restoring euthyroidism with L-T4 therapy (29-34). Ultrasonic myocardial textural analysis indicates that myocardial composition is altered in patients with Sh (35). Many Sh patients are elderly and the onset or progression of the disease in these vulnerable subjects may precipitate cardiac decompensation and promote congestive diastolic heart failure (5,8).

Overt hypothyroidism is associated with premature atherosclerosis and coronary artery disease. Epidemiological studies of the link between Sh and atherosclerosis have yielded conflicting results (36,37). Compelling evidence of a higher prevalence of atherosclerotic cardiovascular disease in patients with Sh (defined as TSH >4.0 mU/L) emerges from a recent large cross-sectional survey of 1,149 women aged 55 years or more, living in Rotterdam (37). It was shown that Sh patients had a significantly increased age-adjusted prevalence of aortic atherosclerosis on chest radiographs and myocardial infarction compared with controls. The attributable risk percentage for Sh associated with myocardial infarction was within the range of the traditionally recognized risk factors for coronary artery disease, including hypercholesterolemia, hypertension, smoking and diabetes mellitus. Moreover, in a cross-sectional analysis, Sh was associated with ischemic heart disease independent of age, systolic blood pressure, body mass index, cholesterol, smoking, or presence of diabetes mellitus (38).

The mechanisms responsible for atherosclerosis and coronary artery disease in patients with Sh are controversial (8,39). Diastolic hypertension (40,41), dyslipidemia (42-45), endothelial dysfunction (46), elevated C reactive protein levels (47) and coagulation abnormalities (48) are atherosclerotic risk factors associated with Sh and may be reversed after L-T4-induced euthyroidism.

In a recent review of guidelines, treatment of Sh was recommended when serum TSH is >10 mU/L so as to prevent progression to overt disease (3). Treatment of subclinical hypothyroidism based on cardiovascular risk was not recommended because the data available were considered insufficient and unconvincing (3). Furthermore, routine levothyroxine treatment was not recommended when TSH is between 4.5 and 10 mU/L (3).

However, mild hypothyroidism (TSH <10 mU/L) can negatively affect the cardiovascular system, especially diastolic function, endothelial function and systemic vascular resistance. Treatment of this mild form of hypothyroidism may improve cardiovascular function (8, 49) and it may prevent atherosclerosis and coronary artery disease (50) thereby reducing the cardiovascular risk.

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ROLE OF THE ORBITAL FIBROBLAST IN THE DEVELOPMENT OF GRAVES' OPHTHALMOPATHY

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Clinical and histologic hallmarks

Graves' disease is a common disorder with an incidence in women of 1/1000 population/year. In addition to hyperthyroidism, 25-50% of individuals with Graves' disease develop clinical involvement of the eyes (1). While some patients with GO experience only mild ocular discomfort, 3-5% suffer from intense pain and inflammation with double vision or even loss of vision.

The clinical symptoms and signs of GO can be explained mechanically by the increase in tissue volume evident within the bony orbit. The expanded orbital tissues cause forward displacement of the globe and impairment of venous and lymphatic outflow from the orbit. These changes, combined with the local production of cytokines and other mediators of inflammation, result in proptosis, periorbital edema, conjunctival erythema and chemosis (Figure 1).



Computerized tomographic scans show that the majority of patients with GO have enlargement of both the orbital fat and the extraocular muscles, while others appear to have only adipose tissue or extraocular muscle involvement. The extraocular muscles cells themselves are intact in early, active disease, suggesting that they are not themselves the targets of autoimmune attack. Rather, the enlargement of the extraocular muscle bodies results from an accumulation of hydrophilic mucopolysaccharides, including especially hyaluronan, within the perimysial connective tissues (2). In later stage disease, the resolving inflammatory process within the muscles may leave them fibrotic and misaligned.

The increase in the volume of the adipose/connective tissues within the orbit appears to contribute more significantly to the overall expanded orbital tissue volume than does the extraocular muscle enlargement. Computerized tomographic studies show that proptosis measurements in these patients are most closely correlated with the volume of the fat compartment (3). This expanded adipose tissue volume appears to result both from hyaluronan accumulation with attendant edema, and from the emergence of a population of newly differentiated fat cells within these tissues (4).

Thyroid ("pretibial") dermopathy is typically a nodular thickening of the skin on the anterior lower legs. This condition is evident in approximately 15% of Graves' patients with severe GO. The histologic changes in the subdermal connective

tissues in thyroid dermopathy are similar to those within the GO orbit, with lymphocytic infiltration and hyaluronan accumulation (5), but without an increase in adipose tissue volume.

Heterogeneity in the fibroblast phenotype

The characteristic histologic changes within the tissues outlined above suggest that the orbital fibroblast constitutes the target cell in GO. Rather than being a homogeneous population of cells, fibroblasts exhibit phenotypic heterogeneity even within a single tissue (6). Some cells within the orbital fibroblast population are capable of producing hyaluronan and inflammatory prostanoids, while others (termed "preadipocyte fibroblasts" or "preadipocytes") have the capacity to differentiate into mature adipocytes (5). Connective tissues investing the extraocular muscles contain the former, while preadipocytes are found primarily in the orbital connective/adipose tissue depot. These phenotypic differences between fibroblasts within the orbit may help to explain why some patients with GO have predominant eye muscle disease, while enlargement of the adipose tissues is the major disease feature in others (8,9).

Fibroblasts also possess a wide array of tissue-specific phenotypes (10). Early studies of orbital fibroblasts focused on cytokines, their effects on orbital fibroblasts biology, and phenotypic differences between fibroblasts from the orbit and skin (1,6). For instance, orbital fibroblasts treated with IFN- γ or leukoregulin synthesize high levels of hyaluronan, while dermal fibroblasts similarly treated produce only small amounts (11,12). More recent studies have centered on the particular sensitivity of orbital fibroblasts to induction of CD40 expression following IFN- γ treatment. This receptor is bound by the CD154 receptor on activated T lymphocytes. CD40/CD154 ligation results in the production by fibroblasts of several mediators of inflammation, including IL-1, IL-6, and IL-8, and in the synthesis of high levels of hyaluronan (13).

Preadipocyte fibroblasts also show regional differences in the expression of adipocyte-specific genes (14,15), and vary in their adipogenic potential; PPAR- γ agonists enhance differentiation of preadipocyte fibroblasts from subcutaneous sites, while those from omental sites are refractory to these agents (16). The study of such depot-specific differences in fibroblast phenotype may help to explain why patients with GO have expanded orbital adipose tissues, without evidence of involvement of other adipose tissue depots, and why the lower legs are more commonly affected than are other skin regions.

Unique anatomical features of the orbit and lower extremities appear to play a role in their prominent clinical involvement in Graves' disease (17,18). The unyielding confines of the bony orbit predisposes to compression of low-pressure lymphatic and venous channels, increasing retroocular pressure and periorbital edema. Similarly, prolonged standing contributes to compromise of these channels in the lower extremities, likely contributing to the dependent edema seen in thyroid dermopathy. Moreover, individual anatomic variability, such as the shape of the orbits or variations in venous or lymphatic vessels, may place some individuals with Graves' disease at special risk for the development of severe GO or dermopathy.

Extrathyroidal TSHR expression

The close clinical relationship between Graves' hyperthyroidism and GO (18), and the finding of a correlation between thyroid-stimulating autoantibody levels and the clinical activity of GO (19), suggest that immunoreactivity against TSHR may underlie both conditions. The concept that TSHR-expressing orbital adipose tissue may be targeted in GO evolved from early studies showing TSH binding to guinea pig adipose and retro-orbital tissues, or to porcine orbital connective tissue membranes (20,

21). The expression of this receptor in human fat tissue was first suggested by studies showing regulation of lipolysis by physiologic levels of TSH in human fetal and newborn, but not adult, adipocytes (22). These results implicated TSH and its receptor in the normal regulation of thermogenesis in early post-natal life.

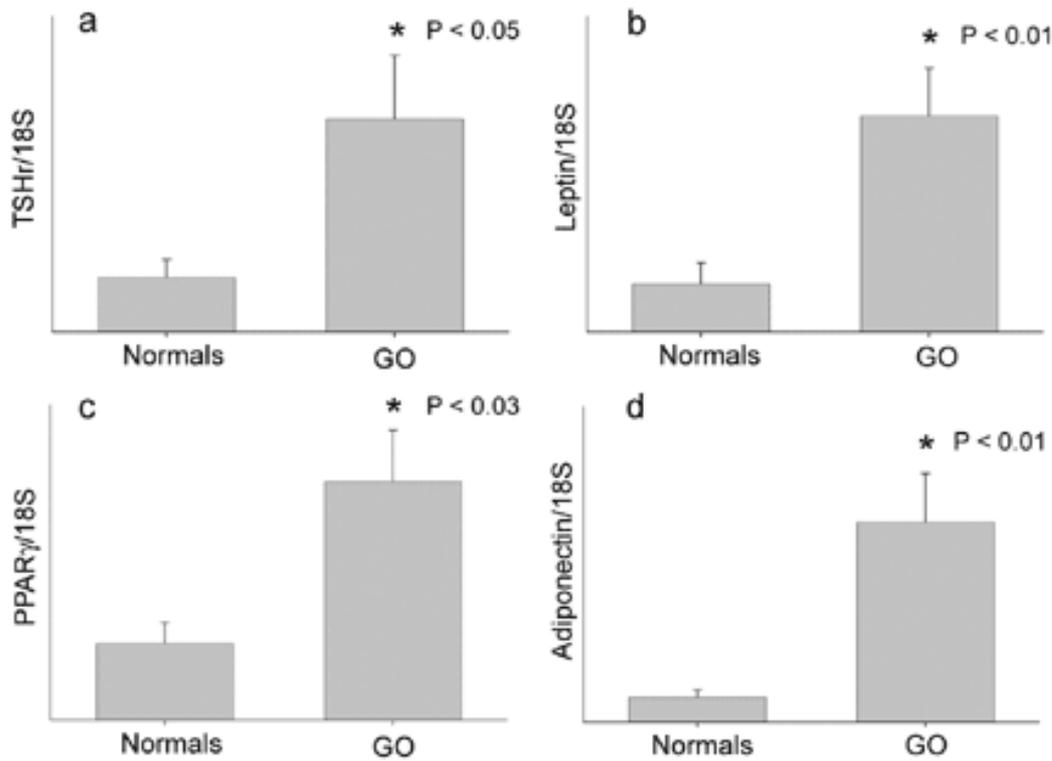
A prerequisite for involvement of TSHR as an autoantigen in GO is that it be expressed in affected orbital tissues. Studies aimed at identifying TSHR in the orbit tissues have been performed by several laboratories using many different approaches. Results of these studies are in general agreement, demonstrating the presence of TSHR mRNA and protein in both GO and normal orbital adipose tissues and derivative cultures (23-28). In addition, our laboratory reported that TSHR expression is up-regulated in GO orbital fat compared with normal orbital adipose tissues (29). A recent study by another group supported these findings and additionally showed a positive correlation between TSHR mRNA levels in GO orbital adipose tissues excised during decompression surgery and the patients' clinical activity score (30). Similarly, TSHR appears to be more abundant in PTD (pretibial dermopathy) skin than in normal pretibial skin (17).

The only animal model of Graves' disease in which ocular changes suggestive of GO have been reported to date was developed by Marian Ludgate and colleagues (31). This group transferred T cells primed in animals immunized with a TSHR fusion protein or vaccinated with TSHR cDNA. While thyroiditis and antibodies directed against the TSHR were reported in these animals, hyperthyroidism and thyroid-stimulating autoantibodies were not produced. The authors described tissue edema, dissociation of muscle fibers, minimal lymphocytic infiltration, and the presence of TSHR immunoreactivity within the orbital adipose tissues in the majority of immunized animals. However, while the histopathology appeared promising, the mice did not develop any of the characteristic clinical signs of GO. Of particular interest is a recent publication by this same group in which they brought into question the interpretation of the thyroid and ocular changes reported in their original study (32). Nevertheless, the partial success of this model suggests that transfer of TSHR-primed T cells may hold potential for the induction of ocular disease, and supports the concept that TSHR may be an important orbital autoantigen.

Relationship between adipogenesis and TSHR expression

We studied the relationship between adipogenesis and TSHR expression in cultures of orbital preadipocyte fibroblasts undergoing in vitro differentiation. Levels of mRNA encoding TSHR, as well as leptin and adiponectin (genes expressed exclusively by mature adipocytes), were found to be approximately 10-fold higher in differentiated cultures compared with control cultures. In addition, relatively greater expression levels of these genes was apparent in cultures derived from GO orbital tissues than in normal orbital cultures (33, 34).

We also examined expression of these genes in uncultured GO and normal orbital adipose tissue specimens. We found TSHR, leptin, PPAR- α and adiponectin mRNA levels to be several-fold higher in the GO than in the normal tissues (Figure 2), with significant positive correlations noted between levels of TSHR mRNA and mRNA levels of the adipocyte genes (35).



These results suggested to us that adipogenesis may be enhanced in the GO orbit, and that increased TSHR expression is a consequence of this process.

Potential role of the insulin-like growth factor receptor (IGF-1R)

Recent studies by Terry J. Smith and colleagues demonstrated that fibroblasts from patients with Graves' disease are activated by immunoglobulins (IgG) from these same donors to synthesize the RANTES and IL-16, molecules that provide signals for the infiltration of immunocompetent cells into areas of inflammation (36). This activation appears to be mediated through the IGF-1 receptor pathway as it is blocked in these cells by specific IGF-1 antibodies or transfection with a dominant-negative mutant IGF-1R (37). The IgG-stimulated activation of this receptor does not, however, appear to be restricted to fibroblasts from the orbit and pretibial skin, as fibroblasts obtained from diverse sites in these Graves' patients behaved similarly. In contrast, fibroblasts obtained from patients without known autoimmune disease, regardless of their site of origin, do not respond to these IgGs. These findings suggest that IGF-1R may be a novel second autoantigen in Graves' disease, playing an important role in lymphocyte trafficking. The relatively restricted involvement of the orbit and pretibial skin in the extrathyroidal manifestations of Graves' disease may be explained, in part, by the exquisite sensitivity of fibroblasts from these sites to stimulation by cytokines and other immune factors (38).

Summary

Histologic examination of orbital tissues in GO reveals that the characteristic changes result primarily from hyaluronan accumulation with edema, expansion of the fat compartment, and infiltration of the tissues by T lymphocytes. Studies using cells obtained from these tissues have shown that the orbital fibroblast is a resident cell uniquely capable of participating in these diverse cellular processes. These cells are particularly sensitive to stimulation by cytokines and other immune mediators, responding by increasing CD40 expression, synthesizing large quantities of hyaluronan, and secreting

inflammatory cytokines. In addition, the preadipocyte subpopulation of fibroblasts is capable of differentiating into mature adipose cells that exhibit high levels of TSHR. Fibroblasts have also been shown to display IGF-1R. When bound by IgG from Graves' patients, these receptors initiate downstream signaling that results in RANTES and IL-16 production and leads to local lymphocytic infiltration. The relative site-specificity of orbital and pretibial involvement in Graves' disease may be explained both by the relative sensitivity of these fibroblasts to immune mediators, and by the unique anatomical features of these sites that appear to predispose them to compression of low-pressure lymphatic and venous channels.

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Thyroid hormone and major depression

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Introduction

The relationship between mood and thyroid function has been recognized since the 19th century when it became clear that hypothyroidism is associated with a variety of mental disturbances including depression. Nevertheless, it has been only during the past decades that the complex interactions between thyroid hormone and the central nervous system have begun to be elucidated. Direct evidence for the importance of thyroid hormone for the metabolic integrity of the adult human brain was obtained only in the 1990s when studies using magnetic resonance spectroscopy and positron emission tomography in hypothyroid patients showed effects of thyroid hormone supplementation, especially in the frontal lobe, on regional cerebral blood flow, glucose utilization and additional metabolic parameters (1,2). This provided a biological basis for the wide variety of mental symptoms in hypothyroidism including disturbed mood and cognition. In view of these observations it is not surprising that various investigators have focused on the question whether in depressed patients without manifest pathology of the thyroid gland, changes occur in the hypothalamus-pituitary-thyroid axis (HPT axis). Most of these studies have focused on serum thyroid hormone concentrations, but more recently changes in the hypothalamus of patients with depression have been reported as well. Another approach has been the use of thyroid hormones as an adjunct treatment for affective disorders, adding thyroid hormone to antidepressant therapy in an attempt to increase treatment efficacy.

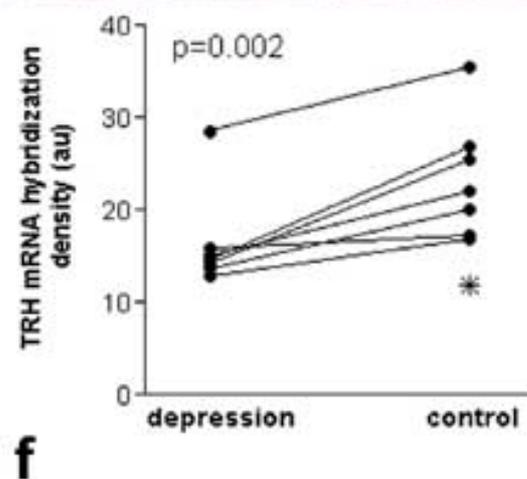
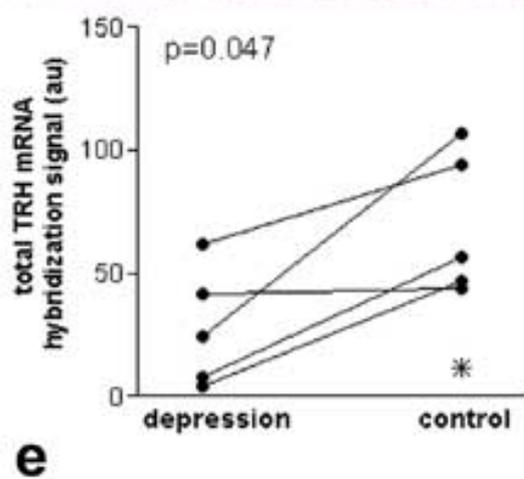
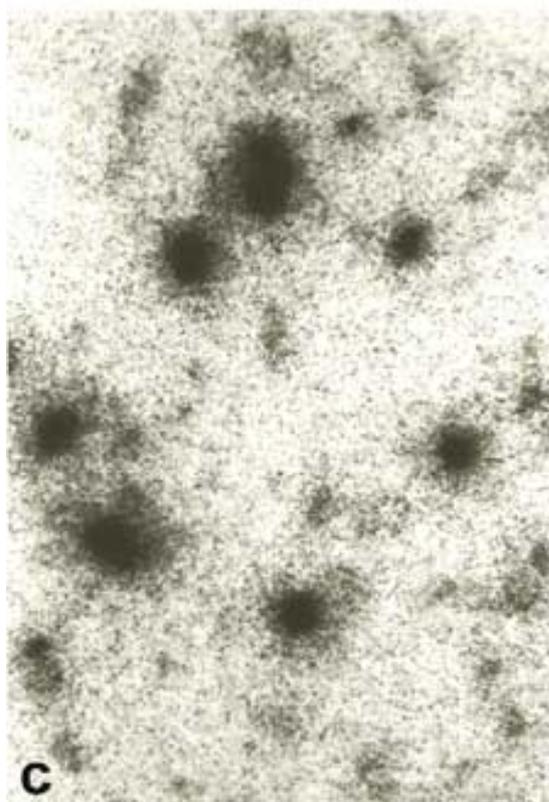
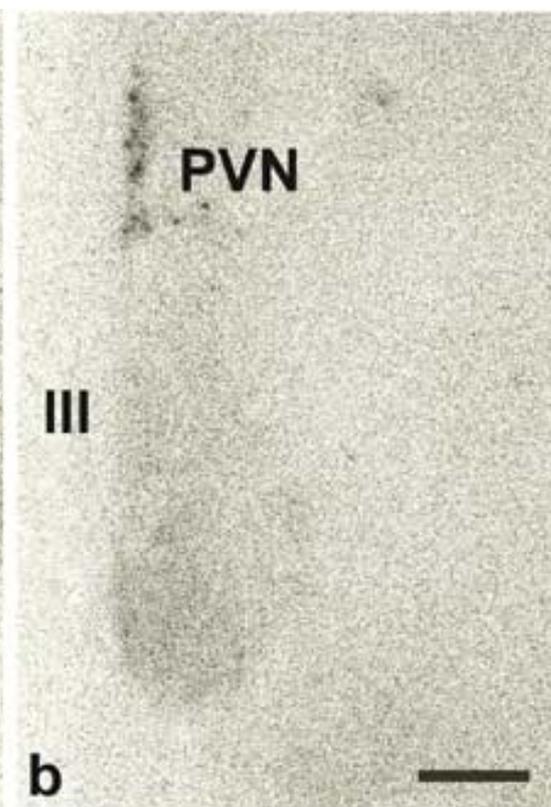
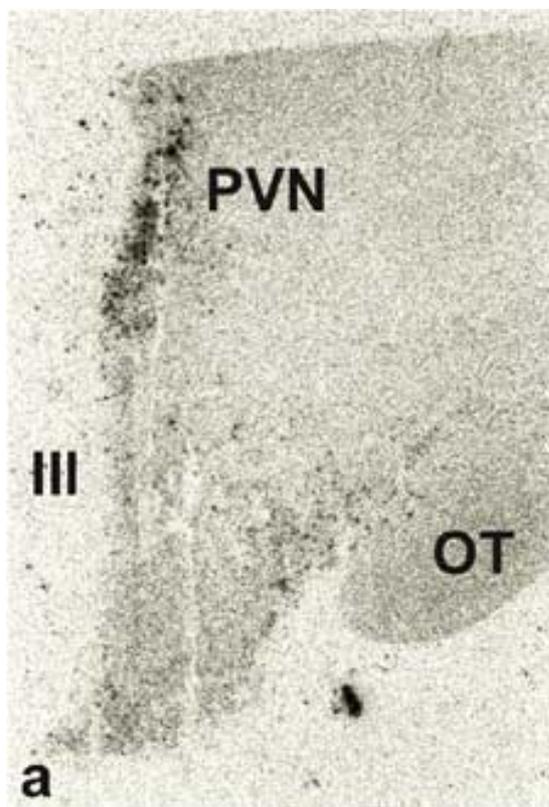
Thyroid hormone serum concentrations in major depression

Major depression has been associated with changes in the HPT axis. Various authors have reported decreased serum TSH with a somewhat blunted response to TRH stimulation, and increased serum thyroxine (T4) or free T4 (FT4). Most of the changes reported were still within the reference range for the particular serum concentration studied. Furthermore, an increased prevalence of subclinical hypothyroidism and thyroid peroxidase (TPO) antibodies has been described. These endocrine changes in major depression may have pathogenetic relevance as they have been proposed to reflect serotonin and/or norepinephrine deficiency which are prominent neurochemical targets for antidepressant therapy (3), or to reflect effects of hypercortisolism within the brain inducing mildly decreased cerebral bioavailability of thyroid hormone (4). However, several other studies did not find any changes in serum TSH and/or FT4 in patients with major depression (e.g., 5). These inconsistencies are probably due to a number of factors. First, studies reported in the literature differ with respect to the in-/outpatient status of the included patients, studies on inpatients being more numerous. This may be somewhat surprising since outpatients comprise the vast majority of patients with major depression. Second, patients were on antidepressant medication in several studies which may influence endocrine parameters even after short-term discontinuation (6). Third, the heterogeneity of depression is a potential bias. Several studies included unipolar as well as bipolar patients, or were designed without stratification for depression subtype such as melancholic depression.

In view of these inconsistencies and the potential clinical importance of HPT axis changes in depression we recently performed a study in 113 outpatients with unipolar major depression who had been free of antidepressants for at least three months and in 113 age- and sex matched controls. To be eligible for the study the patients had to be between 18 and 65 years of age and to fulfill the diagnostic criteria major depressive disorder according to the Structural Clinical Interview for the Diagnostic and Statistical Manual of Mental Disorders (DSM), fourth edition (SCID-IV). Patients were required to have a score of at least 16 on the 17-item Hamilton Rating Scale for Depression (HRSD) which is a commonly used questionnaire to rate depression severity. Surprisingly, the serum concentration of TSH was slightly higher in patients with major depression as compared to controls and this difference persisted after the exclusion of patients with TPO antibodies and/or subclinical hypothyroidism (median TSH 1.70 in major depression versus 1.50 mU/L in controls, respectively). There were no signs of activation of the hypothalamus-pituitary-adrenal axis in the depressed patients as evident from unaltered 24h urinary cortisol excretion (7). Dividing the patients according to depression subtype (e.g., melancholic depression versus other subtypes) or depression severity (e.g., HRSD lower versus higher than 23) did not unmask any endocrine changes in the depressed patients. Therefore, the somewhat higher serum TSH found in the present study in outpatients cannot be attributed to less severe depression in these patients as compared with inpatients. An alternative explanation may be altered circadian structure in depressed patients. Serum TSH shows a clear diurnal variation with a nocturnal TSH surge and this rhythm is driven by the biological clock located in the hypothalamic suprachiasmatic nucleus (SCN) (8). Indeed, changes in circadian rhythms as well as alterations in the SCN have been found in depressed patients (9).

Thyroid hormone and hypothalamic changes in major depression

Thyrotropin-releasing hormone (TRH)-containing neurons in the hypothalamic paraventricular nucleus (PVN) represent the major determinant of hypothalamic setpoint regulation of the HPT axis. In animal experimental studies these neurons have been shown to respond to hypothyroidism with increased TRH mRNA and protein content, thereby increasing TSH release from the anterior pituitary, with opposite changes seen in hyperthyroidism. Thyroid hormone status is sensed by thyroid hormone receptors (TRs) expressed by TRH cells in the PVN. An additional feedback pathway is represented by monosynaptic projections from the hypothalamic arcuate nucleus to TRH neurons in the PVN. In the arcuate nucleus, the blood brain barrier is absent and both TRs and iodothyronine deiodinase type 2 (D2) are abundantly expressed. This pathway has been shown to be implicated in mediating the effects of falling serum leptin concentrations on central downregulation of the HPT axis during starvation. Earlier studies from our group revealed both parvo- and magnocellular TRH neurons in the human PVN (10) and decreased TRH mRNA in direct relation with decreased serum T3 and TSH in the framework of non-thyroidal illness (11), suggesting an important role for TRH neurons in human HPT axis setpoint regulation. In view of several reports of altered serum TSH in depression we performed a study of TRH mRNA expression in the PVN of patients with unipolar depression versus patients without psychiatric disease by quantitative in situ hybridization. Total TRH mRNA signal in the PVN was decreased in depression (figure) (12). Unfortunately, the distinction within the human PVN between neuroendocrine TRH cells projecting to the median eminence and centrally projecting TRH cells cannot be made at present.



depression

control

depression

control

e

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Figure: (a,b) macroscopic photographs of film autoradiograms of representative sections through the PVN of a control subject and weaker hybridization signal in a subject with major depression, respectively. (c,d) TRH mRNA containing cells in the PVN of the same subjects. Bars represent 2 mm (a,b) and 50 μ m (c,d). OT= optic tract. (e,f) Total TRH mRNA hybridization signals in depressed subjects and controls. Subject pairs matched for severity of fatal illness are interconnected with lines. Each dot represents one subject. Note strongly decreased TRH hybridization signal in subjects with depression. Asterisk represents patient with primary hyperthyroidism and without depression. Reproduced with permission from (12)

CRH mRNA was shown earlier to be increased in the PVN of depressed patients (13) which suggests an increased hypothalamic drive of the HPA axis in depression. Since glucocorticoids have been shown to decrease TRH gene expression in the PVN of rats (14), we hypothesized that decreased TRH mRNA in depression might result, at least in part, from HPA axis activation. To obtain circumstantial evidence for this we assessed total TRH mRNA hybridization signal in the PVN of patients treated with pharmacological doses of glucocorticoids just before death and found a clear decrease as compared with untreated patients (15). Thus, our findings in postmortem studies are congruent with central, i.e., hypothalamic, activation of the HPA axis which may lead to decreased hypothalamic TRH stimulation of TSH from the anterior pituitary. Notably, these observations were made in deceased patients who had been admitted to a hospital before death which may explain the discrepancy with the paucity of endocrine changes found in depressed outpatients mentioned above.

Thyroid hormone and treatment of major depression

Selective serotonin reuptake inhibitors (SSRI) are currently favored in therapeutic practice as a first step in the treatment of major depression. SSRI increase the bioavailability of serotonin at the synapse, which is assumed to elicit the therapeutic response. There is evidence from animal experimental studies that triiodothyronine (T3) is also capable of increasing serotonergic neurotransmission by desensitization of inhibitory 5-HT_{1a} autoreceptors in the raphe nucleus, thus disinhibiting cortical and hippocampal serotonin release, and by increasing cortical 5-HT₂ receptor sensitivity, further increasing 5-HT neurotransmission (16). In addition, various antidepressants (including SSRI) stimulate D₂ activity, theoretically leading to increased cerebral T₃ concentrations, thus enhancing serotonergic neurotransmission (3). These considerations have led several investigators to perform clinical studies in patients with major depression using thyroid hormone in combination with antidepressants. A number of rather small clinical trials have been performed aimed at increasing efficacy of treatment with tricyclic antidepressants (TCA) by adding T₃, showing inconsistent albeit encouraging results. A meta-analysis suggested that larger placebo-controlled studies should be performed including patients with the currently favored SSRI (17). Another approach has been to try and accelerate response to TCA by adding T₃ which was shown to be effective (18).

We recently investigated the efficacy of T₃ addition to an SSRI (paroxetine) in patients with major depression in a double-blind, placebo controlled clinical trial in 113 patients with major depression. Patients were randomly assigned to 8 weeks of treatment with low dose T₃ (25 μ g), higher dose T₃ (25 μ g twice daily), or placebo in addition to paroxetine 30 mg daily. Response rate after 8 weeks (defined as a reduction in HRSD score by \geq 50%) was 46% in all three treatment arms ($p=0.99$).

T3 addition did not accelerate clinical response to paroxetine, while patients on T3 addition reported more adverse events than patients on placebo co-medication (19). These results did not support a role for T3 addition to SSRI in the treatment of major depressive disorder. However, there still is a possibility that T3 co-medication is effective in patients with refractory depression.

Conclusion

HPT axis changes in major depression have been studied for many decades and results have been somewhat controversial. Inpatients have been reported to frequently exhibit hypercortisolism which may be explained in part by an increased hypothalamic drive of the HPA axis as suggested by postmortem studies. Hypercortisolism may explain recently reported decreased hypothalamic TRH mRNA expression in major depression, in turn giving rise to lower serum TSH. By contrast, outpatients with major depression exhibit only marginal endocrine alterations, including slightly higher serum TSH, which is not explained by less severe depression. To test the hypothesis that T3 can be used to further enhance serotonergic neurotransmission in patients with major depression treated with an SSRI, we performed the first randomized clinical trial to investigate the efficacy of T3 addition to paroxetine and found no effect on efficacy or on time interval to response. Whether T3 addition to antidepressants may be effective in refractory depression is subject of our current studies.

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UNDERSTANDING THYROID HORMONE ACTION AND THE EFFECTS OF THYROID HORMONE REPLACEMENT – JUST THE BEGINNING NOT THE END.

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BACKGROUND

Thyroid hormone – the easiest hormone to replace?

L-Thyroxine is considered the treatment of choice for hypothyroid patients as it has a long half-life, is inexpensive to produce and provides stable levels of T4, T3 and TSH over 24-hour period. Indeed, this favourable pharmacodynamic profile linked with excellent bioavailability following oral administration and the ability to fine titrate dosing using sensitive TSH assays has led most endocrinologists to believe that, unlike all other hormones, thyroid hormone replacement is straightforward. An unwelcome challenge to this view is the proportion of hypothyroid patients that report that they are not back to their normal self despite doses of thyroxine sufficient to normalise TSH levels (1,2,3,4,5). We recently tried to quantify this problem in a community-based cross-sectional survey and observed a 6.7% absolute excess of psychiatric caseness (32.3 vs 25.6%) in patients on thyroxine compared to an age- and sex-matched control population from the same community. This difference persisted even in individuals on T4 with recent TSH levels in the laboratory reference range (6).

Is dissatisfaction on thyroxine replacement therapy related to thyroxine?

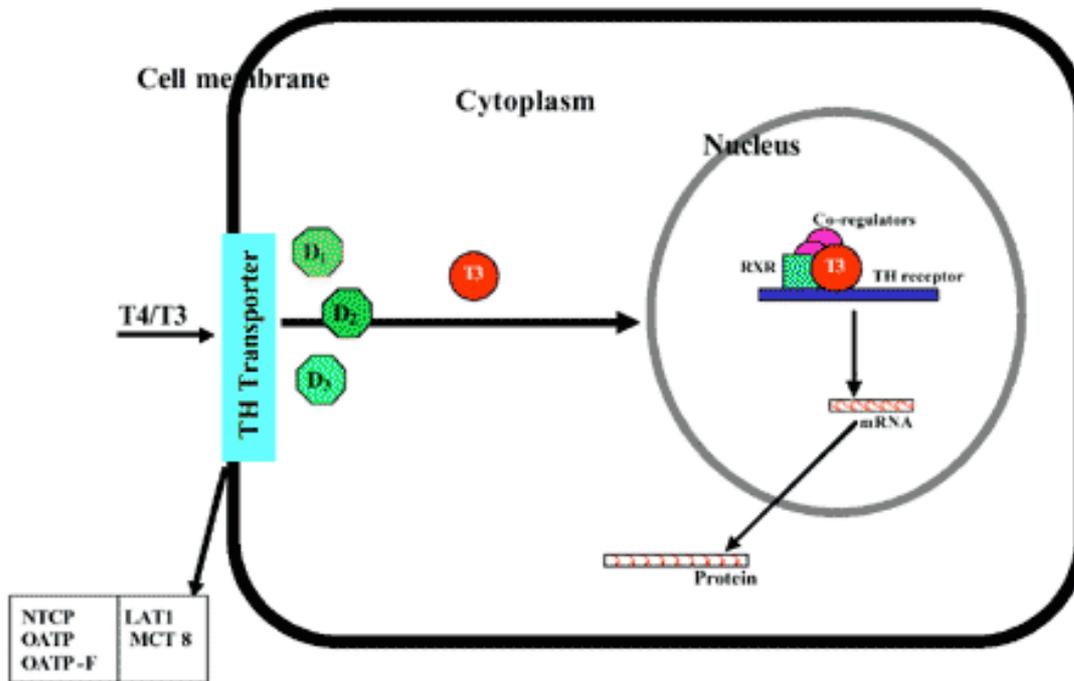
Thyroid dysfunction has two very important clinical features which distinguishes it from dysfunction in other endocrine glands: it is both very common (up to 5% of the population) (7,8) and very easy to detect. As a result there will inevitably be extensive overlap between patients with hypothyroidism and patients with other medical and psychological conditions. Hence one explanation for apparent dissatisfaction with thyroid hormone replacement is likely to arise from dysphoric patients being screened for thyroid dysfunction and then started on thyroxine for minor rises in TSH. If their symptoms were not due to hypothyroidism in the first place, they would not be expected to improve on treatment and the result will be a dissatisfied patient on thyroxine. Undoubtedly this accounts for a proportion of dissatisfaction on thyroxine and is consistent with the strong placebo effect in trials of alternative forms of thyroid hormone replacement (9,10,11). A second explanation may be that thyroid hormone at supraphysiological levels has a useful euphoric effect that is lost on dose reduction (12,13). Thirdly, impaired psychological well-being may be related to thyroid autoimmunity, independent of the patients thyroid status as suggested by some epidemiological studies (14). At present this is difficult to quantify and the possible mechanism remains unclear. However, recent developments in the biology of thyroid hormone action and metabolism indicate that a fourth alternative explanation - relative tissue hypothyroidism – may explain at least some cases.

Possible mechanisms resulting in tissue hypothyroidism despite normal TSH levels

Although it has long been known that T4 needs to be converted to T3 for intracellular action, recent studies have emphasised

the complexity of thyroid hormone action (see figure).

Possible sites of defective thyroid hormone metabolism



abbreviations:

T4 – Thyroxine; T3 – Tri-iodothyronine; TH – Thyroid Hormone; RXR – Retinoid X receptor; D1-3 – Deiodinases – Type 1-3; NTCP – Na⁺/ Taurocholate co-transporting polypeptide; OATP – Organic anion transporting polypeptide; LAT1 – L-type amino acid transporter 1; MCT 8 – Monocarboxylate transporter 8

The presence of membrane thyroid hormone transporters amplifies hormone uptake by up to 10-fold (15,16). Following uptake, deiodination is carried out by not one but three selenium dependent deiodinases (D1 – D3) each with different catalytic specificity, tissue distribution and sensitivity to extracellular influences. As a result, the amount of intracellular T3 derived directly from circulating T4 rather than T3 can vary up to 10-fold between tissues (17). In addition, thyroid hormone receptor action depends on many co-regulators (18) whose levels vary between tissues and operates via 9 different isoforms of 2 different thyroid hormone receptor genes (TR α and TR β) whose levels also vary widely between tissues (19). Hence, although levels of circulating TSH in the reference range typically indicate normal levels of intracellular T3 in the hypothalamus and pituitary, there are several mechanisms by which such a normal TSH level may fail to indicate intracellular euthyroidism in other tissues, for example following thyroid hormone replacement (Table 1).

TABLE 1: MECHANISMS BY WHICH A NORMAL CIRCULATING TSH LEVEL (eg. ON T4 REPLACEMENT) MAY FAIL TO REFLECT HYPOTHYROIDISM IN SOME TISSUES

Level of effect	Mechanism	Evidence
1. Expression of different deiodinases	Pituitary is more sensitive to T4 than to T3 due to high levels of D2 expression. This would result in normal TSH and low normal T3 and fail to reflect true thyroid status in all body tissues.	<ul style="list-style-type: none"> • Titration of T4 to a normal TSH typically results in raised T4/T3 ratio⁽²⁰⁻²²⁾ • D2^{-/-} mouse has high TSH and T4 levels but normal T3 levels⁽⁵⁷⁾ • Polymorphisms in deiodinase affect the T3/rT3 ratio⁽⁵⁸⁾
2. Differential sensitivity of different deiodinases to modulating factors	Certain factors may have a greater effect on T3 levels in tissues, which express lower levels of D2 or higher levels of deiodinase D3 than the pituitary.	<ul style="list-style-type: none"> • D2 and D3 levels vary widely between tissues, e.g. brain regions⁽⁵⁹⁾ • D2 and D3 activity are differently regulated⁽¹⁷⁾
3. Thyroid hormone transporters	Increased expression of thyroid hormone transporters may make pituitary less sensitive to hypothyroidism than other tissues	<ul style="list-style-type: none"> • MCT8 transporter mutations result in only mild TSH elevation and low normal T4 but high T3 and neurological defects^(15,16)
4. Co-regulators	Variation in levels of TR α /TR β co-regulators between tissues may result in tissue specific effects	<ul style="list-style-type: none"> • No specific evidence yet
5. Differential thyroid hormone receptor expression	Important variations in TR α expression between individuals may not affect TSH levels as pituitary expresses predominantly TR β	<ul style="list-style-type: none"> • TRα^{0/0} mouse has normal thyroid blood levels but hypothyroid bone⁽⁶⁰⁾
6. Differential expression of proteins related to thyroid hormone action	If the expression is different from pituitary, may result in tissue specific effects not reflected in changes in TSH	<ul style="list-style-type: none"> • Association of polymorphisms in the calcium channel (Ca(v)1.1) with susceptibility to thyrotoxic periodic paralysis⁽⁶¹⁾

Major abnormalities in these pathways might result in a small subgroup of individuals being very dissatisfied with T4 replacement despite normal TSH levels, while common polymorphisms with more minor effects might result in a range of satisfaction with T4 in treated patients. Also, in the general population with an intact thyroid axis, such common polymorphisms might represent predisposing factors for other conditions such as depression or anxiety.

Evidence that tissue specific variation in T4 action is clinically important – studies of combined T3/T4 replacement

At present, the evidence that mechanisms such as those described in Table 1 have clinically important effects on thyroid hormone replacement is largely circumstantial. Note that in many cases these pathways are not easy to study in vivo and

variations would result in differences in intracellular thyroid hormone levels (difficult to detect) without changes in serum levels. For example, deiodinase activities are located intracellularly and may not be reflected in serum levels. However, several studies have indicated that replacement therapy with T4 alone titrated to achieve normal TSH levels results in levels of T4 in the high reference range while levels of T3 remain in the low reference range (10,11,20,21,22). This is consistent with the relatively high levels of D2 in the pituitary making it sensitive to circulating T4 levels. If the local deiodinase (D2) activity in the pituitary were low, normalisation of TSH production would only be achieved when circulating T3 levels had returned to normal levels, irrespective of T4 levels (mechanism 1, table 1). Interestingly, treatment of patients with subclinical hypothyroidism with T4 alone frequently lowers circulating T3 levels, despite normalising TSH and T4 levels (23). This is almost certainly because the up-regulation of deiodinase levels in the thyroid that occurs in thyroid failure results in increased intrathyroidal T4 to T3 conversion and this effect is lost after exogenous T4.

In the light of these observations, and consistent with mechanisms 1 and 2 in table 1, one explanation for the apparent failure of T4 replacement alone to satisfy some patients is that satisfactory T3 levels are not restored in all tissues, especially those with low deiodinase levels (24,25). This has led to an interest in combined replacement with T3 and T4 to mimic the production by the intact thyroid gland. Earlier studies on combined T3/T4 therapy did not attract attention as many patients felt the side effects of excessive T3. The doses used in these studies would now be considered excessive (26). In 1999, the study of Bunevicius et al rejuvenated the debate in this area with encouraging results (27). However, this has been followed by several further studies published recently refuting this finding (28,29,30,31,32).

Why have these later studies not replicated the findings of Bunevicius et al? One explanation is that these studies used different T3/T4 combinations in different regimes (Table 2).

TABLE 2: COMPARISON OF STUDIES USING COMBINED T4 / T3 THERAPY

TABLE 2: COMPARISON OF STUDIES USING COMBINED T4 / T3 THERAPY

Studies	Mean T4 dose	Mean FT4 Levels (pmol/L)		TSH Levels (mU/L)		T3 dose used (mcg)	Wellbeing	Significance
		Before	After	Before	After			
Bunevicius 1999 (n=33)	175	25.7	23.1	0.8	0.5	12.5 od	Better	0.04 - <0.001
Siegmund 2004 (n=23)	130	22.1	20.1	1.72	0.5	14:1 T4:T3	No difference	Not given
Saravanan 2003 (n=697)	127	21.1	13.73	0.94	2.28	10 od	Better	<0.01*
Bunevicius 2002 (n=20)	115	20.7	12.3	1.02	0.47	10 od	No difference	0.09
Clyde 2002 (n=22)	NA	15.83	10.7	2.6	2.0	7.5 bd	No difference	0.28
Sawka 2003* (n=40)	NA	15.7	10.55	1.75	1.8 – 2.4	19* as bd	No difference	0.28 – 0.35
Walsh 2003 (n=101)	136	15.3	11.4	1.5	3.1	10 od	Worse	0.03 - <0.01

* T3 dose is titrated. Other studies used fixed doses of T3.

In addition, several reviewers have commented that the original study by Bunevicius et al used a heterogenous population on high doses of thyroxine replacement and that the substitution was for a short period of time (5 weeks) without a washout period before the cross-over (33). However, it remains possible that there is beneficial effect of combined substitution that is smaller than that originally suggested by Bunevicius et al or that affects a small subgroup of individuals and hence was missed in the other studies (3). Our own study based on power calculations from our previous cross-sectional study (6), involved 697 individuals and showed a modest beneficial effect after 3 months (10). Interestingly, thyroid function "drifted" between 3 and 12 months of follow-up (with a fall in the T3/T4 ratio) and the effect was lost (11).

What might be the effects of hypothyroidism in some tissues despite normal TSH?

It is well known that thyroid hormone is important for a variety of bodily functions including thermogenesis, basal metabolic rate, memory, skeletal and myocardial muscle contractility and sleep. In subclinical hypothyroidism problems with lipid

profiles (34,35,36), left ventricular contractility (37), neuromuscular conduction (38) and psychological well-being are well established. It is not clear whether these functions are completely normalised after thyroxine replacement. Small defects in any or all these functions could result in significant morbidity.

Evidence that thyroid hormone levels are important determinants of mood?

The motivation for reconsidering our approach to thyroid hormone replacement has come from patients who describe low mood and lack of energy on current replacement regimes. Studies in animals indicate some evidence that thyroid hormones can raise cortical serotonin levels (39). Evidence in adult humans is more limited. There is also an extensive though somewhat controversial literature concerning the use of thyroid hormone, often in supraphysiological amounts (up to 300ug/day) in treatment-resistant depression as well as reports relating to impaired response to selective serotonin reuptake inhibitors in hypothyroidism (13,40). A recent large cross-sectional study failed to show an association between thyroid hormone levels and depression and anxiety ratings although only TSH and T4 levels were compared. A correlation was observed between previously diagnosed thyroid disease and psychological ratings independent of thyroid function, which may represent evidence for a link with thyroid autoimmunity (41).

Is it safe to "over-replace" thyroid hormone?

While it is clear that endogenous TSH suppression in thyrotoxicosis is harmful (42,43,44), evidence is more limited if the TSH is suppressed due to exogenous thyroxine. However, several studies have shown deleterious changes in echocardiographic parameters on suppressive doses of thyroxine which are reversed by dose reduction (45,46,47,48,49,50). Recent evidence also suggests that at least in the post-menopausal women, who are the majority of patients on thyroxine replacement, bone density is reduced (51,52) and the fracture risk increases significantly on suppressive doses of thyroxine despite correcting for related variables (53). This might also be true in men on thyroxine replacement (54). However, the relationship with TSH was not studied in that epidemiological study. There are no long-term studies of patients on T3. These studies provide evidence that increasing thyroid hormone levels to "compensate" for relative tissue hypothyroidism in some tissues, is not without risks.

FUTURE DIRECTIONS

Where do we go from here?

New developments in thyroid hormone biology have indicated multiple levels at which variations in the pathway of thyroid hormone action shown in figure 1 could have clinically important effects but at present evidence of clinical relevance is limited. To make progress in this area and determine whether inter-individual variations in the pathway of thyroid hormone action contribute to psychological morbidity, predispose to other conditions and/or determine failure to respond adequately to thyroid hormone replacement in some individuals is a complex task. Progress is required in 4 areas: (1) There is a need for new markers of thyroid action in different tissues. In particular, it will be important to determine whether individuals who respond poorly to thyroid hormone in terms of psychological well-being fail to improve in any more objective measures that could relate to thyroid hormone action e.g. sleep pattern or serotonergic responses. These could then be used to monitor

response to intervention more objectively. (2) Studies are required to identify any variations or polymorphisms in elements of the pathway of thyroid hormone action- e.g. T3/rT3 ratio, deiodinase or transporter polymorphisms - which predict the psychological response to thyroid hormone or correlate with other potentially thyroid hormone related effects (eg sleep parameters, echocardiographic changes or changes in bone turnover). (3) Future intervention studies with T4 alone or in combination with T3 should be large in order (a) to carry sufficient power to see any clinical significant effect, (b) to allow correlations to be drawn between response to therapy and baseline measures of thyroid hormone action or metabolism and (c) to be sufficiently long-term enough to enable assessment of the risk to the heart and skeleton of potential overplacement. Such studies also need to be very carefully blinded to distinguish placebo effects from effects attributable to the intervention. (4) Future studies involving T3 replacement will require careful attention to dosing, dose titration and dosing ratios with T4. We have shown that despite chronic combined T3/T4 therapy wide fluctuations persist in the free T3 levels (55). Thus, use of new low-dose and slow-release preparations to allow careful monitoring and physiological replacement will be particularly valuable (56,33)

CONCLUSIONS

Despite 100 years of thyroid hormone replacement, controversy still exists about the optimum replacement therapy for hypothyroid patients. Several recent studies have given insight in to the complex thyroid hormone metabolism. These support the hypothesis that serum and tissue levels of thyroid hormones may diverge significantly and vary between tissues. The dissatisfaction experienced by some individuals on thyroxine replacement despite normal TSH levels may in part relate to this. If so, it should be seen as a pointer to greater understanding of the action of thyroid hormone and its predisposing effects on morbidity in many conditions rather than an unwelcome clinical frustration. If so, we are the beginning of a road of discovery rather than at the end of an unsuccessful chapter in thyroid hormone replacement.

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Association of autoimmune thyroiditis with other autoimmune diseases

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We recently published an overview of disease associations with autoimmune thyroid disease (AITD), based on a PubMed search of the literature from 1965-2000 (1). This editorial provides an update on subsequent studies in this area, again derived from a PubMed search up to June 2004. As with the previous overview, considerable selection has been necessary, with many case reports and small scale, uncontrolled studies having to be omitted. The well known contribution of AITD to type 2 autoimmune polyglandular syndrome (APS) has been reviewed extensively elsewhere and will not be covered here (2). AITD is less common in patients with type 1 APS (2-10%) but a novel mutation of the aetiological AIRE gene has been reported which closely cosegregates with AITD in some patients (3).

Organ-specific autoimmune disorders

Several large recent studies have clarified the strength of the association between AITD and type 1 diabetes mellitus. The prevalence of thyroid peroxidase and/or TG antibodies in 197 recent onset German diabetic patients was 18.4%, 7.8% in their first degree relatives and 3.2% in 150 controls, indicating a strong genetic component to this association (4). Generally higher figures of 27% (n = 109), 28% and 10% respectively were reported in similar patient groups from Turkey (5). Up to half of the diabetic children with thyroid antibodies in two studies from Germany and Denmark developed elevated TSH levels or goitre (6), or an abnormal echogenic pattern on thyroid ultrasound (7), within 4 years of follow-up. Diabetic patients who are older, female, have concurrent parietal cell antibodies and are glutamic acid decarboxylase antibody-positive have the greatest risk of AITD (8). Latent autoimmune diabetes of adults (LADA) is also associated with a 2-fold increase in TPO antibody prevalence, compared to matched type 2 diabetic patients (9).

The strong association of AITD with autoimmune Addison's disease has been long documented (10). Details of a uniquely large Italian cohort have recently been published; of over 4000 patients with AITD , 1% had antibodies to the adrenal cortex (11). In a series of 94 nationally recruited Norwegian Addison's patients, 29% had autoimmune hypothyroidism and 6% had Graves' disease (12).

Tissue transglutaminase antibodies were found in 3.2% of 220 Italian patients with AITD, and all had coeliac disease on biopsy; even given recent reports of a population prevalence of 1%, this represents at least a 3-fold excess of coeliac disease (13). A huge survey of 2624 vitiligo patients from N.America and the UK found that 17% had AITD (21% in women, 6% in men), compared with 5.7% of first degree relatives and an expected control population prevalence of 1.9% (14). These figures are similar to those above for diabetic families. In a much smaller series of 106 Austrian vitiligo patients, AITD was found in 21% compared to 3% of controls; of interest, the presence of antinuclear antibodies in these patients was associated with atrophic thyroiditis (15). In 200 UK patients with primary biliary cirrhosis, 21% had clinically significant AITD and 34% had thyroid autoantibodies (16). Taking into account that the famous Wickham study of the prevalence of AITD in the healthy population originated from the same area, and found much lower figures of AITD in healthy subjects (17), it seems so far that this is a strong and validated association with primary biliary cirrhosis.

The relationship between AITD and multiple sclerosis is much less clear, yet more intriguing in the light of the frequent emergence of Graves' disease after treatment of the disorder with lymphocyte-depleting monoclonal antibodies (18). In a survey of 571 UK patients with multiple sclerosis, an excess of associated autoimmune disorders was found in the first-degree relatives, especially those with multiplex families (27%, compared to 15.7% in simplex families and 11.7% in controls); AITD made the major contribution to these associated disorders but was not increased in the probands themselves (19). Furthermore, there was no excess of subclinical AITD or TPO antibodies in 152 Italian patients with relapsing-remitting multiple sclerosis, compared to 437 healthy controls, although there was a trend to an increased frequency of TPO antibodies in the men (20). This trend was confirmed in a larger series of 96 consecutive German men with multiple sclerosis, in whom 9.4% had AITD compared to 1.9% of controls, but there was no increase in AITD in 257 female patients (21).

Lymphocytic hypophysitis (LH) is a rare condition often associated with other autoimmune disorders including AITD (22). The autoantigens involved and the pathogenesis remain obscure, although antibodies to the ubiquitous enzyme, α -enolase, have been proposed as a marker. These antibodies were detected in 70% of biopsy-proven LH patients, 15% of patients with AITD and 10% of controls (23). Rather similar findings have been reported from Japan, with 22% of Graves' disease patients, 19% of Hashimoto's patients and 6% of controls being positive for antipituitary antibodies (24). Unsuspected growth hormone deficiency was found in patients with AITD who had pituitary antibodies (25), suggesting a potentially important and novel

association that requires confirmation and further follow-up.

Non-organ-specific autoimmunity

Although less strongly associated than the organ-specific disorders, recent studies have confirmed the increased frequency of AITD in systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA). In 300 SLE patients in the UK, the prevalence of autoimmune hypothyroidism was 5.7% and 14% had thyroid autoantibodies; the prevalence of hyperthyroidism was not increased (26). The frequency of AITD and TPO antibodies in SLE is around twice that seen in RA but in the latter still exceeds that expected in the population (27). Indeed, the association of RA with AITD has led to spurious associations being reported between RA and polymorphisms of the CTLA-4 gene; the association disappears once those RA patients with AITD are removed from analysis (28). Primary Sjögren's syndrome is also associated with AITD; 30% of 137 patients had AITD compared to 4% of controls in a recent French survey (29) and of 25 Brazilian patients, 52% had thyroid antibodies compared to 4% of controls (30).

The reality of an association between chronic urticaria and AITD has been debated but two recent studies support this association. Of 187 children with chronic urticaria, 4.3% had thyroid antibodies, around three times higher than controls derived from the literature (31). In 45 adults with chronic urticaria, 27% had thyroid antibodies compared to 3% of controls, although none had clinical evidence of thyroid disease (32).

A novel association with AITD was recently reported in 16 patients with chronic periaortitis, in whom 3 (19%) had TPO antibodies, and one of whom had hypothyroidism (33). Replication is clearly needed.

Summary

There are an ever-increasing number of studies confirming expected associations (Table) and demonstrating possible new associations between AITD and other autoimmune diseases.

Summary of main disease associations with AITD

Disease	% with AITD	% with TPO Ab
Addison's disease	30 - 35*	greater than 11*
Alopecia areata	0 - 15	5 - 14
Coeliac disease	3 - 6	3 - 48
Diabetes mellitus type 1	5 - 10	20
Pernicious anaemia	up to 25	50
Primary biliary cirrhosis	15 - 21	30 - 35
Rheumatoid arthritis	up to 10	11 - 32
Sjögren's syndrome	20 - 30	50
Systemic lupus erythematosus	5 - 10	15 - 50
Vitiligo	8 - 21	30
* not associated with APS type 1 or 2		

The associations are important clinically, although there is as yet no cost-benefit analysis of screening programmes for these disorders based on the known associations. This is an area ripe for large, multicentre studies as the focus increases on using autoantibodies as predictors of disease and new developments in array technology offer new dimensions in assays for screening (34).

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TRANSTHYRETIN MUTATIONS IN FAMILIAL AMYLOIDOTIC POLYNEUROPATHY

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Introduction

Transthyretin - TTR (OMIM 176300) is a well characterized molecule that consists of a tetramer of identical subunits of 127 amino acids each; the molecular structure has been determined by X-ray analysis (1). TTR is a plasma transport protein for thyroxine - T₄ - and for retinol, through the association with retinol binding protein (RBP).

Over 80 different mutations in transthyretin (TTR) have been reported. The vast majority is inherited in an autosomal dominant manner and is related to amyloid deposition, affecting predominantly peripheral nerve and/or the heart. A small portion of TTR mutations is apparently non-amyloidogenic. Among these are mutations responsible for hyperthyroxinemia, presenting high affinity for T₄ (a TTR ligand). Compound heterozygotic individuals for TTR mutants have been described; noteworthy is the clinically protective effect exerted by a non-pathogenic over a pathogenic mutation explained by stabilization of TTR tetrameric native structure. This fact lead to possible avenues of therapeutic intervention including stabilization via the central hydrophobic channel that runs through the molecule where thyroid hormones bind.

Amyloidogenic TTR mutations

Familial amyloidotic polyneuropathy - FAP - was first described by Andrade (2) in 1952 in the Northern area of Portugal; kindreds had an age of onset of clinical symptoms in the third to fourth decade of life. Early impairment of temperature and pain sensation in the feet and autonomic dysfunction leading to paresis, malabsorption, sphincter dysfunction, electrocardiographic abnormalities, emaciation and death were typical clinical features. The genetic defect in these Portuguese FAP kindreds was ascribed to a valine to methionine substitution at position 30 (3) – TTR Val30Met - resulting from a single A to G nucleotide change (4). Over 500 kindreds have been identified in Portugal, constituting the largest focus of FAP worldwide; the patient prevalence rate in the area where FAP is common in Portugal has been estimated as 105×10^{-5} (5), and the gene carrier frequency as 1 in 625 (6). The second largest known Val30Met focus is Northern Sweden where more that 350 families have been diagnosed (7); other relevant foci include Japan and the Island of Maiorca (8,9). A few cases of homozygosity for the Met 30 gene occur but do not lead to a more severe form of the disease (10).

Many other TTR mutations associated with FAP clinically not differing from the original description by Andrade have been described; others, give rise to variable phenotypes such as the presence of both neuropathy and cardiomyopathy, presentation of carpal tunnel syndrome, predominant vitreous TTR deposition and leptomenigeal involvement. A few TTR mutations are related to cardiomyopathy without neurological symptoms. The most common TTR mutation associated with

cardiac amyloidosis is Val122Ile, described in the Black population; after the age of 60, isolated cardiac amyloidosis is four times more common among blacks than whites in the United States and 3.9 percent of blacks are heterozygous for Val122Ile; a few cases of homozygosity for this mutant have been found (11). A list of TTR mutations associated with amyloidosis is presented in Table 1 (bibliographic references can be found at: <http://www.ibmc.up.pt/~mjsaraiv/ttrmut.html>)

Table 1 - Transthyretin mutations in amyloidoses

Mutation	Codon change	Predominant Clinical Features	Origin
Cys10Arg	TGT → CGT	PN, AN, Eye	Hungary
Leu12Pro	CTG → CCG	LM, PN, AN	UK
Asp18Glu	GAT → GAG	PN, AN	Columbia
Asp18Gly	GAT → GGT	LM	Hungary
Asp18Asn	GAT → AAT	Heart	USA
Val20Ile	GTC → ATC	Heart	Germany
Ser23Asn	AGT → AAT	Heart	Portugal
Pro24Ser	CCT → TCT	Heart, CTS, PN	USA
Ala25Thr	GCC → ACC	LM, PN	Japan
Ala25Ser	GCC → TCC	Heart, PN	USA
Val28Met	GTG → ATG	PN, AN	Portugal
Val30Met	GTG → ATG	PN, AN, Eye	Several
Val30Ala	GTG → GCG	Heart, AN	Germany
Val30Leu	GTG → CTG	PN, AN	Japan
Val30Gly	GTG → GGG	LM, Eye	France
Phe33Ile	TTC → ATC	PN, Eye	Poland
Phe33Leu	TTC → CTC	PN, AN	Poland
Phe33Val	TTC → GTC	PN, AN	UK
Phe33Cys	TTC → TGC	CTS, Heart	USA
Arg34Thr	AGA → ACA	PN, Heart	Italy
Lys35Asn	AAG → AAC	PN, AN, Heart	France
Ala36Pro	GCT → CCT	PN, Eye	Greece
Asp38Ala	GAT → GCT	PN, Heart	Japan
Trp41Leu	TGG → TTG	Eye	Russia
Glu42Gly	GAG → GGG	PN, AN	Japan
Glu42Asp	GAG → GAT	Heart	France
Phe44Ser	TTT → TCT	PN, AN, Heart	Ireland
Ala45Asp	GCC → GAC	Heart	Italy
Ala45Ser	GCC → UCC	Heart	Sweden
Ala45Thr	GCC → ACC	Heart	Italy
Gly47Arg	GGG → CGG	PN, AN	Japan
Gly47Ala	GGG → GCG	Heart, PN, AN	Italy
Gly47Val	GGG → GTG	PN, AN, Heart	Sri Lanka
Gly47Glu	GGG → GAG	PN	Germany
Thr49Ala	ACC → GCC	Heart, PN	Italy
Thr49Ile	ACC → ATC	PN, Heart	Japan
Ser50Arg	AGT → AGG	PN, AN	Japan/Portugal
Ser50Ile	AGT → ATT	Heart, PN, AN	Japan
Glu51Gly	GAG → GGG	Heart	USA
Ser52Pro	TCT → CCT	PN, AN, Heart	UK/Portugal
Gly53Glu	GGA → GAA	LM, Heart	France
Glu54Gly	GAG → GGG	PN, AN	UK
Glu54Lys	GAG → GAA	PN, AN, Heart	Japan
Leu55Arg	CTG → CGG	LM, PN	Germany
Leu55Pro	CTG → CCG	PN, Heart, AN	Taiwan
Leu55Gln	CTG → CAG	AN, PN	USA
His56Arg	CAT → CGT	Heart	USA
Leu58His	CTC → CAC	CTS, Heart	Germany
Leu58Arg	CTC → CGC	CTS, AN, Eye	Japan
Thr59Lys	ACA → AAA	Heart, PN	Italy
Thr60Ala	ACT → GCT	Heart, CTS	Ireland
Glu61Lys	GAG → AAG	PN	Japan
Phe64Leu	TTT → CTT	PN, CTS, Heart	Italy
Phe64Ser	TTT → TCT	LM, PN, Eye	Italy

Thr59Lys	ACA → AAA	Heart, PN	Italy
Thr60Ala	ACT → GCT	Heart, CTS	Ireland
Glu61Lys	GAG → AAG	PN	Japan
Phe64Leu	TTT → CTT	PN, CTS, Heart	Italy
Phe64Ser	TTT → TCT	LM, PN, Eye	Italy
Ile68Leu	ATA → TTA	Heart	Germany
Tyr69His	TAC → CAC	Eye	Scotland
Tyr69Ile	TAC → ATC	CTS,Heart	Japan
Lys70Asn	AAA → AAC	CTS, PN, Eye	Germany
Val71Ala	GTG → GCG	PN, Eye	Spain
Ile73Val	ATA → GTA	PN, AN	Bangladesh
Ser77Phe	TCT → TTT	PN	France
Ser77Tyr	TCT → TAT	PN	Germany
Tyr78Phe	TAC → TTC	Heart, PN	Italy
Ile84Ser	ATC → AGC	Heart, CTS, Eye	Switzerland
Ile84Asn	ATC → AAC	Eye, Heart	Italy
Ile84Thr	ATC → ACC	Heart, PN, AN	Germany
Glu89Gln	GAG → CAG	PN, Heart	Italy
Glu89Lys	GAG → AAG	PN, Heart	USA
Ala91Ser	GCA → TCA	PN, CTS, Heart	France
Gln92Lys	GAG → GCT	Heart	Japan
Ala97Gly	GCC → GGC	Heart, PN	Japan
Ala97Ser	GCC → TCC	PN, Heart	France
Ile107Val	ATT → GTT	Heart, CTS, PN	Germany
Ile107Met	ATT → ATG	PN, Heart	Germany
Ala109Ser	GCC → TCC	PN	Japan
Leu111Met	CTG → ATG	Heart	Denmark
Ser112Ile	AGC → ATC	PN, Heart	Italy
Tyr114Cys	TAC → TGC	PN, AN, Eye	Japan
Tyr114His	TAC → CAC	CTS	Japan
Tyr116Ser	TAT → TCT	PN, CTS	France
Ala120Ser	GCT → TCT	Heart, PN, AN	Africa
Val122Ile	GTC → ATC	Heart	Africa
Val122del	GTC → loss	Heart, PN, CTS	Equador/Spain
Val122Ala	GTC → GCC	Heart, Eye, PN	UK

AN - autonomic neuropathy; CTS - carpal tunnel syndrome; Eye - vitreous deposition; PN - peripheral neuropathy; LM - leptomeningeal amyloid; Heart - cardiomyopathy

Table 2 - Non-amyloid TTR mutations and compound heterozygotes

Mutation	Codon change	Frequency*
Gly6Ser	GGT → AGT	33/558
Met13Ile	ATG → ATC	nd
Asp74His	GAC → CAC	nd
His90Asn	CAT → AAT	16/12,400
Gly101Ser	GGC → AGC	nd
Pro102Arg	CCC → CGC	1/8,000
Arg104Cys	CGC → TGC	nd
Arg104His	CGC → CAC	nd
Ala108Ala**	GCC → GCT	nd
Ala109Thr	GCC → ACC	1/10,000
Ala109Val	GCC → GTC	nd
Thr119Met	ACG → ATG	35/10,000
Pro125Ser	CCC → TCC	nd
Compound heterozygotes		
Gly6Ser/Val30Met		7/160
Gly6Ser/Phe33Ile***		nd
Gly6Ser/Ala45Asp		nd
Gly6Ser/Ser77Tyr		nd
Gly6Ser/Tyr114Cys		nd
Gly6Ser/Thr119Met		nd
Gly6Ser/Val122/Ala		nd
His90Asn/Val30Met		nd
His90Asn/Glu42Gly***		nd
His90Asn/Thr119Met		nd
Arg104His/Val30Met		nd
Arg104His/Thr59Lys		nd
Thr119Met/Val30Met		nd

* Refers to mutant allele frequency; ** Silent mutation

*** Mutations on the same allele

Non-amyloidogenic TTR mutations

Several TTR mutations without pathogenic consequences and compound heterozygotes carriers of TTR mutations have been described and are presented in Table 2 (bibliographic references can be found at: <http://www.ibmc.up.pt/~mjsaraiv2ttrmut.html>)

The allele frequency has been estimated in screening studies in different populations; this is the case of Gly6Ser present in about 12% of the Caucasian population (12) and the Thr119Met mutation found in about 0.8% of Portuguese and German populations investigated (5). Of particular importance is compound heterozygosity of non-amyloid and amyloid mutations usually occurring in different alleles. Thus, the polymorphic Gly6Ser mutation has been described in association with different amyloid mutants as documented in Table 2; this mutation does not influence the clinical outcome of Val30Met carriers (13), whereas the Thr119Met and the Arg104His mutations do. Thus, differences in clinical presentation and severity of symptoms among Portuguese and Japanese Val30Met patients carrying respectively the Met119 and the His104 mutations are observed with a clear protective effect exerted by the nonpathogenic mutation (14,15), which confer more stability to the molecule (see below). Substitutions in position 109 have been found in individuals with euthyroid hyperthyroxinemia and lead to an increase in the affinity for T4 (16).

Aggregation pathway

The three dimensional structure of TTR revealed an extensive β -sheet structure. Each monomer contains two β -sheets, composed of strands DAGH and CBEF, which interact face-to-face through hydrogen bonds between strands HH' and FF' to form a dimer (represented in Fig. 1). In the tetramer, hydrogen bonds between main chain atoms belonging to loop AB of one monomer and strand H' from the other monomer as well as hydrophobic contacts are important.

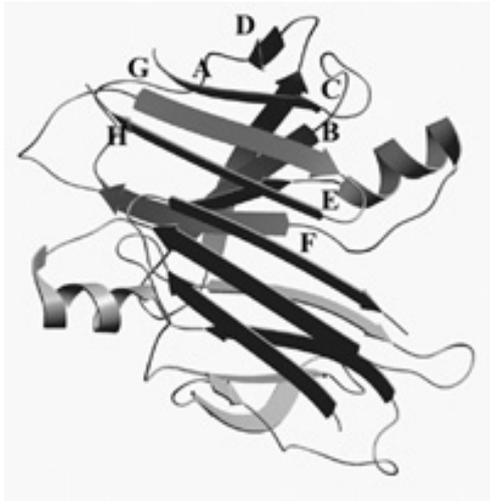


Fig. 1 Title

The effects introduced by amyloidogenic mutations have been the subject of intensive study mainly by X-ray crystallography but, with the exception of the Leu55Pro mutation, did not reveal drastic changes; so far, the solved structures point to a clear destabilization of the tetrameric structure of the protein. The structural studies by X-ray diffraction on the particularly aggressive mutant TTR - Leu55Pro revealed aggregation of monomeric TTR, consistent with data from synchrotron analyses of "ex vivo" fibrils (17); important changes in secondary structure by the disruption of strand D which becomes part of a long loop that connects strands C and E were observed. Disruption of the D strand affects the hydrogen bonding with the A strand, exposing new surfaces involved in aggregation; in particular, the contacts of the β -helix and the AB loop are different, suggesting these regions are important in amyloidogenesis. In fact, deletion or multiple substitutions in the D strand lead to highly amyloidogenic mutants (18); when monoclonal antibodies were generated against these mutants, they recognize circulating TTR form carriers of FAP mutations, pointing out that modified tetrameric species circulate in FAP patients (19). These species have a higher tendency to dissociate into monomers, which then polymerize into amyloid fibrils.

The Leu55Pro structure also pointed out for an important bond between Tyr78 and the AB loop, in stabilizing the quaternary structure. Based on this information a putative TTR mutant Tyr78Phe was constructed, leading to a highly amyloidogenic TTR recognized in its soluble tetrameric form by monoclonal antibodies specific for the amyloid fold (see above) and forming amyloid fibrils readily at neutral pH (20). In summary, mutations that destabilize the D strand, or that loose the AB loops of the tetramer and dimer-dimer interactions increase the susceptibility of amyloid formation.

Possible therapeutic strategies in FAP: TTR stabilizers and fibril disrupters

Based on our knowledge of the aggregation pathway, possible therapeutic strategies in FAP encompass either preventing dissociation of the native tetrameric structure into monomers, the building blocks of fibrils, through tetrameric stabilizers or disrupting the fibril structure through fibril disrupters.

Development of TTR stabilizers derives for most part from early work on the protective Thr119Met TTR mutation. X-ray analysis of the TTR Thr119Met:T4 complex demonstrated that this variant presents alterations in the T4 binding channel including dimer-dimer contacts. When compared to the wild type protein or to the amyloidogenic TTR Val30Met, the TTR Thr119Met variant shows new atomic interactions involving hydrogen bonds occurring within monomers, dimers and the tetramer (21). These additional interactions explain the higher stability of the variant and its protective effect over the Val30Met mutation in compound heterozygotic carriers of TTR Val30Met / TTR Thr119Met. Thus, tetrameric stabilizers can act either by binding TTR in the central hydrophobic channel that runs through the molecule, where the hydrophobic hormone T4 binds, to prevent dissociation into monomers (as documented in figure 2) or by affecting interactions within the monomer.

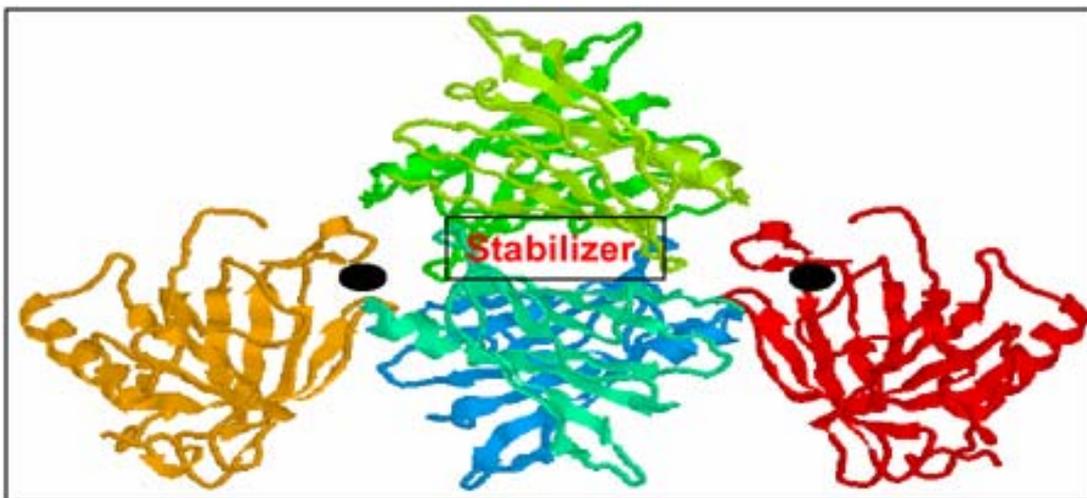


Fig. 2 – Complex of TTR tetramer and retinol binding protein with stabilizer in TTR central hydrophobic channel

Several compounds have been reported as amyloid fibril formation inhibitors, «in vitro», as is the case of some NSAIDs including flufenamic acid, diflunisal and diclofenac (22,23). For TTR amyloidoses it has been proposed that they would exert their effect through binding to T4 binding sites in TTR as demonstrated by the crystal structure of some of the complexes, in particular the TTR-diclofenac complex (23). However, all the assays reported were performed using isolated recombinant TTR «in vitro», in the absence of physiological modulating factors, namely other plasma binding proteins. Therefore, it is of most importance to characterize the interaction of the different compounds with TTR concerning binding specificity and selectivity to assure their preferential binding to TTR in plasma over other thyroxine binding proteins, in particular the highly abundant albumin and the most potent T4 binding protein of human serum, thyroxine binding globulin, TBG. Binding by proteins other than TTR leads to decreased drug availability in plasma and compromise their use as therapeutic agents for

TTR amyloidosis; this fact led to the development of novel derivatives that show TTR binding in «ex vivo» assays for T4 and for tetrameric stability. For instance, an iodinated diflunisal derivative – IDIF – has been shown to present high specificity and high binding affinity to TTR as shown in the T4 binding protein profile obtained after electrophoresis of plasma. Unlike diclofenac, diflunisal and flufenamic acid, which bound to albumin and to TBG, IDIF and BrDIF, displaced T4 preferentially from TTR (24).

IDIF disclosed not only selective TTR binding affinity but also efficiently stabilized TTR from dissociation into monomers in plasma from heterozygotic TTR Val30Met carriers and from controls. This was demonstrated by plasma TTR resistance to dissociation, after incubation with the compounds, in isoelectric focusing experiments. Similar «ex vivo» results were obtained using TTR Val30Met transgenic mouse plasma. In this "ex vivo" assay diclofenac and flufenamic acid, previously reported as inhibitors of TTR fibril formation did not seem to stabilize the TTR tetramer. Taken together, "ex vivo" and "in vitro" studies present evidence for the selectivity and efficiency of novel diflunisal derivatives as TTR stabilizers and inhibitors of fibril formation vis a vis reported TTR fibril inhibitors. The criteria of: (i) "ex vivo" TTR binding selectivity in T4 binding sites; (ii) "ex vivo" TTR tetrameric stabilization; and (iii) definition of the inhibitory step of fibrillogenesis, must be taken into consideration in further testing of drugs with therapeutic interest in TTR amyloidosis.

As for TTR fibril disrupters, the effect of the drug 4'-iodo-4'-deoxydoxorubicin (I-DOX) on the in vitro assembly of TTR Leu55Pro fibrils has been investigated by following the fibril growth over a 15 day period in the presence and absence of this drug. I-DOX did not inhibit fibril formation up to 10 days since fibrils of approximately 7- 8 nm width, identical to the fibrils present in the non-treated sample were observed. However, after 15 days of I-DOX treatment, only round particles approximately 5- 6 nm wide, resembling soluble native TTR were observed. A series of other compounds including tetracyclines, and nitrophenols have also been studied for their effects on amyloid fibril formation/disaggregation (25). Among these, doxycycline resulted in the complete disaggregation of fibrils. In addition, the species generated upon I-DOX or tetracyclines were non-toxic, as revealed by the lack of significant caspase-3 activation on a Schwannoma cell line, making this class of compounds, in particular doxycycline, of potential use in the treatment of TTR amyloidoses.

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SCREENING FOR THYROID FUNCTION?

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WHAT IS SCREENING?

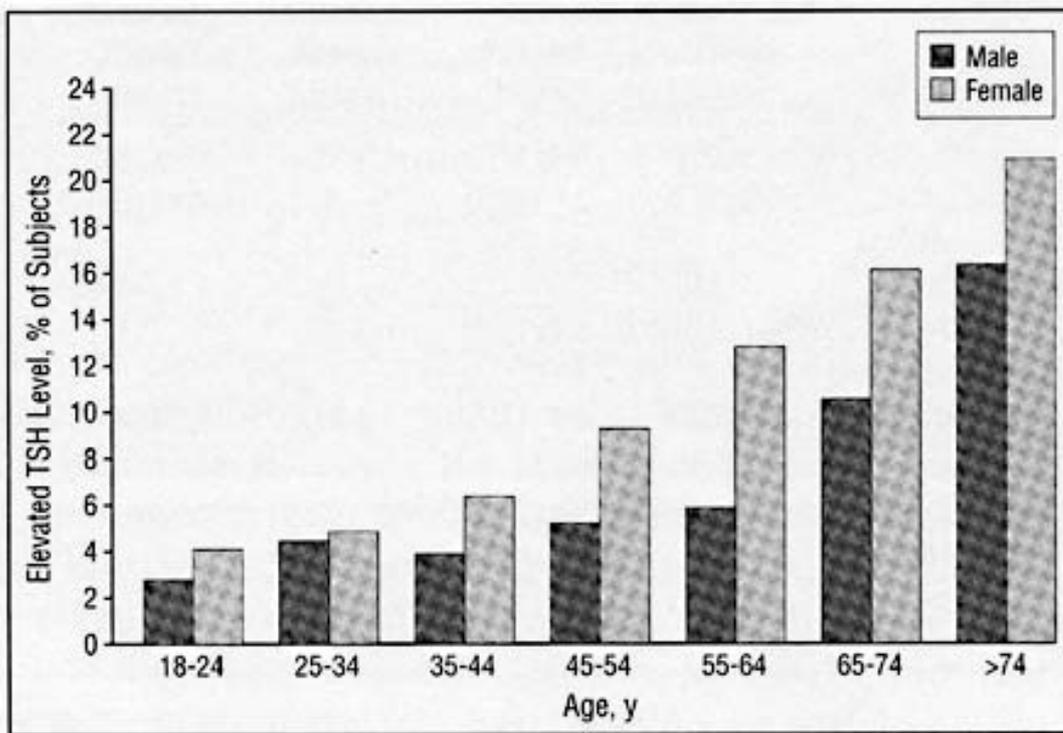
Screening was first defined as “the presumptive identification of unrecognised disease or defect by the application of tests, examinations, or other procedures which can be applied rapidly to sort out apparent well persons who probably have a disease from those who probably do not” (1). Screening is justified when 1) a disease is common and associated with serious morbidity and mortality; 2) screening tests are sufficiently accurate in detecting early stage disease, are acceptable to patients, and are feasible in general clinical practice; 3) treatment after detection by screening has been shown to improve prognosis relative to treatment after usual diagnosis; and 4) evidence exists that the potential benefits outweigh the potential harms and costs of screening (2). These criteria were recently updated by a National Screening Committee from the UK Health Department (3).

Three tiers of screening exist and must be considered in a screening programme (4):

1. The nanolevel of care delivered to an individual patient by a medical practitioner in the office or out-patient clinic
2. The microlevel of care delivered usually by an individual practitioner or practitioners within a specialty to a community population served by a district hospital
3. The macrolevel of care delivered to a whole population by a regional or national organisation.

EPIDEMIOLOGY OF THYROID DYSFUNCTION

The earliest biochemical abnormality in hypothyroidism is an increase in serum thyrotrophin (TSH) concentration associated with normal serum thyroxine (T_4) and triiodothyronine (T_3) concentrations (subclinical hypothyroidism), followed by a decrease in serum T_4 concentration, at which stage most patients have symptoms and benefit from treatment (overt hypothyroidism). The prevalence of spontaneous hypothyroidism is between 1% and 2%, and it is more common in older women and ten times more common in women than in men (5). In cross-sectional community studies, the prevalence of newly diagnosed overt hypothyroidism is 3-4 per 1000 (6,7,8). The cause is either chronic autoimmune disease (atrophic autoimmune thyroiditis or goitrous autoimmune thyroiditis (Hashimoto's thyroiditis)) or destructive treatment for thyrotoxicosis, which may account for up to one-third of cases of hypothyroidism in the community. Subclinical hypothyroidism is found in 8% of women (10% of women over 55 years of age) and 3% of men (6,7,8) (see Figure) and can progress to overt hypothyroidism, particularly if antithyroid antibody positive (9).



LEGEND TO FIGURE

The percentage of 25,682 subjects with a high serum thyrotropin (TSH) concentration, by sex and decade of age, in the Colorado Thyroid Study.

(From Canaris GJ, Manowitz NR, Mayor G, et al. The Colorado Thyroid Disease Prevalence Study. Arch Intern Med 160:526-534,2000)

The prevalence of a history of thyrotoxicosis in women is between 0.5 and 2%, and it is also ten times more common in women than in men (10). In cross-sectional community studies, the prevalence of undiagnosed thyrotoxicosis is 1-2 per 1000 (7,8) and the most common causes are Graves' disease, followed by toxic multinodular goiter, whilst rarer causes include an autonomously functioning thyroid adenoma, or subacute thyroiditis. Subclinical hyperthyroidism is defined as a low serum TSH concentration and normal serum T_4 and T_3 concentrations, in the absence of hypothalamic or pituitary disease, non-thyroidal illness, or ingestion of drugs that inhibit TSH secretion. The available studies differ in the definition of a low serum TSH concentration and whether the subjects included were receiving T_4 therapy for hypothyroidism. The reported overall prevalence ranges from 0.5% to 6.3%, with men and women over 65 years having the highest prevalence; approximately half of them are taking thyroxine (7,8,9).

RECOGNISED INDICATIONS FOR SCREENING

Screening programmes for congenital hypothyroidism were developed in the 1970s in which TSH or T_4 were measured in heel-prick blood specimens to detect this condition as early as possible. Certain groups within the population who should have an assessment of thyroid function at least once include those with atrial fibrillation, dyslipidaemia, subfertility and osteoporosis and those patients who present with a suspected goitre (5). Routine testing of thyroid function in patients

admitted acutely to hospital is not warranted unless specific clinical indications exist (11). Surveillance of thyroid function is recommended following destructive treatment for thyrotoxicosis by either radioiodine or surgery, in women with a past history of post-partum thyroiditis, patients with diabetes particularly women with type 1 diabetes, Down Syndrome and Turner's Syndrome, post neck irradiation and patients receiving lithium and amiodarone therapy (5).

POPULATION SCREENING?

Controversy exists as to whether healthy adults in the community would benefit from screening for thyroid disease (10). The prevalence of unsuspected overt hypothyroidism or thyrotoxicosis is low but a significant proportion of subjects tested will have evidence of mild thyroid failure or excess. Different recommendations and position papers have been reported by various physician organisations as to whether subclinical thyroid disease is of sufficient clinical importance to warrant screening (12-18). A recent cost-utility analysis using a computer decision model has however suggested that the cost-effectiveness of screening for mild thyroid failure compares favourably with other preventive medical practices (19). These data led the ATA to recommend population-based screening for thyroid dysfunction by measurement of serum TSH, beginning at age 35 years and every five years thereafter (20). However, a recent scientific review from representatives of the ATA, the Endocrine Society and AACE concluded that there was insufficient evidence to support population screening (21).

CRITERIA FOR POPULATION SCREENING

1. The Condition

Hypothyroidism is an insidious condition with a significant morbidity and the subtle and non-specific symptoms and signs may be mistakenly attributed to other illnesses, particularly in post-partum women and the elderly. Thyrotoxicosis has a significant short-term morbidity and long-term morbidity and mortality. Apart from progression to overt hypothyroidism, subclinical hypothyroidism may result in non-specific symptoms, dyslipidaemia and increased risk of cardiovascular disease. The possible consequences of subclinical hyperthyroidism include progression to overt thyrotoxicosis, systemic symptoms, atrial fibrillation and adverse cardiac end points and osteoporosis. The quality of evidence on the strength of association with these outcomes in subclinical hypothyroidism and hyperthyroidism has recently been reviewed (21).

2. The Screening Test

The first test in any screening programme is that which identifies those within the population who would benefit from screening. In the absence of the confounding effects of non-thyroidal illness or drugs, a normal serum TSH concentration has a high negative predictive value in ruling out thyroid disease in the healthy ambulant subject (22). Normal serum TSH concentrations may be recorded in hypothyroidism secondary to pituitary or hypothalamic disease but this is rare. In nearly all populations screened a raised serum TSH above 5mU/l is accepted as being unequivocally raised. A simple blood test is usually acceptable in most populations and is often included in a health screening process.

The standard follow-up investigation for subjects with a raised serum TSH greater than 5mU/l is a repeat measurement plus

a serum FT₄. Measurement of thyroid peroxidase antibodies in subjects with a borderline raised serum TSH that is found by screening the general population may be justified (10). If there is no intervention, then an annual test of thyroid function is warranted.

Few subjects screened will have overt hyperthyroidism but the consequences of finding a suppressed serum TSH have to be addressed when using a TSH assay. Subjects with subclinical hyperthyroidism can be categorised into those with low but detectable serum TSH (0.1-0.4mU/L) and those with a clearly low serum TSH (less than 0.1mU/L). If a subject has a low serum TSH value between 0.1 and 0.4mU/L and is not on thyroxine therapy, then the first step is to repeat the measurement together with free T₄ and free T₃ to exclude overt hyperthyroidism (and also central hypothyroidism) within one or two months. In most circumstances serum TSH will have returned to within the reference range. If the repeat serum TSH measurement remains between 0.1-0.4mU/L with normal free T₄ and/or free T₃ concentrations, then repeat testing every 12 months is all that is required. In those subjects with a serum TSH less than 0.1mU/L, free T₄ and free T₃ should be measured to exclude overt hyperthyroidism. Usually, subclinical hyperthyroidism will be confirmed, and although no consensus exists, it has been strongly argued that further diagnostic testing is warranted (21,23).

3. The Treatment

Treatment of overt hypothyroidism with T₄ is cheap and usually effective. The risks of T₄ therapy include the long-term consequences of inadequate or over-treatment with T₄. There is considerable evidence for poor compliance with T₄ therapy and community studies consistently show that 50% of treated hypothyroid patients have serum TSH levels either above or below the reference range (7,9,24). There has been some concern that T₄ given in doses that suppress serum TSH to undetectable concentrations might promote osteoporosis (25-27) or atrial fibrillation (28). In a recent observational 10-year cohort study from Birmingham, a low serum TSH (<0.05mU/l) but not a raised serum TSH was associated with an increased risk of all cause mortality and cardiovascular mortality (29).

Evidence that T₄ therapy to normalise serum TSH in subjects identified with subclinical hypothyroidism at screening improves symptoms or cardiovascular outcomes is lacking (21). There is observational data suggesting that subclinical hypothyroidism during pregnancy may be associated with sub-optimal intellectual performance in children but these are based on relatively small numbers of cases (30). Some studies have even suggested that the maternal serum free T₄ level is more sensitive than the serum TSH in predicting the likelihood of adverse intellectual outcomes in the offspring (31). No intervention data on the effect of T₄ therapy in pregnancy exist. Most clinicians (at the nanolevel) treat those patients who have both raised serum TSH concentrations and positive thyroid-antibody tests, even if symptoms are absent, provided that no contraindication is present, in view of the annual risk of developing hypothyroidism of approximately 5% (10). If serum TSH alone is raised the

annual risk of developing hypothyroidism is approximately 3% per year. The higher the serum TSH level, the greater is the prognostic significance for the development of overt hypothyroidism. No consensus exists regarding the treatment of subclinical hyperthyroidism but any potential benefits of therapy must be weighed against the substantial morbidity associated with the treatment of thyrotoxicosis.

4. The Screening Programme

No high quality randomised controlled trials of a complete screening programme for thyroid disorders exist. A case can be argued from a cost-utility analysis using a computer decision model that has assessed the consequences and costs of including serum TSH screening with cholesterol screening (19,32). It concluded that testing women aged 35 years and older with repeat serum TSH every five years for 50 years would be beneficial. The cost-effectiveness of screening for mild thyroid failure or subclinical hypothyroidism was comparable with that of other commonly performed preventive and therapeutic health practices such as hypertension, exercise, breast cancer screening and oestrogen replacement therapy in women. The costs of identifying and treating subclinical hyperthyroidism were not considered in this study.

Healthy subjects are exposed to adverse effects of screening without a guarantee of benefit and may be harmed by a screening programme. Subjects identified as having subclinical thyroid disease may suffer from the labelling effect described among hypertensive patients (33). Other costs include further investigations in those with borderline results and those who are falsely reassured and therefore fail to realise the significance of symptoms occurring later. Subjects with positive screening tests do not always comply with treatment. It is also essential that any screening programme be evaluated within a randomised controlled trial that has been designed with mortality as the outcome (5).

CONCLUSION

Screening for thyroid disease is clearly warranted in certain patient groups. Case-finding for thyroid disease in women older than 50 years or if visiting a doctor in primary care with non-specific symptoms might be justified in view of the high prevalence of mild thyroid failure. There is an urgent need for long-term studies of the effects of treatment of both subclinical hypothyroidism and hyperthyroidism, to determine if there is indeed benefit from screening for thyroid dysfunction in adults.

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THYROID HORMONE AND BONE DEVELOPMENT

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Introduction

Normal bone development and linear growth depends on the co-ordinated contributions of various genetic, environmental, endocrine and nutritional factors and continues until closure of the epiphyseal growth plate following puberty.

Thyroid hormone (T3) is essential for normal skeletal development. Childhood hypothyroidism results in growth arrest, delayed bone age, epiphyseal dysgenesis and short stature (1-3). Thyroid hormone replacement induces catch-up growth, though maximum predicted height may not be reached and any height deficit correlates with the duration of untreated hypothyroidism (3;4).

Untreated childhood thyrotoxicosis causes accelerated growth and advanced bone age with premature closure of the growth plate and short stature (5). Resistance to thyroid hormone (RTH) is associated with a variable phenotype and, although only a few patients have been investigated in detail with regard to skeletal phenotype, features including advanced bone age, delayed bone age, short stature, craniofacial abnormalities and fractures have all been described (6;7).

The molecular mechanisms of action of thyroid hormone within developing bone are currently incompletely understood. This short paper will describe the normal epiphyseal growth plate and briefly summarize available information on the role of thyroid hormone in ensuring normal skeletal development.

Bone formation and the normal growth plate

Bone formation begins when mesenchymal cells form condensations or clusters of cells (8). These cells either differentiate directly into bone forming osteoblasts (intramembranous ossification of flat bones cf. skull, scapula, ileum) or into chondrocytes that lay down a cartilage mould that is subsequently replaced by ossified bone (endochondral ossification of long bones cf. tibia, humerus, femur).

The epiphyses and metaphyses of long bones originate from separate ossification centres that are separated by a growth plate (Figure).

Figure: View the figure

Within the normal growth plate, chondrocytes are organised into layers. At the epiphyseal end chondroblast progenitor cells

occur singly and in small clusters to form the reserve zone. Progressing towards the metaphysis flattened chondroblasts undergo clonal expansion forming discrete columns that constitute the proliferative zone. These proliferative cells then lose their ability to divide and differentiate to form hypertrophic chondrocytes, which secrete type X collagen. Chondrocytes of the hypertrophic zone enlarge and finally undergo apoptosis to leave a cartilaginous framework that forms a scaffold for invading osteoblasts to lay down newly mineralised bone within the primary spongiosum. These osteoblasts are derived from bone-marrow stromal cells that enter the primary spongiosum via capillaries that advance from the adjacent bone marrow cavity.

Thyroid hormone receptors in the developing bone growth plate

Thyroid hormones act via two thyroid hormone receptors (TRs), TR α and TR β , which act as hormone inducible transcription factors (9). Each TR is expressed as multiple isoforms. TRs are normally expressed by chondrocytes of the reserve, proliferative and prehypertrophic zones, as well as by osteoblasts of the primary spongiosum (Figure). Differentiated hypertrophic chondrocytes do not express T3 receptors suggesting that reserve zone and proliferating chondrocytes are primary T3 target cells but that differentiated chondrocytes are unresponsive to T3 (10;11).

The hypothyroid growth plate

The tibial growth plate of thyroparathyroidectomized rats is disorganized with reductions in the numbers of reserve zone chondrocytes, proliferating chondrocytes and bone marrow stromal cells (11;12). Proliferating chondrocytes fail to form discrete columns and the hypertrophic zone is diminished in width and morphologically indistinct. Expression of collagen X, a specific marker of hypertrophic chondrocyte differentiation, is undetectable in the hypothyroid growth plate, indicating that hypertrophic chondrocyte differentiation is severely impaired. The growth plate is separated from the primary spongiosum by a mineralised interface, essentially sealing off the growth plate from vascular invasion and preventing further bone lengthening, leading to growth retardation. There is disruption of the normal functional continuity between maturing chondrocytes and mineralizing osteoblasts with markedly reduced osteoblast invasion and fewer, thinner bone trabeculae. An increased concentration of TR-expressing mast cells is also found in the bone marrow adjacent to this abnormal growth plate (13).

The growth plates of hypothyroid rats also have abnormal cartilage matrix deposition. Normal cartilage matrix is composed of proteoglycans containing chondroitin and heparan sulfates and hyaluronic acid residues. In hypothyroid rats, critical electrolyte staining studies using alcian blue have revealed an abnormal increase in sulfation of heparan sulfate proteoglycans in proliferating chondrocytes. This abnormal matrix is deposited in a patchy irregular fashion suggesting that thyroid hormones influence extra-cellular matrix biology as well as cellular activity of the growth plate. Treatment of these rats with thyroid hormone reverses these changes and studies have shown that this is through the direct actions of T3 on bone, and is not growth hormone (GH) mediated (12).

Tibial dyschondroplasia (TD), a disorder of broiler chickens, associated with avascular non-mineralized cartilage extending

from the epiphyseal growth plate, results from the inability of proliferating chondrocytes to undergo terminal differentiation to hypertrophic chondrocytes. This disorder has been shown to be associated with a markedly reduced expression of iodothyronine deiodinase type 2 (DIO2) in the growth plate (14). DIO2, by catalysing the conversion of T4 to T3, is the key enzyme that determines the availability of T3 to target cells, including growth plate chondrocytes. The fact that circulating thyroid hormone levels (primarily determined by activity of the DIO1 enzyme) are normal in these chickens suggests that growth plate specific hypothyroidism, caused by reduced conversion of T4 to T3 by DIO2, is a major aetiological factor in the development of TD and provides further evidence of the essential role of T3 in terminal chondrocyte differentiation. Activity of DIO2 has also been suggested in neonatal rat tibia cultures and a mouse chondrogenic cell line (ATDC5) (15). Higher levels of DIO2 activity at the later stages of development in the organ-cultured tibias again support the hypothesis that local T3 production contributes to hypertrophic chondrocyte differentiation.

Thus T3 is necessary to stimulate resting zone cells to differentiate to proliferating chondrocytes, for chondrocyte hypertrophy and differentiation and for vascular invasion of the growth plate.

The Ihh/PTHrP signalling pathway in the bone growth plate

Indian hedgehog (Ihh) is a member of the hedgehog family of secreted ligands and is a master regulator of bone development. Ihh is synthesized by prehypertrophic and hypertrophic chondrocytes (8;16).

Ihh stimulates production of parathyroid hormone-related peptide (PTHrP) from cells at the periarticular ends of bones (Figure). PTHrP acts on the PTH/PTHrP receptor (PPR) to keep proliferating chondrocytes in the proliferative pool. When the source of PTHrP is sufficiently distant the chondrocytes are no longer stimulated by PTHrP, they stop proliferating and start to synthesize Ihh. In addition, Ihh stimulates chondrocyte proliferation directly and also controls the differentiation of osteoblasts from perichondrial cells during the formation of the bone collar.

Thus interactions between Ihh and PTHrP determine the lengths of proliferating columns of chondrocytes in the growth plate and hence the pace of bone growth.

Changes in the Ihh/PTHrP signalling pathway in hypo- and hyperthyroidism

The distribution of Ihh mRNA within the proliferative, prehypertrophic and hypertrophic zones of the growth plate is similar in euthyroid and thyrotoxic rats. In contrast, in hypothyroid animals Ihh is mainly located within the upper regions of the proliferative zone and the reserve zone (11).

PTHrP mRNA expression is also altered in the hypothyroid growth plate. Levels of expression are increased and include expression by chondrocytes extending throughout the proliferative and reserve zones. In euthyroid, thyrotoxic and hypothyroid-T4 treated animals PTHrP mRNA expression is restricted to a discrete layer of prehypertrophic and hypertrophic chondrocytes.

PTH/PTHrP receptor (PPR) is also altered by thyroid status. It is expressed throughout all zones of the growth plate in euthyroid and hypothyroid animals, but is completely absent in the thyrotoxic growth plate and restricted to proliferative and prehypertrophic chondrocytes in hypothyroid-T4 treated rats.

Thyroid hormone has been shown to stimulate terminal differentiation of growth plate chondrocytes by down regulation of Sox9, a transcription factor present in cells of mesenchymal condensations and proliferating chondrocytes but not in hypertrophic chondrocytes (17). This terminal differentiation process is associated with expression of cyclin-dependant kinase inhibitors known to regulate the cell cycle checkpoint (18).

This data strongly supports a role for thyroid hormone in regulating components of the Ihh/PTHrP feedback loop in the growth plate and thus the pace of chondrocyte differentiation and bone growth.

Fibroblast Growth Factor Receptor-1 (FGFR1) as a T3-target gene in bone

FGFRs are membrane tyrosine kinase receptors that are widely expressed during embryogenesis (19;20). Three FGFRs (1-3) are known to be essential for skeletal development. Mutations of all three FGFRs can cause premature fusion of skull sutures and other variable bony abnormalities, while an activating mutation of FGFR3 is the cause of achondroplasia, the most common genetic form of dwarfism (20).

FGFR1 has been identified as a T3-target gene in osteoblasts (21). T3 acting via the thyroid hormone receptor- α (TR α) enhances FGF stimulation of FGFR1 activity. TR α ^{0/0} mice, that display a hypothyroid phenotype of delayed endochondral ossification, have abnormalities of cartilage matrix similar to those described above, namely an increase in heparan sulfate proteoglycans (22). It is known that heparan sulfate is required for binding of FGF to FGFR and for ligand-induced receptor activity (23). Therefore T3-regulated production of heparan sulfate, or modification of its structure, might be the mechanism by which T3 regulates FGFR1 signalling.

Thyroid status and mast cells in the bone marrow

As mentioned above, an increased concentration of TR-expressing mast cells is also found in the bone marrow adjacent to the abnormal growth plate in hypothyroid rats, suggesting a possible involvement of mast cells in thyroid hormone dependent endochondral bone formation (13). Mast cells and chondrocytes can communicate with one another and mast cells can regulate chondrocyte proteoglycan production and matrix deposition. Mast cells are also implicated in matrix degradation, angiogenesis, the release of bound growth factors from extracellular matrix stores and FGF signalling (24;25). A number of matrix degrading enzymes are induced by T3 and thyroid hormone is necessary for the proteoglycan degradation that occurs before endochondral ossification (26;27). Thus mast cells and thyroid hormones have the opportunity to interact on several important processes to regulate endochondral ossification and skeletal development, though the precise mechanisms for these likely interactions remain poorly understood.

Angiogenesis of the developing growth plate and thyroid hormone

Vascular invasion of the growth plate is essential for normal longitudinal bone growth (28). Angiogenesis is intimately linked to chondrocyte hypertrophy and when hypertrophy is inhibited angiogenesis and subsequent endochondral ossification is blocked (29). Thus hypertrophic, but not resting or proliferating chondrocytes, express vascular endothelial growth factor (VEGF), a key regulator of angiogenesis (28). T3 is also known to be required for normal endochondral angiogenesis, although the molecular mechanisms involved are not yet understood (11;12). This may occur via modulation of the *Ihh*-PTHrP loop to control the pace of chondrocyte differentiation, via alterations in FGF signalling or by T3-mediated degradation of the cartilage matrix to release sequestered FGFs and VEGFs.

The role of thyroid hormone in skeletal development in genetically modified mice

In order to further our understanding of the role of T3 in skeletal development a number of mouse models in which components of the T3 signalling pathway in bone have been altered have been generated. These include the *Pax8*^{-/-} mouse, a genetic model of congenital hypothyroidism due to thyroid gland agenesis, the *TR*^β^{PV/PV} mouse, a model of human RTH, a thyroid stimulating hormone receptor (TSH) knockout mouse and a number of mice with knockouts of one or more TR isoforms (30-35). The Table summarises some of these genetic models of thyroid hormone deficiency, excess and resistance in terms of effects on skeletal development and growth.

Table: Genetically modified mouse models of altered thyroid hormone signalling

Pax8^{-/-} mice have no thyroid and thus no circulating thyroid hormone (30;31). They have a severely abnormal skeletal phenotype with growth retardation and die at weaning unless rescued by replacement with thyroid hormone. This phenotype is more severe than that seen in double null *TR*^α^{0/0}*TR*^β^{-/-} mice, which have no thyroid hormone receptors, suggesting that a total lack of thyroid hormone, resulting in persistent expression of unliganded or apo-TRs, is more detrimental to skeletal development than a complete deficiency of TRs (22). Interestingly, the skeletal phenotype of *Pax8*^{-/-} mice can be partially rescued by crossing them with *TR*^α^{0/0} mice, which lack only *TR*^α receptors (but not *TR*^β^{-/-} mice, which lack only *TR*^β receptors), indicating that apo-*TR*^α is responsible for the potent detrimental effects on bone development seen in congenital hypothyroidism (30).

The predominant TR mRNA expressed in bone is *TR*^α which is present in 12-fold greater concentrations than *TR*^β (32). Although they have normal thyroid hormone, GH and IGF-1 levels, *TR*^β^{0/0} mice have a skeletal phenotype that includes failure of hypertrophic differentiation, impaired mineralization, delayed endochondral bone formation and growth retardation (22). In contrast, skeletal development in *TR*^β^{-/-} mice appears to be normal (36;37).

However, *TR*^β^{PV/PV} mutant mice, which have a point mutation in *TR*^β and autosomal dominant thyroid hormone resistance with very high circulating thyroid hormone levels have pronounced skeletal abnormalities (32). These include accelerated growth in utero with premature ossification. In the postnatal period, growth rate slows, bone mineralization increases as the

skeleton matures early and the growth plate becomes quiescent leading to shortened limb length and ultimately growth retardation. Such a phenotype is similar to that seen in childhood thyrotoxicosis and indicates that the skeleton of TR $\beta^{PV/PV}$ mice is thyrotoxic. FGFR1 mRNA expression has also been shown to be increased in the perichondrial region, growth plate and osteoblasts of TR $\beta^{PV/PV}$ mice, further evidence to strengthen the hypotheses that thyroid status regulates FGFR1 signalling in bone and that the skeleton in TR $\beta^{PV/PV}$ mice is thyrotoxic (32).

The thyrotoxic skeletal phenotype of the TR $\beta^{PV/PV}$ mice can be explained as follows. Bone is a TR α sensitive organ, whereas the pituitary is a TR β sensitive tissue. Mutations in TR β cause resistance to the normal negative feedback effects of thyroid hormone on the pituitary, resulting in elevated circulating thyroid hormone levels. These act on TR α , which is predominant in bone, to generate a TR α -mediated thyrotoxic skeletal phenotype. In contrast, TR $\alpha 1^{PV/PV}$ mice, which harbour a similar mutation in TR $\alpha 1$, have severe growth retardation and a hypothyroid phenotype, providing further support that TR α is the predominant functional TR isoform in bone.

Recently a TSHR $^{-/-}$ mouse has been generated (34). Homozygous mice were hypothyroid, underweight, growth retarded and died by 10 weeks of age. Thyroid hormone replacement at weaning normalized body weight but did not correct bone length, bone weight or bone mineral density. It was suggested therefore that TSH acts as a direct inhibitor of bone turnover. However, two alternative possibilities need to be considered. Firstly, it is possible that the mice in this study, which were analysed at seven weeks of age, had not had sufficient time following hormone replacement for catch-up growth to occur. Alternatively, untreated hypothyroidism in utero or in the first few weeks prior to weaning and hormone replacement may result in permanent irreversible effects on skeletal development and bone mineral density. At this stage it is not clear whether the skeletal effects of TSH are direct and independent of thyroid hormone or whether the influence of hypothyroidism during development irreparably impairs skeletal function in later life and the effects of thyroid hormone on bone predominate over the actions of TSH.

The future: many unanswered questions, scope for further investigation

In summary, T3 effects on the developing skeleton are complex. T3 is essential for normal skeletal development, with roles in chondrocyte differentiation, hypertrophy and angiogenesis. The skeletal response to T3 is accelerated in thyrotoxicosis and decelerated in hypothyroidism, indicating the exquisite sensitivity of the growth plate to T3 and the necessity for euthyroidism to ensure linear growth progresses normally.

There are many unanswered questions about the signals that influence endochondral bone formation. The full details of how T3 interacts with these multiple pathways and switches from the primarily anabolic role it adopts in utero and childhood to its predominantly resorptive actions seen in adulthood remains unclear. Do the actions of T3 in fetal life, childhood and early adulthood influence the development of peak bone mass? If so, could subtle genetic variations in the components of this pathway predispose to later osteoporosis? Are there sex specific differences in the role of thyroid hormone in fetal life? Newborn boys with congenital hypothyroidism are twice as likely as girls to have absent knee epiphyses at birth, suggesting that the impact of thyroid hormone on fetal skeletal maturation is gender specific (38). What are the potential mechanisms for

this?

We do not yet know the physiological function of the apo-TR in bone. Nor do we have a clear understanding of the role of TR β in bone – in mice at least TR β does not appear to play a primary role in normal skeletal development although there is evidence that it can partially compensate for the absence of TR α . We also do not know whether TR α and TR β are co-expressed in the same cells. The availability of genetically modified mice together with the development of selective TR agonists provides us with tools to investigate these questions further. GC-1 is a synthetic thyroid hormone analogue selective for binding and activation of TR β . Compared with the bone loss seen in T3-treated thyrotoxic rats, rats treated with GC-1, exhibit predictable thyrotoxic effects on organs such as the liver and pituitary but preserve their bone mass, further confirmation that TR α is the predominant isoform in bone and potentially a very useful mechanism for examining receptor/organ selective effects of T3 (39;40).

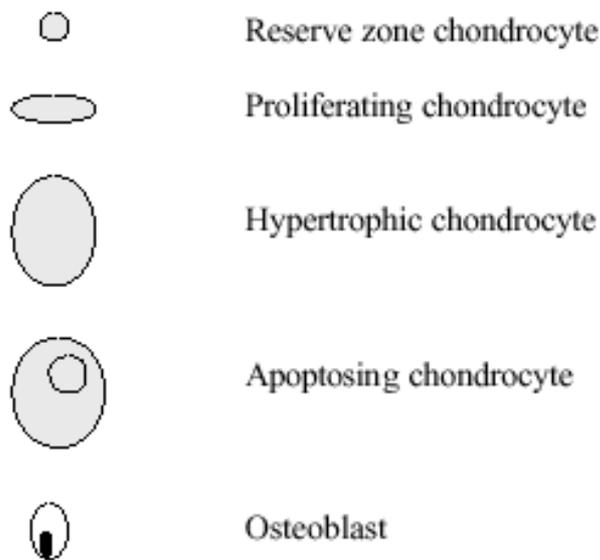
We know that liganded-TRs act as hormonal inducible transcription factors. FGFR1 has recently been identified as a T3 target gene in bone. What are the as yet other unidentified target genes? What are the molecular mechanisms that lead to their modulation by T3?

In addition to the thyroid hormone receptors, receptors for growth hormone (GH), insulin like growth factor-1 (IGF-1), and glucocorticoid (GC) are also expressed by growth plate chondrocytes (41-45). T3 influences expression of several components of GH/IGF-1 signalling in bone and can regulate hepatic 11 β -hydroxysteroid dehydrogenase, the enzyme that is responsible for maintaining circulating concentrations of GC (46). In return GC are known to regulate thyroid hormone deiodinase activity in renal tubular cells, and as the deiodinases are known to be expressed in the growth plate, this may be a mechanism whereby GC control local levels of available T3 (15;47). Understanding the mechanisms behind the interactions between the T3 signalling pathway and the systemic and paracrine effects of GH/IGF-1 and GC will be important in teasing out the molecular biology of thyroid hormone-dependent skeletal development.

Our whole outlook on the role of thyroid hormones in bone has recently been challenged by the suggestion that T3 may not be responsible for all the actions of thyroid hormones in bone and that TSH itself may have direct effects on skeletal turnover. Clarification of the relative roles of TSH and T3 in bone will provide a better understanding of bone turnover changes associated with hypo- and hyperthyroidism and the relative contributions of TSH and T3 to skeletal development.

The last few years have seen an increasing interest in the molecular actions of T3 within bone and its influence on skeletal growth and homeostasis, and this remains an emerging and exciting area of clinical and scientific research.

Legend Figure The euthyroid and hypothyroid growth plates, with a summary of T3 actions.



The anatomical features of the upper region of a long bone (a) are shown together with an expanded view of the euthyroid growth plate (b) and an outline of changes which occur in the hypothyroid growth plate (c). Reserve cells undergo clonal expansion to form columns of proliferating chondrocytes. Prehypertrophic chondrocytes differentiate into hypertrophic chondrocytes, which enlarge and finally undergo apoptosis. The resulting lacunae form the scaffold for new trabecular bone formation. Vascular invasion facilitates the migration of osteoblasts.

Also outlined is the *Ihh*/*PTHrP* negative feedback loop. *PTHrP* is secreted from perichondrial cells and cells at the end of long bones (1). *PTHrP* acts on proliferating chondrocytes to keep them in the proliferative pool and thereby delay the production of *Ihh*. When the source of *PTHrP* is sufficiently distant then *Ihh* is expressed. *Ihh* acts on chondrocytes to increase their rate of proliferation (2) and stimulate the production of *PTHrP* (3). *Ihh* also acts on perichondrial cells to convert them into the osteoblasts of the bone collar (4).

The location of various TR isoforms within bone and a summary of T3 actions in bone are also given.

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THE IMPACT OF MATERNAL THYROID DISEASE ON THE DEVELOPING FETUS : IMPLICATIONS FOR DIAGNOSIS, TREATMENT AND SCREENING.

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The title of this editorial is the title that was given to a two-day workshop jointly organized in January 2004 in Atlanta by the Center for Diseases Control and Prevention (CDC) and the American Thyroid Association (ATA) to review the available data and discuss perspectives on "maternal hypothyroidism & potential consequences for the offspring". The ultimate goal is to promote the health of mothers and their babies, as well as their ideal development during the life span. Participants were limited to a highly selected group of well known experts in the field, including endocrinologists, pediatricians, pediatric endocrinologists & psychologists, gynecologists-obstetricians, epidemiologists & experts in occupational & environmental health. Most participants were from the USA & Canada; Europe was represented by Daniel Glinoyer, John Lazarus, Gabriella Morreale de Escobar, Victor Pop and Tom Vulsma. This Editorial is not the official synopsis of the meeting but merely represents our personal views on the topics that were discussed in Atlanta during these two days.

In her opening remarks, Dr Coleen Boyle from the CDC indicated that the objectives of the workshop were to assess the prevalence of thyroid deficiency in reproductive age women, evaluate the weight of evidence to suggest that maternal thyroid disease (MTD) has an adverse impact on pregnancy outcome and child development, examine the ability to accurately detect and treat MTD, and finally consider recommendations for clinical and public health practice. When considering for instance the issue of systematic screening for MTD, C. Boyle stressed keeping in mind that in the USA 60% of pregnancies are unplanned, 16% of women do not receive prenatal care until the second trimester and 4% do not have prenatal care. No equivalent epidemiologic global data are available in Europe (to the best of our knowledge), but it is likely that the situation in our continent is broadly similar (or perhaps even worse in some countries ?).

1. The iodine nutrition status during pregnancy

Recent data from the "NHANES 3" study indicated that iodine nutrition was adequate in the overall population in the U.S. of A., with a median urinary iodine concentration (UIC) of 160 µg/L (1). However, the same national survey also showed that the iodine intake tended to be somewhat lower in women in the child bearing age, with a median UIC slightly above 100 µg/L. Furthermore and importantly, among the women aged 15-45 yrs, 5-12 % had a UIC below 50 µg/L. Therefore, and although complete data are not yet fully available, this indicates that a significant fraction of young women must have a UIC below 100 µg/L. Thus, the iodine nutrition status is probably marginally restricted or even moderately deficient in a non negligible fraction of the " to-be-pregnant " female population in the United States.

Concerning the iodine nutrition situation in Europe, several studies have confirmed the risks associated with iodine deficiency (ID) in women during pregnancy. Altogether, these studies have emphasized the need to assess the actual iodine nutritional status regionally, because mild ID tends to present as a "geographical pocket" condition, with wide variations between different areas within a given country. Conceptually, one important medical aspect of this problem is that the threshold range for ID to induce maternal thyroidal consequences (hypothyroxinemia & raised serum TSH, maternal & fetal goiter formation, consequences for fetal development, etc) is relatively narrow, corresponding to a daily iodine intake between 75-100 µg in the pregnant state (see recent Reviews in 2-4).

When feasible, primary prevention of ID is preferable. This implies, however, the provision of sufficient iodine to the general population in order for a pregnancy to start with an "at ease" situation for the maternal thyroid economy (i.e. replete

intrathyroidal iodine stores). When primary prevention is not possible (and this is usually a public health & political issue), it is necessary to employ simple ways to increase the iodine intake during pregnancy as early as possible. The Recommended Daily Allowance (RDA) is ~225 µg of iodine/day during pregnancy (5). Thus, after assessing the iodine nutritional status in any concerned area, simple but effective measures should be implemented. In Belgium for instance, 50-75 µg of iodine/day are obtained naturally from the diet. With iodised salt now freely available, and even though the salt intake ought to be restricted during a pregnancy, 4 gr of salt will bring another 60 µg of iodine/d. However, since the use of iodised salt is not mandatory in most European countries, this measure can be implemented only on a voluntary basis and hence, constant and proactive education of the public (and the doctors) is necessary. Also, it is advisable to inform pregnant women to eat more fish, etc, perhaps contributing another 10-15 µg of iodine/day. Together, therefore, a quantitatively important complement must come from multivitamin pills specifically prepared for pregnancies. Such pills have now replaced most older formulae in Belgium and they contain 125-150 µg of iodine. By combining relatively simple measures, it should be easy to reach the RDA for iodine. Finally, it is important to keep in mind that reassessments are needed to ensure that the ultimate goal has been achieved (and this is rarely done) and also to continue monitoring the iodine nutrition status in the pregnant populations. In the United States, iodine-containing multi-vitamin formulae represent only one-third of prescription prenatal vitamins and two-thirds of shelf-available preparations but, from the presently available (anecdotal) information, it seems that pregnant women may not all presently use them.

Our first conclusion from the workshop in Atlanta was that the use of iodine supplements should be promoted during pregnancy in the U.S. of A. and women advised to use multivitamin pills containing 150 µg of iodine during pregnancy. Only the future will tell how our this proposal will be implemented in the United States. Concerning most European countries where ID is even more prominent than in the U.S. of A., iodine-containing "pregnancy" pills should be made available in every country (which is presently not yet the case). Furthermore, the medical community, as well as young women, should be duly informed of the necessity to prescribe and use them.

2. The outcome of pregnancy in mothers with thyroid dysfunction (overt & subclinical hypothyroidism)

From a substantial body of data available from both retrospective and prospective studies, it appears clearly that 6-12 % of child bearing age women have thyroid antibodies, 1-2.5 % of pregnant women have subclinical hypothyroidism (SCH) and 0.3-0.5 % of unselected pregnancies have clinical hypothyroidism (CH), with/without symptoms, that are undiagnosed before pregnancy. New epidemiological data were presented at the CDC meeting by Dr Ken Leveno (from Dept Ob-Gyn., Univ. Texas). Among 17.000 women enrolled at their prenatal clinic before 20 wks gestation, their results showed that 2.5% had a supranormal serum TSH. Among them, over 90% had a normal serum free T₄ (SCH) and 10% a lowered serum free T₄ (CH). It is of interest to note that these prevalences, obtained recently in a prospective study in an iodine-sufficient area, were quite similar to those obtained ten years ago in Europe in an area with mild iodine restriction (6).

The next important question pertains to the frequency and importance of obstetrical complications in women with SCH/OH and the beneficial impact of early detection and treatment. The study from Texas (referred to above) also showed that the

prevalence of preterm birth, intensive neonatal care admission and respiratory distress syndrome were significantly increased in pregnancies with SCH & CH, with a relative risk that was almost double, compared to healthy control pregnancies. Dr Jorge Mestman (from USC in Los Angeles) also presented new data on 143 hypothyroid pregnant women. Among them, 11 were newly diagnosed cases, 35 women were known to have hypothyroidism but had stopped taking I-T₄ (corresponding to CH), and 40 women were treated for hypothyroidism but their replacement dose had not been adequately adjusted (corresponding to SCH). Their study showed that the frequency of both gestational hypertension and prematurity were markedly increased and that there was a trend for the risk of obstetrical complications to be increased with the absence of (or a delay in) re-establishing normal thyroid function.

Our second conclusion from the workshop was that the frequency and type of adverse effects on the outcome of pregnancy, related to both SCH and CH, may vary amongst different studies (mainly miscarriages, low birth weight with or without associated prematurity, gestational hypertension, and fetal death). However, there was a good consensus among the members of the panel that the evidence supporting the notion that both SCH and CH are associated with adverse effects on the outcome of pregnancy was strong. Hence, thyroid dysfunction should be detected (by thyroid function testing) and treated (with I-T₄).

With regard to I-T₄ treatment of hypothyroid pregnant women, recent data from Reed Larsen's group in Boston indicate that I-T₄ requirements are increased in almost all of them, as early as 5-8 weeks gestation, with a plateau ~50% higher reached by mid-gestation (7). These results (first presented at the 2003 ATA meeting) confirm occasional observations already reported by other investigators and emphasize the need to adjust the dosage of I-T₄ as early as possible during pregnancy, and thereafter monitor thyroid function until at least mid-gestation (we would advise until the end of the 2nd trimester).

With regard to screening, it was considered that universal screening before pregnancy was not practically feasible, except in selected high risk patients who plan for their pregnancy. Thus, thyroid function tests should be carried out as soon as possible after the first missed menstrual period and, after SCH or CH has been confirmed, the patients should be referred to an endocrinologist or at least have the possibility to contact experienced consultants (by telephone or e-mail, for instance). The panel came to the conclusion that it was probably too early to propose a systematic national screening of thyroid dysfunction in the U.S. of A., but that large prospective and randomised pregnancy studies should be undertaken to assess: a) the clinical importance of SCH; and b) whether treatment of SCH improves pregnancy outcome.

Practically, four clinical conditions can be recognized. The first is **clinical hypothyroidism** (i.e. elevated TSH & lowered free T₄ with/without thyroid antibodies).

This condition requires active treatment with ~150 µg I-thyroxine/day (2-2.4 µg/Kg/d), followed by close monitoring of thyroid function and adequate adjustment of the dosage to maintain euthyroidism. The second is **subclinical hypothyroidism** (i.e. raised TSH & "normal" free T₄ with/without thyroid antibodies). Even though the evidence is slightly less strong for SCH than for CH, it is still good enough to warrant I-thyroxine treatment in these women (8). The third is **thyroid autoimmunity (TAI)**

features with euthyroidism (i.e. normal TSH & free T₄ with thyroid antibodies). There is limited - but reasonably good - knowledge of what happens to such women when followed during gestation. A fraction of them are able to maintain euthyroidism, while the others progressively develop SCH (or even CH). The evolution depends on underlying factors that are not entirely understood, among which are the duration and intensity of the autoimmune attack, the residual functional capacity of the maternal gland to adapt, and finally perhaps also superimposed iodine deficiency (9). TAI with normal thyroid function can be diagnosed only by systematic screening programs, and arguments can be defended both in favour and against such screening. This question therefore needs further study and, in the mean time, the best attitude is probably to refer women with thyroid autoimmunity and normal thyroid function to experienced endocrinologists and use common sense to decide on whether to treat such women or simply monitor the evolution of thyroid function during later gestational stages (10). It is worth mentioning that if the need for national screening programs is presently undecided and more research obviously needed on the potential maternal and fetal consequences, one can already strive today for a better education and information of the public and medical community. Screening programs can also be organized locally (as they already are in several European hospitals nowadays). Finally, it is perhaps worth considering voluntary screening, whereby pregnant women would accept to pay the cost of measuring serum TSH, free T₄ and TPO-Ab in early pregnancy. Finally, the fourth category is the most controversial and difficult to apprehend. It concerns the pregnant women with an early (1st trimester) **low free T₄ and a strictly normal serum TSH in the absence of thyroid antibodies**. As of today, it is only safe to state that not enough is known about the reality, importance, and the underlying causes of such biochemical abnormality. More research is therefore needed to examine whether this condition truly exists, what these subjects really have and what the risks are for both the mother and offspring. Taking the lower percentile of a normal free T₄ range is, by definition, not sufficient to define a true abnormality or a disease. This can only be done within the framework of a research project to assess potentially deleterious associations with disease in such women. Studies are currently ongoing on this topic, as in the "CATS" study, recently undertaken in Wales by Dr John Lazarus. For the moment, all we can say is that we need to keep an open mind, since studies such as those of Dr Victor Pop have indicated that isolated abnormally low maternal free T₄ values (between 5-7 pmol/L) at 12 weeks gestation may be associated in some cases with a lower index of development, up to 3 years of age (11, 12).

3. Neuropsychological performance of the offspring

The study by Haddow et al., published in the New England Journal of Medicine in 1999, shows very clearly that maternal hypothyroidism, when present already in the first half of gestation and not adequately corrected thereafter, is associated with a lower global IQ in the school-age children of these mothers (13). Because of the complex processes that take place to ensure the progressive and normal maturation of the fetal brain, it is accepted that any cause of significant maternal hypothyroxinemia and, in turn, significant reduction in the transfer of maternal thyroid hormones to the fetal compartment, may be associated with deleterious and perhaps irreversible effects. What remains to be better appreciated by future studies is " what is really responsible for what " ? For instance, global IQs measured at 5-6 years of age seem to be more directly related to elevated maternal serum TSH values during late gestation, while other cognitive defects (such as poorer visual

performance or delayed responses to various stimuli, etc) seem to be more directly related with earlier (i.e. first trimester) maternal TSH alterations. Thus altogether, the evidence available today points again to the crucial importance of detecting early and treating adequately MTD.

Having reviewed the evidence, it was the feeling of the participants to the workshop that the neuropsychological consequences of maternal hypothyroxinemia in the progeny probably represent a multifactorial condition. Presently available observations may be explained in part by the obstetrical consequences associated with undiagnosed hypothyroidism during pregnancy (for instance premature delivery, gestational hypertension, lower birth weight, smaller head circumference, etc), in part by the direct consequences of an insufficient maternal transfer of thyroid hormones to the developing brain, and finally in part also by crucial environmental factors among which one factor (but it is not the sole one) is undiagnosed hypothyroidism during several years in the postpartum with all its familial implications (postnatal maternal care of the infant, psychostimulating its intelligence in the first years of life, etc). Thus in summary, our conclusions are that the evidence is presently insufficient - or unconvincing - to directly relate maternal hypothyroxinemia to the neuropsychological performances in the offspring and that further research is needed in this field.

SUMMARY

1) The iodine nutrition status during pregnancy

- A- THE MEDIAN URINARY IODINE CONCENTRATION IN THE USA HAS DECREASED FROM OVER 300 μ G/L IN THE 1970'S TO 160 μ G/L PRESENTLY.
- B- MILD TO MODERATE IODINE RESTRICTION MAY BE PRESENT IN 4-8% OF THE YOUNG FEMALE POPULATION IN THE US OF A.
- C- THE RECOMMENDED DAILY ALLOWANCE FOR IODINE DURING PREGNANCY IS 225 μ G/DAY. IN ORDER TO ACHIEVE THESE RECOMMENDED AMOUNTS DURING THE PREGNANT STATE, IN MOST OF OUR EUROPEAN COUNTRIES WITHOUT MANDATORY NATIONAL PROGRAMMES OF IODINE SUPPLEMENTATION IN THE DIET OF THE POPULATION, IT IS NECESSARY TO COMBINE DIFFERENT MEASURES, SUCH AS THE (MODERATE) USE OF IODISED SALT, PUBLIC INFORMATION ABOUT EATING PRODUCTS FROM THE SEA AND, MOST IMPORTANTLY, THE THOROUGH USE OF MULTIVITAMIN PILLS CONTAINING ADEQUATE AMOUNTS OF IODINE.

2) The outcome of pregnancy in mothers with thyroid dysfunction (overt or subclinical hypothyroidism)

- A- SIX-TWELVE % OF CHILD BEARING WOMEN HAVE THYROID AUTOANTIBODIES; 1-2.5% OF PREGNANT WOMEN HAVE SUBCLINICAL HYPOTHYROIDISM (SCH); 0.3-0.5% OF UNSELECTED PREGNANCIES HAVE OVERT HYPOTHYROIDISM (OH); AND FINALLY, A MAJORITY AMONG WOMEN WITH EITHER SCH OR OH HAVE CHRONIC AUTOIMMUNE THYROIDITIS.
- B- OBSTETRICAL COMPLICATIONS ARE ASSOCIATED WITH SCH/OH AND THERE ARE GOOD DATA TO UNDERSCORE THAT EARLY DETECTION AND TREATMENT GREATLY IMPROVE THE OUTCOME OF SUCH PREGNANCIES.
- C- THE MAIN OBSTETRICAL COMPLICATIONS ASSOCIATED WITH MATERNAL THYROID DISEASE (MTD) ARE INCREASED MISCARRIAGE RATE, GESTATIONAL HYPERTENSION AND PRETERM DELIVERY.
- A- UNIVERSAL SCREENING FOR MTD IS PRESENTLY PRACTICALLY NOT FEASIBLE. THEREFORE, DOCTORS & THE PUBLIC SHOULD BE BETTER INFORMED ABOUT THE NEED TO IDENTIFY WOMEN WHO HAVE A POTENTIAL HIGH RISK (I.E., INFERTILITY, PREVIOUS MISCARRIAGES, TYPE I DIABETES MELLITUS, JUVENILE THYROIDITIS, AUTOIMMUNE THYROIDITIS IN CLOSE RELATIVES, ETC).

3) Neuropsychological performance of the offspring

- A- THE NEUROPSYCHOLOGICAL CONSEQUENCES OF MATERNAL HYPOTHYROXINEMIA PROBABLY REPRESENT A MULTIFACTORIAL CONDITION.
- B- REDUCED IQ AND IMPAIRED NEUROPSYCHODEVELOPMENT IN CHILDREN BORN TO MOTHERS WITH MTD MAY BE EXPLAINED IN PART BY THE OBSTETRICAL CONSEQUENCES OF UNDIAGNOSED HYPOTHYROIDISM DURING PREGNANCY.
- C- THE DELETERIOUS DIRECT ROLE OF AN INSUFFICIENT TRANSFER OF MATERNAL THYROID HORMONES TO THE DEVELOPING FETAL BRAIN IN THE EARLY STAGES OF GESTATION CAN, HOWEVER, NOT BE IGNORED.
- D- FINALLY, CRUCIAL ENVIRONMENTAL FACTORS MUST ALSO BE TAKEN INTO ACCOUNT, SUCH AS THE FACT THAT MANY WOMEN WITH MTD MAY REMAIN UNDIAGNOSED FOR SEVERAL YEARS (AS WAS THE CASE IN THE HADDOW STUDY OF 1999); PROLONGED UNDISCLOSED, AND HENCE UNTREATED, MATERNAL HYPOTHYROIDISM - PER SE - MAY PLAY A ROLE IN EXPLAINING THE IMPAIRED NEUROPSYCHOLOGICAL OUTCOME IN THE PROGENY.

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STRUCTURE OF THE THYROID HORMONE AND THYROID HORMONE BINDING-PROTEINS**Vivian Cody**

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REFERENCES**Introduction**

The thyroid hormones (thyroxine or 3,3',5,5'-tetraiodo-L-thyronine [T4], and its metabolites) are synthesized and secreted from the thyroid gland and circulate throughout the body bound to transport proteins (transthyretin TTR; thyroxine-binding globulin TBG; serum albumin). They are also protein-bound when transported through the cell membrane and into the nucleus. Studies of thyroid hormones provide insight into the molecular events that control their biosynthesis, transport, and mechanism of action. Therefore, their molecular interactions with proteins are of paramount interest. Currently, structural data have elucidated thyroid hormone-protein interactions for the ligand-binding domain of nuclear thyroid hormone receptors, and the serum transport proteins, TTR and serum albumin. Structure-activity relationships among the metabolites of T4 and its deiodination products have shown that specific stereochemical features of the thyronine nucleus are crucial in determining protein binding specificity and binding affinity (Table) (1).

Analogue	TTR binding affinity (%)
L-T4	100
D-T4	2.4
T4-propionic acid	298
T4-acetic acid	676
L-T3	9
rT3	33
3,3'-T2	0.6
3',5'-T2	3

Table. Relative Binding Affinities of Selected Thyroid Hormone Analogues (1).**Thyroid hormone structure and stereochemical characteristics**

Structural data for the thyroid hormones showed that the minimal steric interaction between the tyrosyl ring 3,5 iodine atoms and the phenolic ring 2',6' hydrogen atoms is maintained when one ring is coplanar with, and the other perpendicular to, the plane of the two ether bonds (2,3). These observations showed the hormone to have a skewed conformation and the concept of preferred, if not somewhat rigid, orientations of the molecule. Thus, the 3'-iodine of the hormone 3,3',5-triiodothyronine (T3) can exist in two positional isomers - distal and proximal - depending on whether the phenolic ring iodine atom is oriented away from, or near, the tyrosyl ring (4). Activity measurements of rigid analogues revealed that a distal T3 conformation was the more active analogue (1).

Crystallographic analysis of thyroid hormones showed that a skewed diphenyl ether conformation is observed in the structures of all 3,5-disubstituted hormone analogues (2-4). As mentioned, the bulk of the tyrosyl ring substituents forces the diphenyl ether to adopt a skewed conformation, whereas removal of one of these substituents releases this constraint, permitting an antiskewed conformation (Fig. 1), as observed in the crystal structure of 3,3',5'-triiodothyronine (rT3) (5).

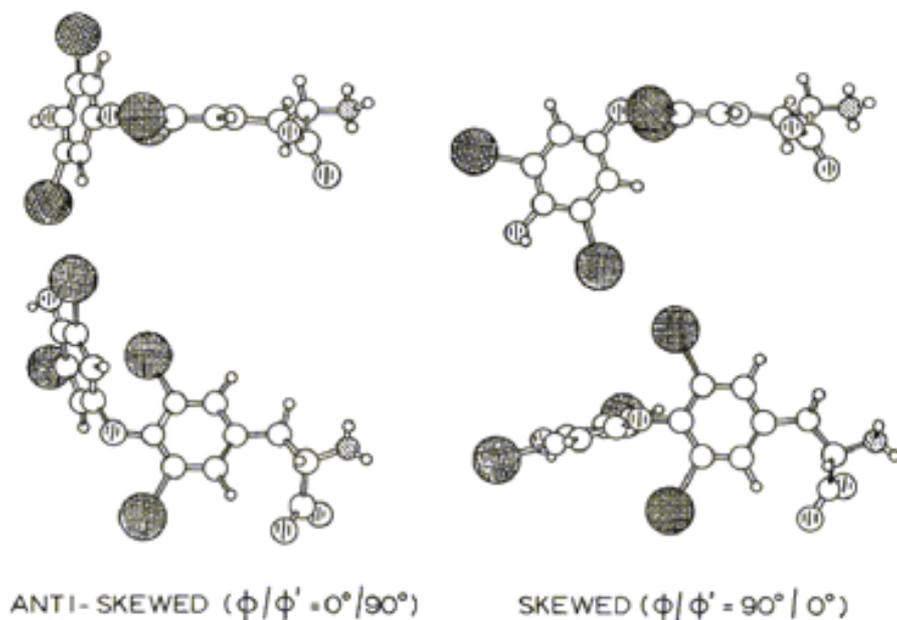


Fig. 1. Two examples of the skewed and antiskewed diphenyl ether conformations of T4. In each case the molecule is viewed parallel and perpendicular to the tyrosyl ring plane (2).

T4 cannot adopt an antiskewed conformation because the bulk of the tyrosyl ring iodine atoms would be placed into the electron density of the phenolic ring. The conformational space available to the thyroid hormone side chain is defined by three torsion angles from which only specific subsets are predicted to be favored energetically, and most of these have been observed (2,4,6). As a result of these stereochemical features, the overall hormone conformation can be either cisoid or transoid, depending on whether the phenolic ring and side chain are on the same or opposite side of the plane of the tyrosyl ring (2). These features play a role in differentiating the binding affinity of hormone analogues for their various hormone-binding proteins. For example, T4 has the strongest binding affinity for thyroxine-binding globulin, whereas the metabolite tetraiodoacetic acid (T4Ac) has the strongest binding affinity for TTR (Table 1).

Protein binding interactions

TTR

Transthyretin is responsible for the serum transport of thyroid hormone and for the binding of retinol-binding protein that binds vitamin A. TTR is a 127-amino acid monomer that forms a homotetrameric structure that has two hormone-binding domains that are partially occupied under normal physiological conditions (7,8). Structure-activity data show that thyroid hormone analogues have different binding affinities for TTR, depending on their substituent patterns (Table 1). In addition, many pharmacologic agents and natural products, such as plant flavonoids, non-steroidal analgesic drugs, inotropic bipyridines and organohalogen environmental agents, are strong competitors for T4 binding to TTR with binding affinities much greater than T4 (9). Studies of T4 displacement from TTR revealed that a synthetic plant flavonoid, EMD 21388 (3-methyl-4',6-dihydroxy-3',5'-dibromo-flavone), is the strongest competitor for T4 binding to human TTR (10,11) and showed that this T4 antagonist alters the circulating total and percentage of free thyroid hormones and serum thyrotropin concentrations (12).

To understand the structure-activity correlations observed for thyromimetic ligand binding, a number of ligand complexes with human and rat TTR have been studied (13-28). Most crystals of human TTR ligand complexes are isomorphous with the orthorhombic lattice reported for the native structure (29,30) and has two independent monomers in the asymmetric crystal lattice. The presence of the crystallographic two-fold axis through the center of the binding domains requires that the ligand either possess two-fold symmetry, or occupy two statistically disordered positions within the binding sites. Because T4 does not have such symmetry, it must bind with a statistical disorder. On the other hand, crystals of rat TTR crystallize such that there is a unique tetramer in the asymmetric tetragonal crystal lattice and these data provided the first observation of the unique binding environment for thyroid hormone (31). When structures of human TTR-T4 complexes were crystallized in space groups that did not require the crystal symmetry, binding occurred with the ligand occupying multiple positions within

the hormone binding domain (18). These data further revealed that there was significant flexibility in the binding preferences for thyroid hormones.

Ligand binding modes for TTR

Structural data for the human TTR-T4 complex (30) revealed that T4 binds in a "forward" mode, with its 4'-OH buried deep within the channel running through the tetrameric protein and has its amino acid side-chain near the channel entrance interacting with Lys-15 and Glu-54. Recently, the TTR-T4 complex crystal structure was determined as a co-crystallized hormone complex (17). These data showed that T4 binds deeper in the channel and displaces the bound water observed in the crystals soaked with T4 (30). Although the orientation is similar, the hormone is rotated such that it shares common binding sites for the 3- and 3'-iodine atoms. These data verify that T4 binding does not affect the main chain conformation significantly but results in local rearrangements of residue side-chains in the binding channel.

The orientation of the weak binding metabolite 3,3'-T2 differs significantly from that of T4 as it binds deeper in the channel than T4, and in this orientation, 3,3'-T2 occupies the binding domain in a completely different manner from T4 (15). The binding affinity of 3,3'-T2, which is 100-fold lower than that of T4, reflects the lack of the second pair of iodine atoms interacting in the channel. The thyroid hormone metabolite T4Ac (Table 1), a tight binder of TTR, shows multiple binding modes in structures with human and rat TTR (13,20). These data reveal the hormone metabolite binds with a mixed population of both "forward" and "reverse" orientations in one binding domain, and two, alternate "forward" binding positions in the second domain (Fig. 2).

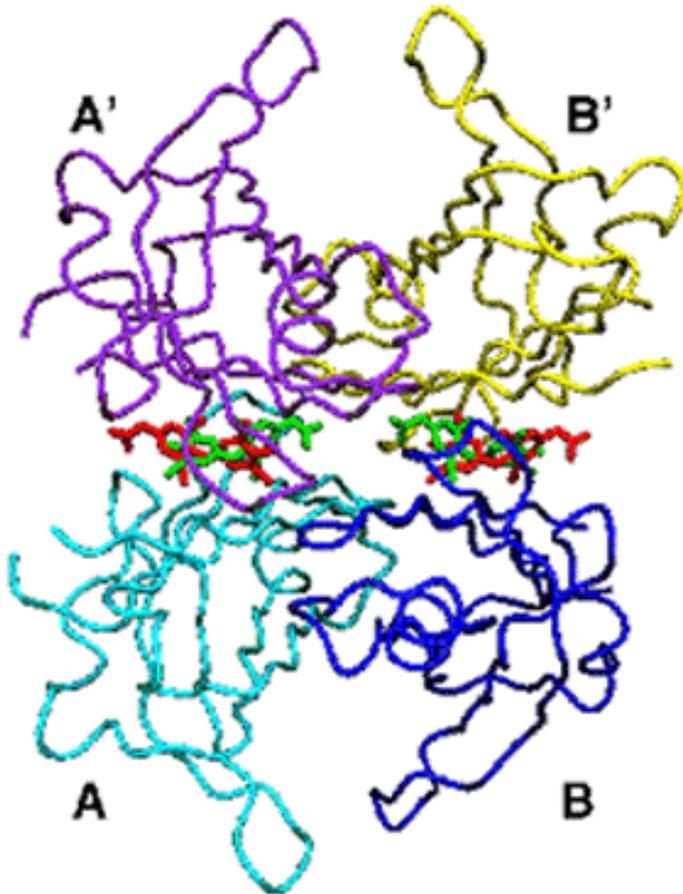


FIG. 2. Position of T4Ac (red) in forward binding mode in domains A and B of rat TTR complex (20). Also shown is the reverse binding mode of T4Ac (green) in the same complex.

The added intermolecular interactions of T4Ac, as reflected in the multiple binding contacts that result from the partial

occupancy of two different orientations, may be indicative of the tight binding affinity observed for this metabolite for TTR.

A comparison of the structures of TTR ligand complexes reveals differences in the channel geometry and in the diameter of the two hormone binding domains of TTR. The analysis of the ligand occupancy permits the identification of the primary site that binds ligand with higher affinity (22). The ligand-induced changes result in a lower binding affinity of ligand to the second binding domain. These data suggest a mechanism to explain the negative cooperativity observed in the binding of hormone to the second binding domain (7,8).

Structural results show that flavones bind to transthyretin in a manner different from T4. Data for the structure of the hTTR-EMD21388 complex revealed that bromoflavone binds deeper in the channel than T4; the bromine atoms occupy symmetric sites in a "forward" mode (that is positioned near the channel center at the tetramer interface) and in a "reverse" mode, with the bromophenolic ring near the channel entrance in TTR (13,21). In this binding, there is partial occupancy of each molecule that bind in opposite directions along the binding domain channel axis. A bromoaurone analogue binds in a similar manner (14). The observation of two alternative binding orientations for EMD 21388 may explain its greater binding affinity for TTR (11). Similar results have been observed for the organohalogens, tri- and penta-bromophenol that were observed to bind exclusively in the "reverse" mode (24).

TTR variants and amyloidic disease

Increased efforts have been made to understand the mechanisms underlying TTR tetramer stability and its relationship to the formation of amyloid fibrils that characterize amyloid diseases including familial amyloid polyneuropathy (FAP), senile systemic amyloidosis, and Alzheimer's disease (32). Recent data show that wild-type and variant TTR form amyloid fibrils that are the causative agents in familial amyloid polyneuropathy (FAP) and senile systemic amyloidosis diseases (33-44). To date, more than 70 single amino acid variants of the 127 residue monomer of TTR have been implicated in FAP disease (33); however, structural studies of TTR variants have failed to identify major differences that could explain their amyloidogenicity (35-38). Several hypotheses have been proposed based on monomeric or dimeric amyloidogenic intermediates to explain fibril formation from TTR monomers. One model proposes head-to-tail polymerization of monomeric intermediates (33,42). Another model is based on the formation of linear aggregates of TTR molecules, each linked by a pair of disulfide bonds involving Cys-10 (35,37,44). In this case, the intermediate is a dimer. A third model is based on data from two engineered amyloidogenic mutants and requires dimers that are associated by antiparallel organization of the F and H strands of the native protein. This model requires the destabilization of the tetramer prior to fibril formation (33,34). Yet another model invokes proteolytic cleavage as the initiation step in fibril formation (34,41).

Although the mechanism of tetramer stabilization is still unclear, it has been shown that ligand binding stabilizes tetramer formation in all variant species (33,39). Therefore, one means of intervention for disease treatment could involve binding of nonthyromimetic analogues that can stabilize the TTR tetramer and possibly delay the onset of fibril formation. To this end, numerous compounds have been screened (27,28,39). Structural data for the TTR complex with flufenamic acid show that the ligand mediates intersubunit hydrophobic interactions and hydrogen bonds that stabilize the normal tetrameric fold (39). Data for the TTR complex with another analgesic analogue, VCP-6, that has 6 times the affinity of T4 (45) show that the molecule forms strong hydrogen bond interactions of its 2-carboxylate with Lys-15 and with the 3,5-dichloro atoms in symmetric hydrophobic pockets near the channel center at the tetramer interface (25,26). These results suggest that the strategy of stabilization by strong competitors may prove fruitful. Comparisons of the environment near the 22 residues in the rat sequence that differ from those in human TTR also permit evaluation of their influence on hormone-binding interactions and tetramer assembly. There are small differences in the local environment of the changed residues that affect ligand binding interactions. Understanding the influence of these residue changes upon binding affinity and tetramer stability may help understand the significance of point mutations in human TTR that result in amyloid fibril formation.)

Human Serum Albumin

Human serum albumin (HSA) is the major protein component of blood plasma and serves as a transporter for T4 and other hydrophobic compounds, including fatty acids (46). Structural data show that HSA is a globular protein of 585 amino acids that is composed of three similar domains, each of which contains two subdomains (47). Structural data for the T4-HSA complex reveals that there are four T4 binding sites in HSA located in subdomains IIA, IIIA, and with two adjacent sites in IIIB (48). These data further reveal that the binding orientation of thyroxine differs in these sites with the hormone binding with a cisoid conformation in some sites and transoid in others. In addition, it was shown that myristate competes for the hormone binding sites, as the fatty acid binding sites partially overlap those of the hormone. Comparison of the crystal structures of HSA crystallized in the presence of both myristate and T4 reveals the presence of a fifth hormone binding site that is formed as a result of fatty acid-induced conformational change.

Naturally occurring mutants R218H or R218P of HSA can give rise to enhanced T4 binding affinity and are the cause of

autosomal-dominant condition of familial dysalbuminemic hyperthyroxinemia (FDH) (46). Structural analyses were carried out on the R218H and R218P mutations in complex with T4 (48). The residue R218 is located in the center of a helix near the entrance to the hormone binding site in subdomain IIA that shows conformational flexibility caused by disorder in the local environment give rise to a mixture of protein conformational states that results in a partial occupancy of the hormone binding site. These mutant albumin structures reveal that T4 binds with 100% occupancy in contrast to the 50% occupancy observed for the wild-type protein. The replacement of arginine with small side chains, coupled with movement of surrounding residues, removes the steric strain present in the native protein complex.

Nuclear Receptor Binding

Biochemical data have shown that the site of action of T3 is the nuclear receptor which is a member of the large nuclear receptor superfamily that contains a central conserved DNA-binding domain, a less conserved carboxy terminal ligand binding domain, and an amino-terminal domain that varies in size and composition among family members (46). Structural data for the thyroid hormone ligand binding domain reveal a largely helical structure with a unique internal-binding mode for T3 that is completely buried in a hydrophobic core (49). Two different thyroid hormone receptor genes (TR α and TR β) have been identified that show differential hormone binding characteristics for these subtypes of receptor. The TR α .1 product represents a functional receptor that responds to thyroid hormone, whereas the TR α .2 isoform does not bind thyroid hormone but can antagonize thyroid hormone action. The TR β .1 and TR β .2 isoforms differ in their amino termini, but both can bind and respond to thyroid hormone (50).

Structural data have been reported for rat TR α ligand binding domain in complex with the non-iodinated thyromimetic, 3,5-dimethyl-3'-isopropyl thyronine (DIMIT) (49), as well as human TR β ligand-binding domain in complex with T3 acetic acid and GC-1 analogue (50). These results identify differences in the ligand-binding pocket between the a and b subtypes involving the single amino acid difference (Ser-277 in TR α or Asn-331 in TR β) that could be used in pharmaceutical design. More recent efforts have extended this concept to the design of thyromimetics with tissue-selective actions. For example, thyromimetics have been synthesized that are selective for TR β (51) while other analogues elicit a T3 response from mutant forms of the thyroid receptor (TR β R320C) (52). These analogues suggest that such "pharmacological rescue" agents have potential for the treatment of thyroid disease.

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NEW DEVELOPMENTS IN THYROID HORMONE RESISTANCE

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1. Introduction

Thyroid hormone receptors (TRs) are ligand-dependent transcription factors that mediate the biological activities of the thyroid hormone (T3). There are two TR genes, α and β , that are located on two different chromosomes. Alternative splicing of the primary transcripts gives rise to four major T3-binding TR isoforms- $\alpha 1$, $\beta 1$, $\beta 2$ and $\beta 3$. These TRs regulate the expression of T3 target genes by binding to the thyroid hormone response elements (TREs) on the promoters. The expression of these TR isoforms is both tissue- and development-regulated (1). The regulation of the transcriptional activity of TRs is complex in that it depends on multiple factors including the types of TREs, the promoter context and many coregulatory proteins (coactivators and corepressors) (1).

Resistance to thyroid hormone (RTH) is a syndrome characterized by reduced sensitivity of tissues to the actions of thyroid hormone (2, 3). Refetoff et al. first described RTH in 1967 (4). But it was not until the tight linkage between the affected RTH family members and the thyroid hormone receptor β (TR β) gene was discovered in 1988 (5) did it become possible to study this syndrome at the molecular level. The identification of a Pro453His mutation in the TR β gene of one kindred (6) established that RTH is caused by mutations of the TR β gene. To date, about 100 different mutations in the TR β gene have been reported in more than 300 families (2, 3).

The hallmark of RTH is elevated thyroid hormone associated with nonsuppressible thyroid stimulating hormone (TSH). Other clinical signs are goiter, short stature, decreased weight, tachycardia, hearing loss, attention-deficit hyperactivity disorder, decreased IQ, and dyslexia (2, 3). The clinical manifestations vary between families with different mutations, between families with the same mutation, and also between members of the same family with identical mutations. Most patients are heterozygous with only one mutated TR β gene, and the clinical symptoms are mild (2, 3). Only one patient homozygous for a mutant TR β has been reported (7). This patient, who died at a young age, displayed an extraordinary and complex phenotype of extreme RTH with very high levels of thyroid hormones and TSH (7).

In the past decade, novel biochemical techniques and the availability of various genetically engineered mouse models have advanced the understanding of the physiological functions of TRs in vivo and the underlying molecular mechanisms for RTH. This article will focus on new developments in RTH.

2. GENERATION OF MOUSE MODELS OF HUMAN RTH: THE TR β PV MOUSE

Earlier work on the characterization of TR β mutants and elucidation of their molecular actions in RTH mainly used in vitro

biochemical methods. It soon became clear that these approaches have limitations when one tries to extrapolate to the physiological context. Such limitations led to the creation of two knock-in mouse models of RTH, one harboring a carboxyl-terminal 14 amino acid frame-shift mutation (TR β PV mouse) (8) and the other harboring a Δ 337T mutation (TR β Δ 337T mouse) (9) in the TR β gene. The two mutations identified in RTH patients were targeted to the TR β gene locus via homologous recombination. These two knock-in mice exhibit RTH phenotypes including dysregulation of the pituitary-thyroid axis and neurological dysfunction (8 -10). Consistent with phenotypes of RTH patients, TR β PV mice also exhibit growth retardation (8), abnormal regulation of serum cholesterol (11), hearing defects (12) and thyrotoxic skeletal phenotype (13). These phenotypes indicate that the TR β PV mouse is a valid model to study the molecular basis of human RTH.

2.1. Molecular mechanisms of the dominant negative activity of TR β mutants in vivo

Genetic analyses indicate that almost all RTH patients are heterozygous for the mutant TR β allele, a finding consistent with the autosomal dominant pattern of inheritance (2, 3). Early in vitro studies suggested four possible mechanisms to account for the dominant negative activity of TR β mutants: (i) formation of inactive dimers between mutant and wild-type TRs (w-TRs), (ii) competition between mutant and w-TRs for binding to TREs, (iii) competition for limited amounts of auxiliary proteins, such as the retinoid X receptors (RXRs), and (iv) stable association of TR β mutants with corepressors, resulting in repression of T3 target genes (14).

Using TR β PV mice, Zhang et al determined which of these possibilities operate in vivo (15). In the liver nuclear extracts of TR β ^{PV/+} mice, PV forms not only TRE-bound homodimers, but also TRE-bound heterodimers with w-TR β 1, w-TR α 1, or RXR. In TR β ^{PV/PV} mice, in addition to PV/PV homodimers, the lack of w-TR β 1 facilitates the formation of TRE-bound PV/TR α 1 and PV/RXR heterodimers. Therefore, in vivo, PV competes with w-TR β or w-TR α 1 for binding to TRE and for heterodimerization with RXRs (15). Such competition leads to repression of the positively T3-regulated target genes-S14, malic enzyme, and type 1 deiodinase-in the liver of TR β PV mice. These studies demonstrated that one of the molecular mechanisms by which TR β mutants exert their dominant negative activity in vivo is through competition (i) of inactive PV dimers with w-TRs for binding to TRE and (ii) of the mutant PV with w-TRs for binding to RXR to bind to TRE of T3-target genes.

2.2. The molecular basis of variable clinical manifestations in RTH

Although most heterozygous RTH patients are clinically euthyroid, some are hypothyroid and some may appear thyrotoxic (2, 3). Intriguingly, the same individual may present evidence of hypothyroidism in one tissue, while showing signs of thyrotoxicosis in other tissues (2, 3). Using TR β PV mice, Zhang et al. showed that differential expression of TR isoforms in tissues contributes to variable clinical manifestations in RTH (15). Since inactive PV/TR α 1 and PV/TR β 1 heterodimers and PV/PV homodimers compete with w-TRs for binding to TRE, in tissues, such as liver and pituitary, in which the major TR isoform is TR β 1, the positive regulated genes are repressed owing to the more effective competition of PV with w-TRs for binding to TREs. The result is tissue hypothyroidism. In tissues, such as heart and bone, in which TR α 1 is the major TR isoform, PV cannot compete effectively with the more abundantly expressed TR α 1 for binding to TREs. Thus the positively

regulated genes are activated by the elevated serum thyroid hormone in TR β PV mice, thereby resulting in a thyrotoxic phenotype (15).

Differential expression of coactivators such as the steroid hormone receptor coactivator-1 (SRC-1) also contributes to the variable clinical manifestations in RTH. Using mice from the cross of TR β PV mice and SRC-1-deficient mice, Kamiya et al. showed that lack of SRC-1 modulates the degree of resistance to thyroid hormone in a target-tissue-dependent manner and alters abnormal expression patterns of several T3 target genes in tissues (11). Thus, complex regulation of actions of TR β mutants leads to varied manifestations of RTH phenotypes.

2.3. Compensatory role of TR α 1 in heterozygous patients with RTH

Most heterozygous RTH patients are clinically euthyroid (2, 3). One possible explanation is that TR α 1 plays a compensatory role in maintaining the normal physiological functions of T3 in these patients. To test this hypothesis, Suzuki and Cheng crossed TR β PV mice with mice deficient in TR α 1 (16) and compared the phenotypes of TR β PV mice with or without TR α 1 (17). The lack of TR α 1 worsened the dysregulation of the thyroid-pituitary axis in TR β PV mice and resulted in more severe impairment of postnatal growth. Furthermore, abnormal expression patterns of T3-target genes in TR β PV mice [e.g., the TSH β , glycoprotein common α -subunit, α -myosin heavy chain, and β -myosin heavy chain genes] were altered by the lack of TR α 1. These results show that the lack of TR α 1 intensifies the manifestations of RTH in TR β PV mice. One can deduce, therefore, that TR α 1 plays an important and previously unrecognized compensatory role in maintaining the physiological functions of T3 in heterozygous patients with RTH.

3. NOVEL PHENOTYPES MEDIATED BY TR β MUTANTS

The TR β PV mouse provides a valuable model to uncover novel phenotypes due to mutations of the TR β gene. Indeed, an unexpected, but remarkable discovery was that TR β ^{PV/PV} mice, but not TR β ^{PV/+} mice, spontaneously develop follicular thyroid carcinoma with sequential capsular invasion, vascular invasion, anaplasia, and, eventually, metastasis (18). The molecular genetics of follicular thyroid carcinoma is not well understood. TR β ^{PV/PV} mice provide an unprecedented opportunity to study gene alterations that contribute to tumor progression and metastasis and to identify potential molecular targets for prevention and treatment. Using cDNA microarrays, Ying et al. have identified altered expression of ~100 genes involved in tumor induction and progression, cell proliferation, metastasis, and cell cycle regulation (19). Furthermore, these investigators further demonstrated that the repression of the signaling pathways of the peroxisome proliferator-activated receptor γ (PPAR γ) is associated with thyroid carcinogenesis (20). This finding is consistent with the frequent occurrence of the PAX8-PPAR γ fusion gene in human follicular thyroid carcinomas, its less frequent occurrence in adenomas, and its absence in papillary thyroid carcinomas (21, 22). The fusion of PAX8, a thyroid transcription factor, to the amino terminus of PPAR γ results in the loss of the transcriptional activity of PPAR γ . Moreover, PAX8-PPAR γ protein acts to inhibit thiazolidinedione-induced transactivation by PPAR γ in a dominant negative manner (21). These studies suggest that PPAR γ could potentially be tested as a molecular target for prevention or treatment of follicular thyroid carcinoma (20).

4. MUTATIONS OF THE TR α GENE DO NOT CAUSE RTH

Given the extensive sequence homology in the functional domains of TR α 1 and TR β and their similar in vitro functional characteristics (1), it is perplexing that no mutations of the TR α gene have ever been found in RTH patients. It has been postulated that mutations of the TR α gene could be embryonically lethal, inconsequential, or not associated with abnormalities of RTH. To test these possibilities, Kaneshige et al. created another mutant mouse by targeting the same PV mutation as that in TR β PV mice to the TR α gene locus via homologous recombination (TR α 1PV mice) (23). That TR α 1PV mice are viable at birth indicates that the mutation of the TR α gene is not embryonically lethal. TR α 1^{PV/PV} mice were rarely obtained and died shortly after birth. In contrast to TR β PV mice that show a hyperactive thyroid, TR α 1^{PV/+} mice do not exhibit such abnormalities. The different phenotype in the pituitary-thyroid axis of TR α 1^{PV/+} and TR β ^{PV/+} mice explains why no TR α mutations could be identified in RTH patients. TR α 1^{PV/+} mice are dwarfs and exhibit reduced fertility, survival, and glucose utilization in the brain (23-25). No such abnormalities were observed in the TR β PV mice (8, 23). The abnormal regulation patterns of T3 target genes also differed in the tissues of these two knockin mutant mice, indicating that the signaling pathways mediated by TR mutants are isoform-dependent (23). Mutations of the TR α genes clearly result in a phenotype distinctly different from that of RTH.

5. RTH WITHOUT MUTATIONS OF THE TR β GENE

A subset of patients with RTH phenotype exhibited no mutations of either the TR β or TR α gene (non-TR-RTH) (26, 27). The clinical and biochemical phenotypes of RTH patients with or without TR β gene mutations have so far been indistinguishable. Because mice deficient in SRC-1 exhibit mild resistance in the pituitary-thyroid axis (11, 28), searches for abnormalities of nuclear corepressors and coactivators were made in non-RT-RTH families. So far, no defects in several of the coregulatory proteins (SRC-1, AIB1, NCoR, SMRT and RXR γ) have been identified.

Recently, several male patients with abnormal thyroid hormone levels and severe mental retardation were reported (29 - 32). They had relatively low TT4, FT4, rT3, high TT3 and FT3, and normal or elevated TSH. These patients were found to have mutations in the monocarboxylate transporter 8 gene (MCT8; 29-32). MCT8 is a specific and active thyroid hormone transporter and is highly expressed in the liver, kidney, heart and brain (33). The association of mutations of MCT8 with severe neurological abnormalities raises the possibility that defects in the uptake of thyroid hormone could underlie abnormal brain development and functions in these patients. Furthermore, the relatively high serum T3 and TSH in these patients suggest that mutated MCT8 could mediate resistance phenotype that is independent of the mutations of the TR β gene. These observations suggest that the molecular genetics of RTH could be more complex than currently thought.

6. SUMMARY AND CONCLUSIONS

The availability of the TR β PV mouse has advanced our understanding of the molecular basis of RTH. This mouse made it possible to clarify the following clinically relevant issues that previously could not be studied *in vivo*.

- One of the mechanisms underlying the dominant negative activity of TR β mutants involves competition of inactive mutant with w-TRs for binding to TREs on the promoters of T3 target genes.
- The dominant negative activity of TR β mutants is modulated by multiple combinatorial factors including the differential expression of TR isoforms and coactivators in target tissues. This complex regulation underlies the varied clinical manifestations in patients with RTH.
- No mutations of the TR α gene have ever been found in RTH patients. This is because mutations of the TR α gene lead to phenotypes distinct from those of RTH.
- In addition to the mutations of TR β genes, other genetic events yet to be identified could also cause RTH.
- The TR β PV mouse provides an opportunity to uncover novel phenotypes due to mutations of the TR β genes, for example, the discovery of thyroid follicular carcinoma in TR β ^{PV/PV} mice.

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FUNCTION RELATIONSHIPS OF DEIODINASES

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Three deiodinase enzymes are involved in regulation of thyroid hormone availability

Three deiodinases regulate local and systemic availability of thyroid hormones, which are produced and secreted by the thyroid gland in higher vertebrates including humans. The physiological role of the deiodinases has been reviewed in recent editorials of this journal (1;2). Deiodinase enzymes are intracellular, integral membrane proteins, which either activate the prohormone L-thyroxine (T₄) to the thyromimetically active 3,3',5-triiodo-L-thyronine (T₃), a reaction catalyzed by the type I (5'DI) and type II 5'-deiodinases (5'DII) , or which inactivate T₄ or T₃ by generation of iodothyronine metabolites such as 3,3',5'-triiodo-L-thyronine (reverse T₃, rT₃) from T₄ or 3,3'-diiodo-L-thyronine from T₃, reactions catalyzed by the Type III 5-deiodinase (5DIII) and the type I 5'-deiodinase (3). The latter enzyme has limited, promiscuous substrate specificity. The type I and type III enzymes are also involved in further degradation of iodothyronines to other di- or mono-iodinated metabolites, which similar to rT₃ and 3'-T₂ do not bind to T₃-receptors (TRs), the ligand activated transcription factors. Sulfated T₄ metabolites are solely deiodinated by the type I 5'-deiodinase (4).

Thyroid hormone availability is regulated in time, space and concentration

The three deiodinases show a remarkably specific expression pattern with respect to developmental stage, tissue and cell types, that provides the basis for a sensitive fine tuning of T₃ provision to target cells in concert with the expression and activity of several specific transporters (5;6) for the charged amino acid-derived thyroid hormones. Recent data suggest distinct compartmentalization of deiodinase-, transporter- and TR-expressing cells, especially in the developing organisms but also in adult differentiated tissues and individuals (7;8). For example glial cells and tanocytes expressing the T₃ generating 5'DII are localized adjacent to TR-positive neurons which also express the T₃ degrading 5DIII (9). Similar cell type-specific and time-dependent distinct expression patterns and levels of activity have been observed for the three deiodinase enzymes during placental implantation of the embryo (7), in human placental layers (10;11), or during the amphibian metamorphosis (1). 5DIII expression is assumed to limit T₃ availability and to prevent exposure of target cells to T₃ inappropriate in concentration, location and time in developing and adult organisms (12). Thyroid hormone levels and deiodinase expression are also modified by several drugs as well as by hypo- and hyperthyroidism, which differently affect the three deiodinase enzymes (13). Apart from cellular proteins involved in modulating thyroid hormone availability, serum contains the highly specific thyroid hormone transport, distribution and binding proteins thyroxine-binding globulin (TBG), transthyretin (TTR), albumin and some lipoproteins, which have evolved to generate a buffer system for the highly hydrophobic iodothyronines, covering almost 6 orders of magnitude in their binding affinity (K_a for T₄ range from 10¹⁰ to 10⁵ M⁻¹).

5'DII knockout mice are viable and show remarkably mild phenotype

All three deiodinases belong to the small and exclusive class of selenocysteine-containing proteins (3). Selenocysteine, the 21st proteinogenic amino acid, located in the active site of these enzymes, is

essential for their proper function (14). Recently, models of mechanism of reaction and inhibition by the antithyroid drug PTU have been proposed based on the active site selenocysteine of the 5'-deiodinase enzymes (15;16). So far no reports on major genetic defects associated with deiodinase mutants have been reported for humans but polymorphisms have been linked to altered thyroid hormone metabolism (see below). Knockout mouse models for the single deiodinase enzymes revealed markedly mild phenotypes (17), suggesting some redundancy and/or functional compensation by the unaffected family deiodinase members, thyroid hormone transporters and/or T3-receptors. This is somewhat reminiscent of many TR β -receptor mutants and some TR-transgenic mouse models, which also indicate a complex network of fine-tuned fail-safe control mechanisms of thyroid hormone action even in the case of thyroid hormone resistance (18). In mice the locus of the 5DIII gene appears to be genomically imprinted and preferentially expressed from the paternal allele and might be linked to phenotypic abnormalities associated with uniparental disomy (19). Targeted disruption of the 5'DII gene in the mouse model reveals serum hormone constellations similar to those observed in pituitary resistance to thyroid hormone with elevated T4 and TSH, normal T3 and altered tissue levels of type 5DIII activities (17). In addition, an impaired thermoregulation has been observed, compatible with 5'DII expression in brown adipose tissue of rodents. No full report on the phenotype of knockout mouse models for 5'DI or cross breeding of the individual knockout mouse models has been published yet. Inbred mouse strains with impaired 5'DI expression due to CGT repeats in an altered promoter structure of this gene have normal serum TSH and T3, but elevated rT3 and free T4 levels (20), again indicating the potential of functional compensation (21) for impaired hepatic, renal and thyroidal expression of 5'DI, even under conditions of streptozotocin-induced diabetes in this mouse strain (22).

Non-thyroidal illness, the low-T3 syndrome and the deiodinases

Impaired hepatic production of T3 by 5'DI has been one of the hallmarks of this dazzling syndrome frequently encountered in several clinically relevant conditions such as starvation, infection, sepsis, major trauma, and chronic non-thyroidal illness but also after administration of several drugs (23;24). The exact molecular mechanism of decreased hepatic expression of 5'DI has been elusive for three decades of research. Therefore no rationale for thyroid hormone treatment of these patients was given, as indicated by the alternatively used term euthyroid sick syndrome (25). Only few studies actually showed decreased tissue T3 (and T4) levels in humans (26). Remarkably, impaired 5'DI expression has been mainly observed in the liver, while 5'DI activities in the kidney, thyroid and pituitary were rarely affected in most animal experimental models mimicking this human constellation. Selenium deficiency which, due to low serum selenium levels would limit hepatic expression of the selenoenzyme 5'DI could be ruled out as one potential factor contributing to the low T3 syndrome (27;28). Strong evidence for inhibition of 5'DI by proinflammatory cytokines, frequently elevated in serum of these patients, has been presented in several animals and cell culture models and in patients, and even strong inverse correlations to serum T3 levels in patients were demonstrated (24;29;30). Interleukins 1 and 6 (IL-1, IL-6), tumor necrosis factor α (TNF α), and Interferon γ inhibit 5'DI expression and activity by transcriptional and post-transcriptional mechanisms involving activation of the transcription factor NF- κ B, limiting the amount of the nuclear receptor coactivator SRC1 or directly inhibiting 5'DI promoter activity (31). However, many of the later effects are not limited to hepatic 5'DI expression, but also inhibit the enzyme in the kidney, and the thyroid, or even stimulate 5'DI in the pituitary. This indicates a complex disturbance of the homeostasis of thyroid hormone deiodination pathways, an observation supported by studies in several mouse knockout models where individual components of the cytokine network were inactivated (32). A promising recent observation in critically ill patients provides novel insight into an additional mechanism contributing to the pathogenesis of the low T3 syndrome and also offers an explanation for the elevated rT3 levels found in these patients: Their liver re-expresses type III 5-deiodinase similar to the fetal and neonatal organ, while livers of healthy adult mammals including humans solely express 5'DI activity (33). Hypoxia has been suggested as the triggering signal for 5DIII re-expression, reminiscent of high 5DIII expression in some hemangioma (see below) (34).

Deiodinases in tumors

5'DI:

5'DI is a sensitive marker for the differentiation stage of several epithelial tumors. E.g. while 5'DI is

highly active in the healthy thyroid gland, its activity is reduced in differentiated thyroid carcinomas and very low or even undetectable in anaplastic thyroid carcinoma or corresponding cell lines, accompanied by a decrease in the expression of the 27 kDa substrate binding catalytic subunit (35). Using DNA microarray technology, Huang et al. (34) demonstrated a down-regulation of 5'DI mRNA in papillary thyroid carcinoma vs. normal thyroid and Barden et al. (36) in follicular thyroid carcinoma vs. follicular adenoma. Expression of the deiodinase gene also loses responsiveness to physiologic stimulation by TSH and T3, but remains sensitive to retinoic acid in differentiated thyroid carcinoma cell lines, an effect not observed in normal thyroid cell lines, but detectable also in the hepatocarcinoma cell line HepG2 (37). In anaplastic lines, also RA responsiveness is lost.

5'DI expression is also reduced in prostate carcinoma (38), in clear cell carcinoma of the kidney, provided that these tumors do not derive from a cell type that does not express 5'DI (39), and in liver adenocarcinoma, although only one sample was compared to normal liver (40). In the mammary gland of the rat, 5'DI is up-regulated during lactation by suckling-triggered prolactin and β -adrenergic stimulation (41). In 1-methyl-1-nitrosourea (MNU)-induced mammary cancer of female Sprague-Dawley rats, 5'DI activity was at least two orders of magnitude higher than in normal non-lactating mammary gland (42). In striking correspondence to other epithelial carcinoma cell lines, 5'DI gets responsive to RA in the human mammary carcinoma line MCF-7, whereas regulation by physiologic stimuli, T3 or the β -adrenergic agonist isoproterenol, is abolished. In the more dedifferentiated breast cancer cell line MDA-MDB-231, retinoic acid stimulation is not observed (43).

Taken together, these results show a tight linkage of 5'DI expression and regulation to the differentiation stage of various epithelia, suggesting a central role for the enzyme in the proper function of these tissues.

5'DII:

Disturbed activities of 5'DI and 5'DII in the pituitary gland would impair hormonal feedback regulation. As reported by Baur et al. (44), 5'DII activity was higher than 5'DI activity in 40 of 43 adenomas and 2 of 3 normal pituitaries, whereby in 15 tumors, only 5'DII and no 5'DI activity at all was observed. In three adenomas and one normal pituitary, there were higher 5'DI than 5'DII levels. Tannahill et al. (45) reported that pituitary tumors expressed 2.6-fold higher 5'DII and 6.5-fold higher 5DIII mRNA levels as compared to normal pituitaries. 5'DI transcripts were only detectable in a minority of the tumors, and in these cases, overexpression was determined. A correlation between deiodinase mRNAs and enzyme activities that was observed in normal pituitaries was lost in the tumors.

Both 5'DI and 5'DII mRNAs and enzyme expression may be enhanced in benign thyroid diseases, i. e. Graves' disease and thyroid adenomas (46-48).

Huang et al. (49) also reported down-regulation of 5'DII mRNA in PTC, and 5'DII activity is increased in thyroid adenoma, but reduced in papillary thyroid carcinoma (50). Kim et al. (51) recently described increased T3 formation from either endogenous or plasma-derived T4, resulting in a persistently increased ratio of serum T3 to T4, which most probably was due to 5'DII expression in follicular thyroid carcinomas. This was deduced from kinetic parameters and from the observation that the elevated serum T3 was not decreased after administration of the 5'DI inhibitor PTU, respectively. A remarkably high expression of 5'DII has been observed in the mesothelioma cell line MSTO-211H, facilitating the further characterization of this enzyme as a selenoprotein (52). It will be of interest, if primary mesotheliomas also express 5'DII; the transformed mesothelial cell line MeT-5A does not.

5DIII:

Hepatic or cutaneous hemangioma affects 5 to 10 % of one-year-old children. These tumors often express very high levels of 5DIII which mediate enhanced turnover of T3 that cannot be balanced by the synthetic capacity of the thyroid ("consumptive hypothyroidism"). In an adult patient, increased 5DIII activity in a hemangioma caused subclinical hypothyroidism (34;53).

Single nucleotide polymorphisms (SNPs) in deiodinase genes

SNPs in the 5'DI gene associated with altered thyroid hormone metabolism:

So far no deiodinase mutations have been discussed to be involved in thyroid diseases. However, several SNPs have recently been described for the 5'DI gene (D1a-C/T at position 785 and D1b-A/G at position 1814 of the 5'DI cDNA sequence) that were associated with plasma TSH and iodothyronine

levels in a population of 158 healthy persons (54). Interestingly, these SNPs affect the 3'-untranslated region in the vicinity of the selenocysteine insertion element which facilitates the insertion of selenocysteine, encoded by a UGA codon, into the catalytic center of the selenoenzyme 5'DI. The T allele of D1a was very frequent and associated with higher plasma rT3 levels, with a corresponding 33 % increase per allele copy, higher plasma rT3/T4 ratios and lower plasma T3/rT3 ratios. The A allele of D1b correlated with decreased plasma rT3 levels, decreased plasma rT3/T4 ratios and increased plasma T3/rT3 ratios, although this was not significant due to the low frequency of this polymorphism. Of the three haplotype alleles observed in this population, namely 1: aCbA, 2: aT-bA and 3: aC-bG, haplotype 2 was positively correlated with rT3, rT3/T4 and negatively with T3/rT3 ratios, whereas haplotype 3 was negatively correlated with rT3, rT3/T4 and positively with T3/rT3, but this was not significant. Haplotype 1 did not show any correlation to serum hormone levels. A T/G polymorphism in the 3'-untranslated region of DIII also was not correlated with plasma hormone levels.

Thus, SNPs in 5'DI may well be the cause for subtle variations in thyroid hormone levels, which nevertheless can have consequences for quality of life, cognition, heart rate and other physiological processes under the control of thyroid hormones and for set points of endocrine feedback regulation.

5'DII as a candidate gene for Syndrome X:

A SNP within the protein coding region of the 5'DII gene was recently described, featuring either Thr or Ala at amino acid 92. As 5'DII contributes to the feedback-regulation by thyroid hormone in the thyroid gland, a correlation with serum TSH levels is conceivable, but was not observed in the study mentioned above (54).

However, in the context of the so-called Syndrome X, the complex of obesity, hypertension, insulin resistance and glucose intolerance/diabetes, this polymorphism may be of importance (55). The allele frequency of this SNP was 0.35 in a population of morbidly obese caucasians, 0.72 in Pima Indians and 0.42 in Mexican-Americans. Association studies in 135 nondiabetic women showed that the Ala variant was correlated with a lower glucose disposal rate, and a trend to higher fasting insulin levels was also observed. In a large study group of 972 nondiabetic subjects recruited for energy balance studies, there was no correlation of the Ala variant with body weight or body mass index. However, carriers of an additional Trp64Arg polymorphism affecting the β 3-adrenergic receptor, showed significantly higher weight and BMI.

These data strongly suggest an association of the Thr92Ala mutation in the 5'DII gene with glucose intolerance and diabetes, suggesting a role of local T3-production by 5'DII in the regulation of energy metabolism. This would result in a vicious circle: reduced T3 leads to reduced expression of GLUT4 in insulin-sensitive and 5'DII expressing tissues such as skeletal muscle or adipose tissue. As 5'DII is stimulated by cAMP via a cAMP-responsive element in its promoter, this condition might be further aggravated via reduced stimulation by a variant β 3-adrenergic receptor, leading to a decrease in cAMP-stimulated expression of an already functionally impaired 5'DII enzyme.

The association between the Thr92Ala SNP and glucose tolerance/diabetes, however, is challenged by the observation that the Thr92Ala 5'DII variant, expressed in vitro, did not show any difference in the kinetic parameters of T4 deiodination as compared to the wildtype enzyme. If the discussed correlation can be explained, e. g., by association with another polymorphism, will have to be clarified in further studies.

Table: Clinically relevant factors regulating and modulating deiodinase function

	Type I 5' Deiodinase	Type II 5'Deiodinase	Type III 5Deiodinase
Proinflammatory cytokines	▼ liver ▲ pituitary		
selenium	▲	▲	▲
hypoxia			▲ liver
TSH, cAMP		▲	
T3	▲		
Genomic imprinting			▼
tumorigenesis	▼	▲	
SNPs	▼▲	?	?

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The physiological and clinical relevance of the TSH Receptor in the anterior pituitary

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I. INTRODUCTION

Although there usually is a good negative correlation between free T4 and TSH levels, there are some notorious exceptions in which the feedback system between fT4 and TSH seems to be disrupted. Most clinicians will be aware of the fact that TSH levels can remain low, despite clinical euthyroidism and normal concentrations of T4 and T3, in patients treated for Graves' hyperthyroidism.¹ This situation is attributed to a delayed recovery of the pituitary-thyroid axis after a prolonged state of thyrotoxicosis.²

This explanation seemed to us unlikely for various reasons. First, TSH levels increase within weeks after discontinuation of T4 therapy in patients treated with TSH suppressive doses of T4 for thyroid cancer, suggesting that the pituitary-thyroid axis can revive fast. Secondly, not all patients treated for Graves' hyperthyroidism show this phenomenon of long-term TSH suppression. And thirdly, a decreased level of TSH after a one year course of antithyroid drugs is an independent risk factor for recurrence of hyperthyroidism upon discontinuation of antithyroid drugs.³ These clinical observations suggest that the long-term TSH suppression in Graves' disease is a specific feature of a subset of Graves' patients: those likely to relapse after antithyroid drug therapy, e.g. patients with persisting TSH Receptor Stimulating Immunoglobulins (TSI).

We hypothesized that TSI are responsible for the prolonged TSH suppression observed in a subset of Graves' patients. The implication would be that TSI could decrease TSH secretion independently of thyroid status, at the central (hypothalamus/pituitary) level. Because the pituitary gland is outside the blood-brain barrier and the hypothalamus is not, we postulated that the TSI may act on the pituitary level decreasing TSH secretion. A requisite for this hypothesis is that the pituitary contains a TSH R.

II. PHYSIOLOGICAL RELEVANCE OF A PITUITARY TSH-RECEPTOR: A PITUITARY ULTRA-SHORT LOOP FEEDBACK.

The hypothesis of the existence of a pituitary TSH-Receptor implies that this receptor might have a function in human physiology, since it would be present in all subjects and not only in Graves' patients. What would the physiological role of such a receptor be? If present, this TSH-Receptor might sense directly the pituitary TSH secretion. This is logical in the sense that such a receptor would enable fine-tuning of pituitary TSH secretion.

In physiological circumstances a decline in T4 production by the thyroid would be sensed in the pituitary, because of the negative feedback system between T4 concentrations and TSH production. Such a decline would be followed by an increase in TSH production leading to a stimulation of the thyroid gland to produce more thyroid hormones. However, there is a certain lag-time between the increase in TSH and a rise in plasma T4 and T3 levels. If during this lag-time TSH would remain

elevated, an overshoot in thyroid hormone production will ensue. This would not occur, if the pituitary would be able to anticipate this effect of TSH on the thyroid gland, for instance by measuring its own TSH output. For this it would need a TSH-Receptor.

The hypothesis thus is that secreted TSH binds to an intrapituitary TSH-R, which upon activation then signals back (e.g. via a cytokine) to the thyrotroph to diminish its TSH secretion in an ultra-short loop feed-back system. Such a short-loop feedback is not limited to TSH secretion. Prolactin receptors,⁴ and GH Receptors⁵ have also been found in the pituitary, and evidence is accumulating that PRL and GH down-regulate their own secretion.^{6,7}

III. THE TSH-RECEPTOR IS PRESENT IN THE PITUITARY

We therefore embarked on a series of studies to demonstrate the presence of the TSH-R in human pituitaries. We first showed by RT-PCR the presence of mRNA encoding for the receptor in a human anterior pituitary library. This was confirmed by using in situ hybridization on human anterior pituitary slices. The presence of TSH-R protein was demonstrated using immunohistochemistry on human anterior pituitaries and using double labeling techniques we could show that the TSH-R was expressed by folliculo-stellate cells (Fig. 1).⁸



Fig.1

These findings were later confirmed by others, who also found that folliculo-stellate cells contain TSH-R protein.⁹ These folliculo-stellate cells make up 10% of the pituitary cell population. They are a kind of dendritic cells and thought to have a function in the signalling with the other pituitary cell populations.¹⁰ Now that we know that they express the TSH-R, their function becomes more clear in that they possibly form part of the ultra-short loop feed back on TSH secretion. It is likely that they are also involved in a paracrine regulation of the secretion of other hypophyseal hormones, because we later showed that they also contain mRNA coding for the GH receptor and the ACTH receptor (manuscript submitted).

IV. CLINICAL RELEVANCE OF THE PITUITARY TSH-RECEPTOR

As mentioned, the pituitary lies outside the blood-brain barrier and this means that TSI may interact with this pituitary binding

site. We postulated that the physiological ligand, TSH, would downregulate TSH secretion to some extent and hence TSI would do the same (Fig 2).

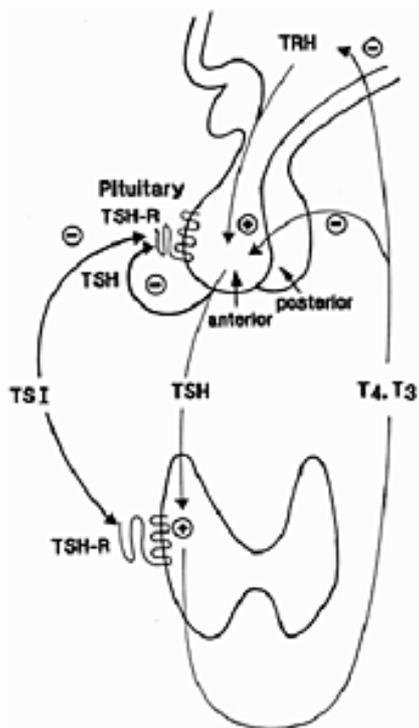


Fig. 2

To test this hypothesis, we treated rats with methimazole and thyroxine (mimicking the block and replacement therapy used in Graves patients) to switch off their thyroid glands, while maintaining euthyroidism. The rats were then infused with human TSI containing IgG's or a control human IgG preparation, in some ways as in the old LATS assay. TSI infusion indeed resulted in a significant decrease in TSH concentrations compared to controls, without differences in T4 or T3 levels between both groups (Fig 3).¹¹

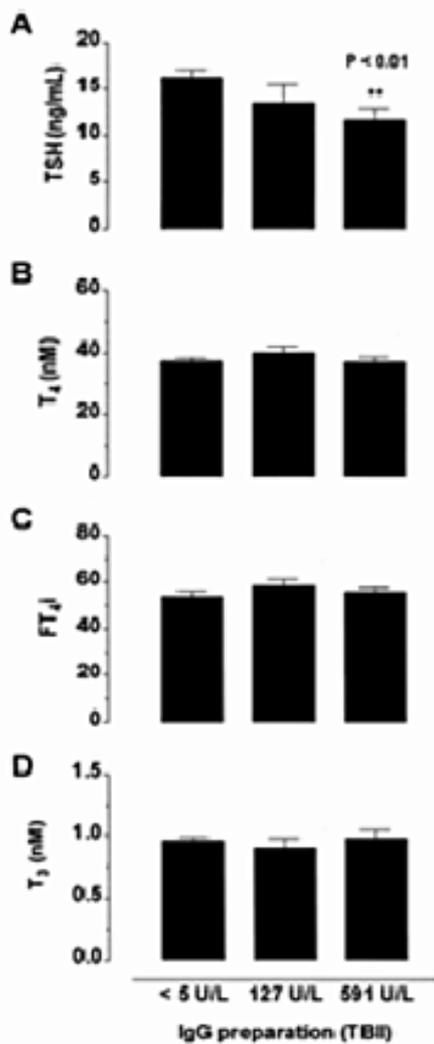


Fig. 3

This study thus showed that TSI is capable of reducing TSH secretion by rat pituitaries independently of thyroid hormone concentrations, indicative of a direct action of TSI on the pituitary TSH-R.

We then put our hypothesis to the proof in patients with Graves' disease treated with block and replacement therapy. We followed a cohort of 45 patients who were rendered euthyroid by methimazole and thyroxine therapy. Three months after having achieved biochemical euthyroidism (defined as normal free T₄ and total T₃ levels), 22 patients still had detectable TSI (measured as TSH Binding Inhibiting Immunoglobulins, TBII assay) levels, whereas in 23 patients TBII levels had become negative. We then compared these two groups, which had similar free T₄ and total T₃ levels by that time, for their TSH values. The TSI positive group had significantly lower TSH values than the group who had become TSI negative (Fig 4).¹²

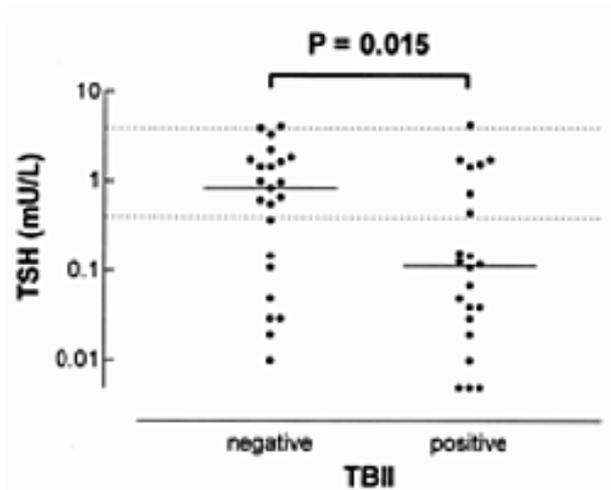


Fig. 4

In addition, we found that TSH levels in these treated Graves' patients only correlated with TBII titers and not with free T4 or total T3 concentrations. These observations clearly support our notion, that TSI suppress TSH secretion via a direct central effect, most likely at the pituitary level when euthyroidism is restored.

V. CONCLUDING REMARKS

Long-term TSH suppression during otherwise successful treatment of Graves' disease has always been attributed to a delayed recovery of the pituitary-thyroid axis. Less experienced clinicians regard it as proof for still existing "subclinical" hyperthyroidism and act accordingly by increasing the methimazole dosage or decreasing T4 substitution.

The above mentioned experiments have clearly shown that prolonged TSH suppression is very likely to be caused by an interaction between the pituitary TSH-R and circulating TSH-R autoantibodies, which can remain present in about half of treated Graves' patients. Low TSH levels in clinically euthyroid patients with normal T4 and T3 levels thus do not indicate persisting low-grade hyperthyroidism, but should instead be seen as an indication for continued TSI activity.

A low TSH value in such patients may be regarded as a positive "bio-assay" for TSI activity and explain why decreased TSH values are an independent risk factor for a relapse of Graves' hyperthyroidism after a course of antithyroid drugs.

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Thyroid hormone and the brain: target cells, role of receptors, and timing of action.

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Introduction

It is now well established that the mammalian brain is a direct target organ of thyroid hormone, both during development and also in adult individuals. Most molecular studies have been directed towards the actions of thyroid hormone during development, and much less is known on its actions in the adult brain. During development, the role of thyroid hormone is the coordination of seemingly unrelated maturational processes. These processes are influenced by the hormone only temporarily during overlapping windows of development with regional specificity. In the brain, as in other systems, the active hormone is T3 which acts by regulating gene expression after binding to specific nuclear receptors. Besides this, there are convincing reports that thyroid hormone also has extra nuclear and extra genomic actions (1), but the extent to which these actions contribute to the general effects of the hormones in the brain is unknown. For details on the physiological and biochemical processes underlying thyroid hormone action in the brain the reader is referred to more extensive recent reviews by the author (2, 3). In this review, I will deal with three important topics, sometimes controversial and which still are not completely settled: what are the cellular targets of thyroid in brain, what is the role of the thyroid hormone receptors, and during which periods of development are thyroid hormones important.

Cellular targets of thyroid hormone in the brain

By situ hybridization analysis, T3 receptor mRNAs are located predominantly, if not exclusively, on neurons (4, 5). Little signal is present in the white matter. T3 receptors in specific neurons likely mediate the effects of the hormone in neuronal cell migration and differentiation, including for example, migration of neurons in the cerebral cortex and in the cerebellum, differentiation of Purkinje cells and cholinergic cells and the control of dendritic spine density in pyramidal cells of the cerebral cortex. T3 receptors are also present in high amounts in neurons in primary culture and in the neuronal line GT1-7 and a number of neuron-specific genes are regulated by T3 directly at the transcriptional level (3). Some of these genes contain thyroid hormone responsive elements (TRE).

It is also beyond doubt that the oligodendrocytes are also cellular targets of thyroid hormone. In vitro, thyroid hormone is required for normal timing of oligodendrocyte differentiation and in vivo thyroid hormone also controls the timing of myelination and the expression of oligodendrocyte-specific genes (6). The myelin basic protein gene promoter contains a TRE, suggesting direct transcriptional regulation. Despite the difficulties in detecting T3 receptor mRNA in brain slice preparations, mature oligodendrocytes in vitro express TRa1 and TRb1, whereas oligodendrocyte precursor cells (OPC) express only TRa1. This receptor isoform mediates therefore the effects of T3 on OPC differentiation (7).

It is controversial whether other cells are direct targets of thyroid hormone. Thyroid hormone has effects on astrocytes in vivo and in vitro; however it is not clear whether these effects are mediated by an action of T3 through the nuclear receptor. There are contradictory reports on the presence of T3 receptors in astrocytes and some evidence has been provided for extra genomic effects of both, T4 and T3 on astrocytes (8). On the other hand, T3 transcriptionally regulates the expression of some astrocyte-specific genes, so that the issue is not entirely settled. The effects of thyroid hormone on other types of cells in the central or peripheral nervous system have not been studied in much detail. Thyroid hormone is needed for proliferation and maturation of microglia (9). Interestingly, these cells in culture express the thyroid hormone receptor isoforms TRa1 and TRb1, but not TRa2. Finally, Schwann cells have been reported to express T3 receptors during development and after nerve regeneration, and T3 acutely increases the expression of early genes in cultured Schwann cells (10).

Role of thyroid hormone receptors

In mammals T3 receptors are the products of two genes known as TRa and TRb. The TRa gene encodes four protein products (TRa1, TRa2, and two truncated products) from which only TRa1 binds T3. The TRb gene encodes four T3 binding proteins, of which TRb1, TRb2 and TRb3 bind also to responsive elements in DNA. In addition, a truncated protein, delta-TRb3 binds T3 but not DNA.

One important question, not entirely settled, is why there are so many receptor isoforms and related proteins. Are the receptors equivalent, or do they regulate different genes and physiological functions? (11). The most prevalent view is that the receptor isoforms are mostly equivalent in their biological activity and that their different physiological role is due to their different patterns of expression and tissue concentrations (12). For example, whereas TRb1 is involved in cochlear development it can be replaced by TRa1 provided it is expressed at sufficiently high levels (13). In the cerebellum TRa1 is expressed in the granular cells, whereas the Purkinje cells express TRb1. Therefore, the effects of T3 on migration of granular cells are mediated by TRa1, whereas those on differentiation of Purkinje cells

are mediated by TRb1(14).

A prominent role of TRa1 in brain development and function may be deduced from its relative expression in cerebrum and cerebellum, accounting for about 70-80% of total T3 receptor binding (15). In addition, TRa1 is expressed earlier in development than TRb1. Therefore it was puzzling that TRa1 null mutant mice did not display obvious signs of developmental abnormalities. One possible explanation for this paradox is that in the absence of ligand, transcriptional repression by the unliganded receptor is responsible for the effects of profound hypothyroidism. According to this, we found that when hypothyroidism is induced in TRa1 mutant mice there is no delay in granular cell migration as observed in the hypothyroid wild type mice (14). Also, congenitally hypothyroid, Pax 8-deficient mice, which die during the first weeks of life, can be rescued by TRa1 deletion (16). It appears therefore that many important effects of profound hypothyroidism may in fact be due to the presence of unliganded TRa1. It follows from this conclusion that many of the biochemical processes influenced by thyroid hormone could really take place in the absence of both, the hormone and the receptor. It is likely that the interplay between the repressor activity of the receptor and the derepressor role of the hormone serves to finely tune the coordinating features of thyroid control on developmental processes.

Timing of thyroid hormone action in brain

An important question is whether thyroid hormone is needed throughout all phases of development or there are limited windows of thyroid hormone action. These questions are important in the analysis of the effects of maternal hypothyroidism and hypothyroxinemia on the fetus, and also on prematurity (17). In the rat model, the peak of thyroid hormone sensitivity for the brain, judging from the highest occupancy of T3 receptors would be around postnatal day 15, and most developmental effects of thyroid hormone action in the brain appear to take place during the first three postnatal weeks. Most thyroid hormone-regulated genes identified to date are sensitive to the hormone at different phases within the period corresponding from about E18 to P25. However, there is strong evidence that the rat fetal brain is under thyroid hormone control before that age and, therefore before onset of thyroid gland function. Receptor mRNAs can be detected as early as E11.5 (5), and receptor protein in nuclear preparations of the whole brain is detectable around E14 (18). Of course, in the absence of the fetal thyroid, maternal hormones would play an important role at these early stages of development. In support of this, the progeny of pregnant dams on low iodine diets had permanent changes in the migratory patterns of cells migrating on E14-E16 in the neocortex and hippocampus (19). It is therefore important to dissect the pathways of thyroid hormone action at these early phases of development and identify the target genes mediating these actions (20).

There is still not much data to understand in molecular terms the role of thyroid hormone in the adult brain (21). Thyroidal status influences neurotransmitter systems, but the mechanisms of regulation are unknown. As during development, thyroid hormone influences gene expression (22) and we have shown recently that deletion of TRa1 leads to alterations of behaviour (23). Expression of a mutated version of TRa1 with dominant negative activity leads to dramatic anxiety-like features, which is normalized by T3 treatment (Collaborative work between Vennström's and Bernal's groups, to be published). The implication of thyroid hormone receptors in behaviour is important, because it may be possible to modulate behaviour by using highly specific agonists of receptor isoforms. Elucidating the mechanisms of thyroid hormone action in the adult brain, including the role of receptor isoforms in behaviour remain therefore an important open field of research for the near future.

Summarizing table:

Thyroid hormone and brain development

A. Role of thyroid hormone

1. Early embryonic brain development:

- a. No effects on neural induction, neurulation, and establishment and polarity and segmentation

2. Cell migration and the formation of layers

- a. Cerebral cortex
 - Contributes to the right position of neocortical neurons, and therefore to the normal layering pattern, and to the distribution of callosal connections.
- b. Cerebellum
 - Controls the rate of migration of granular cells from the external germinal layer to the internal granular layer

3. Neuronal and glial cell differentiation

- a. Specific neuronal types

- Controls dendritic development and number of dendritic spines of pyramidal cells of neocortex and hippocampus. Dendritic spines are important in synaptic plasticity.
- Influences differentiation of cholinergic cells of brain stem and forebrain.
- Maturation of dendritic arborisation of Purkinje cells: in the absence of thyroid hormone, Purkinje cells have elongated primary dendrite, reduced dendritic arborisation and persistence of transient axo-somatic connections.

b. Oligodendrocyte differentiation

- T3 is an instructive factor for oligodendrocyte differentiation from stem cells.
- Thyroid hormones are required for normal myelination. Hypothyroid rats display transiently reduced expression of myelin genes and permanently reduced number of myelinated axons.

B. Role of thyroid hormone receptors

1. Two genes (TRa and TRb) encode four receptor and four non-receptor proteins.
2. TRa1 accounts for 70-80% of total receptor protein present in brain.
3. Cells expressing receptors are neurons and oligodendrocytes. Astrocytes may also express receptors.
4. Functional specificity of receptor isoforms

- Mostly equivalent
- Specificity depends of timing and place of expression
- Cerebellum: granular cell migration: TRa1. Purkinje cell differentiation: TRb1+TRa1
- Cochlear hair cell function: TRb
- Retinal cone photoreceptor development: TRb2
- Oligodendrocyte precursor cell differentiation: TRa1
- Hippocampal GABAergic interneuron function: TRa1

5. Unliganded receptors have intrinsic transcriptional activity. Role of unliganded receptors in normal development is unknown, but many features of hypothyroidism may actually be due to their intrinsic activity.

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Recent developments in the diagnosis and therapy of differentiated thyroid carcinoma (2)

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Introduction

Non-medullary thyroid carcinoma has a low incidence and an overall favorable prognosis. Nevertheless important diagnostic and therapeutic challenges are present. In the Part 1 (Hot Thyroidology, March 2003), new developments in initial diagnosis and therapy have been reviewed. In the present article, recent research on therapeutic targets for recurrent and metastatic disease will be presented.

Therapeutic challenges

Differentiated thyroid carcinoma has an overall 10-years survival rate of 90% (1). This favorable outcome is the combined result of the biological properties of the tumor as well as the effective initial therapy, consisting of near-total thyroidectomy followed by radioiodide ablation therapy.

However, when distant metastases have developed, the prognosis drops dramatically, with a 5 year survival in bone-metastases of follicular thyroid carcinoma of only 5%. Even if death is not imminent, the burden of metastatic disease may hamper quality of life for years. The main problem in metastasized thyroid carcinoma is that the current conventional therapeutic arsenal is limited to radioiodide therapy. When tumors have lost their ability to accumulate radioiodide, which is the case in approximately 50% of the patients with metastases, virtually no therapeutic alternatives are left. The development of new therapies is therefore vital. In the following, recent developments in therapy for differentiated thyroid carcinoma are discussed. These developments can be divided in (a) approaches aimed at improving or re-establishing the potential for radioiodide therapy and (b) targeting other, often non-thyroid specific pathways.

Improving radioiodide therapy

Because radioiodide therapy has such an important role in thyroid carcinoma, many attempts have been undertaken to improve the uptake of radioiodide. The discovery and molecular cloning of the rat and later the human sodium iodide symporter (hNIS) have contributed greatly to the understanding of the physiology and pathophysiology of iodide uptake by the thyroid gland (2; 3).

However, the ultimate dose of radioactivity in thyroid tumors (expressed in Gray (Gy)) is not only determined by iodide uptake but also by the effective half-life, which on its turn is influenced by iodide efflux from the cell. The exact mechanism of iodide efflux remains elusive. Although candidate molecules for apical iodide efflux, pendrin (4) and the apical iodide transporter AIT (5) have been discovered, their exact role in apical iodide transport has not been determined yet.

TSH

The TSH dependency of NIS activity is the base for the long established clinical practice to realize high TSH plasma levels by thyroid hormone withdrawal. The introduction of recombinant human TSH (rhTSH) has offered the possibility to avoid the cumbersome thyroid hormone withdrawal. The value of rhTSH has been demonstrated for diagnostic purposes, as discussed in Part 1 (Hot Thyroidology, March 2003). Although rhTSH has not yet been approved for the preparation of radioiodide therapy, several reports suggest the feasibility of rhTSH as adjuvant to radioiodide therapy (6). It will be difficult however to compare the therapeutic endpoints of radioiodide with rhTSH with classical thyroid hormone withdrawal in patients with metastatic thyroid carcinoma, as the conductance of randomized trials in these patients is hardly impossible.

Iodide deprivation

A well known mechanism to increase radioiodide uptake is to increase the specific activity of iodide, e.g. to decrease the dilution factor of radioiodide with 'cold' inorganic iodide. Although this practice has been established for long by prescribing low-iodide diets, it has only recently been demonstrated that low-iodide diets indeed have benefits for radioiodide therapy (7).

Lithium

The net effect of lithium salts on the thyroid appears to be a decrease in the efflux of thyroid hormone, leading to retention of iodide within the thyroid. Although the mechanism is not clear, this effect led to the application of lithium in hyperthyroidism, both as therapy or as adjuvant for radioiodide (8). It has been used to the same purpose in differentiated thyroid cancer (9). This latter study however is compromised by methodological problems and to date no convincing studies on improved efficacy of radioiodide therapy together with lithium have been published. In addition, the mechanism of radioiodide retention by lithium in thyroid cancer is poorly understood, leading to controversies on optimal dosages and therapy schedules.

NIS

The relation between decreased radioiodide uptake in thyroid carcinoma and decreased NIS activity has been well established. However, controversy exists on the mechanism: Some studies report decreased NIS mRNA and protein in thyroid carcinoma, suggesting that the origin of the problem is at the transcriptional level (10). In other studies however, a defect in targeting of NIS to the cell membrane is reported, which is even accompanied by an intracytoplasmatic overexpression of NIS in about 80% of thyroid tumors (11). These differences have important consequences for interventions aimed at increasing NIS expression.

Gene transfer

Given the importance of NIS, experimental studies have been conducted to enhance NIS expression in thyroid tumors. NIS gene transfer has been performed in a NIS defective thyroid carcinoma cell line. Tumors established with this cellline in mice responded to radioiodide therapy, proving that the concept of reinduction of NIS expression ultimately restores the

susceptibility to radioiodide therapy (12).

However, NIS protein expression is the end-point of complex regulatory mechanisms. It may therefore be assumed that the origin of defective NIS expression is located 'higher up' in the cellular hierarchy.

One of the causal chromosomal rearrangements in papillary thyroid carcinoma involves the *ret* oncogene, leading to constitutive *ret* activation. Introducing this chromosomal rearrangement into a benign thyroid cellline leads to decreased gene expression of the thyroid transcription factors TTF-1 and PAX-8 (13). TTF-1 and PAX-8 are involved in the gene expression of important thyroid proteins, including NIS. As a result, the chromosomal *ret* rearrangement ultimately leads to decreased NIS expression. To underline the importance of this mechanism, it has been reported that experimental gene transfer with PAX-8 leads to re-expression of NIS in a dedifferentiated thyroid cell-line (14). Although these approaches are fascinating from a conceptual viewpoint, a potential clinical application appears not to be within reach.

Pharmacological approaches

Therefore, medical approaches aimed at redifferentiation, or re-induction of thyroid specific proteins have gained much interest. Compounds that have been reported to reinduce NIS expression are retinoids, demethylation inducing substances and histone-deacetylase inhibitors.

Retinoids

Retinoids are vitamin-A derivatives. They influence the transcription of tissue specific gene repertoires, and as such play an important role in embryonic development. The archetypal example of disruption of retinoid signaling leading to cancer is promyelocytic myeloid leukemia, where therapy with retinoids has been highly effective (15). Retinoid therapy has been attempted in other types of cancer with limited success. In thyroid carcinoma, retinoids have been reported to reinduce NIS mRNA expression in cell-lines, although not leading to NIS protein re-expression (16). A clinical study has suggested that 13-cis retinoic acid therapy leads to restoration of sensitivity to radioiodide therapy and tumor regression (17). However, effectiveness parameters in this non-randomized, unblinded study were not uniformly studied. Therefore, the question on clinical validity of retinoids in differentiated thyroid carcinoma still awaits confirmation.

Demethylation and histone-deacetylase inhibitors

One of the mechanisms by which cells can block the expression of certain genes is by enzymes that methylate these genes or de-acetylate the histones that envelope a particular gene. These mechanisms also play a role in the silencing of genes in cancer. Therefore, compounds that can reverse methylation or inhibit histone deacetylation may lead to the reexpression of genes that are silenced in cancer.

Demethylation therapy has been proven successful in leukemia. In an in-vitro study in thyroid carcinoma, the demethylating agent 5-azacytidine led to reinduction of NIS expression, accompanied by radioiodine uptake in thyroid cancer cell lines (18). In parallel, the histone deacetylase inhibitor depsipetide has been reported to reinduce NIS mRNA expression and radioiodine uptake in thyroid carcinoma cell-lines (19). A clinical trial with depsipeptide is now underway (<http://www.nci.nih.gov/clinicaltrials>).

In conclusion, research directed at re-inducing NIS expression has revealed important insights into NIS regulation in thyroid carcinoma. Gene-therapeutic and pharmacological approaches have had anecdotal success in experimental systems. However, their value has been limited in clinical trials or still needs to be confirmed in patients.

Non-thyroid specific targets

Over the last decade, exciting developments have taken place in the identification and molecular dissection of novel pathways involved in cancer. The avalanche of new approaches has led to a considerable number of promising compounds. One of the disadvantages of differentiated thyroid carcinoma is that this low prevalent tumor is usually not included in initial clinical trials with these therapies. However, successful strategies that have survived these initial trials may well become available for thyroid carcinoma. It is not possible to review all candidates for therapy in this article. Ongoing trials in the United States can be viewed at <http://www.nci.nih.gov/clinicaltrials>. The most promising approaches are discussed below.

Cell proliferation

Although differentiated thyroid carcinoma is a low prevalent malignancy, many chemotherapeutic protocols that have been developed over the last decades for more common malignancies have been tried in progressive thyroid carcinoma. Overall, these approaches have been disappointing. Of the classical chemotherapeutic agents, adriamycin, alone or combined with cisplatin and bleomycin may induce temporary remissions or stationary disease in about 30-50% of the patients (20; 21). The same has been reported for paclitaxel (22). Most remissions however, last only a few months and at the cost of a considerable reduction in quality of life.

Neovascularization

Molecular pathways involved in neovascularization have been demonstrated in thyroid carcinoma (23). The cascade of approaches to target tumor-induced neovascularization has led to a number of promising compounds that are now being tested in clinical trials in prevalent tumors. Reports have been published on beneficial effects of anti-VEGF antibodies in thyroid carcinoma cell-lines (24) and endostatin in animal experiments (25). A clinical trial with thalidomide is underway in the United States.

Tyrosine kinase inhibitors

Another intriguing development is the advent of tyrosine kinase inhibitors. The development of imatinib mesylate (Gleevec) is prototypical for the innovative design of modern drugs with the molecular pathogenic defect as a starting point. Following imatinib, other small molecules have been developed, aimed at other tyrosine kinase activated pathways such as the epithelial growth factor receptor activated pathway. Activation of tyrosine kinase pathways is relevant for thyroid carcinoma. The earlier discussed ret chromosomal translocation leads to constitutive activation of ret, which is a tyrosine kinase activating receptor. Tumors established with cell lines in which the ret translocation has been introduced have been successfully treated with the tyrosine kinase inhibitor PP1 (26).

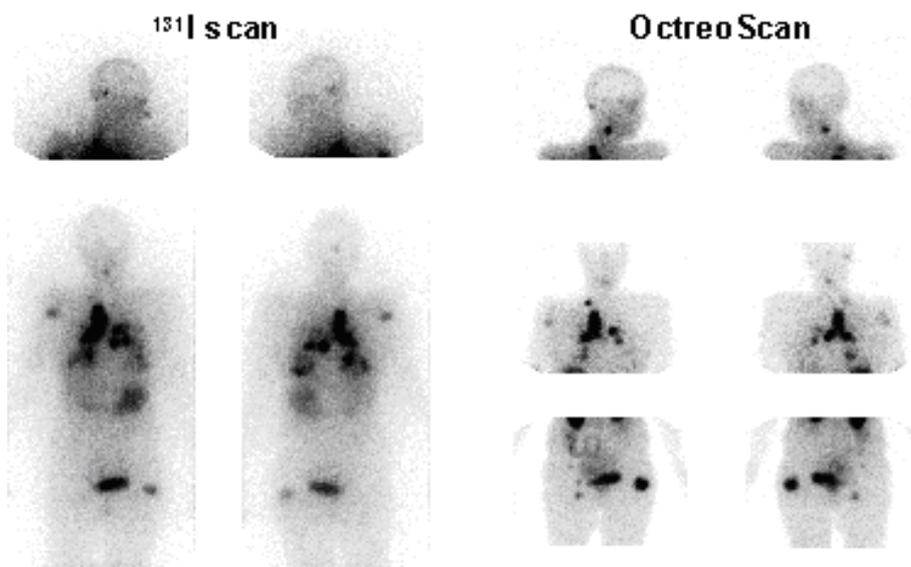
PPAR-g agonists

An interesting new class of drugs are agonists of peroxisome-proliferator activated receptor gamma. (PPAR-g). These drugs have been introduced as anti-diabetic agents. Their proposed mechanism is the differentiation of pre-adipocytes into adipocytes, thereby increasing the fatty-acid storing capacity of adipose tissue. The involvement of PPAR-g in differentiation processes extends beyond the area of adipose tissue. Indeed, altered expression of PPAR-g and in vitro beneficial effects of PPAR-g agonists have been described in a number of malignancies, and recently also in pituitary tumors (27). In thyroid carcinoma, thiazolidinedione treatment induced apoptosis in thyroid tumors and prevented their growth in nude mice (28). Interestingly, a unique chromosomal rearrangement has been described in benign and malignant thyroid neoplasms, involving PPAR-g and PAX-8 (29). This rearrangement acts as a dominant competitive inhibitor of PPAR-gamma and from a theoretical point of view would render these tumors insensitive for PPARgamma agonists.

Radionuclide therapy

Somatostatin Receptor Scintigraphy (SRS)

In part 1, (Hot Thyroidology, March 2003) it has been discussed that the expression of somatostatin receptors (SSTR3 and SSTR5) by differentiated thyroid carcinoma is the base for SRS imaging and therapy. Interestingly, in a considerable number of carcinoma's irresponsive to radioiodide, SRS imaging shows pathological lesions, which has diagnostic and therapeutic consequences (30; 31). A therapeutic trial is currently performed in Rotterdam, the Netherlands, which includes patients with poorly differentiated thyroid carcinoma (32). An interim analysis showed a considerable response rate (seeFig), but definite conclusions have to be awaited until the conclusion of the trial.



¹³¹I scintigraphy and OctreoScan showing multiple metastases in lungs and bone in the same patient.

Courtesy Prof Dr. E.P. Krenning, Rotterdam, NL

Palliative therapy

Surgery, external irradiation, and to a lesser extent radioiodine therapy, are the conventional palliative treatment modalities in patients with metastases of thyroid carcinoma. However, as most metastases do not accumulate iodide and the effect of radioiodide therapy is not rapid, radioiodide therapy is of limited use as a palliative treatment option. Surgery may lead to rapid relief of symptoms, but is only possible when the metastasis is approachable. External irradiation is the most frequently applied palliative therapy in bone metastases of thyroid carcinoma. Although this therapy can be effective, limiting factors may be the radiosensitivity of the tumor and the site of the tumor: in vertebral metastases, the maximal radiation dose is limited by the proximity of the spinal cord. Selective embolization of tumor metastases is another option, which is effective in about 60% of the patients to induce an immediate relief of pain and neurological symptoms (33).

The search for new targets

The recent introduction of high-yield genomic and proteomic techniques has provided enormous perspectives to identify diagnostic and potential therapeutic targets in disease, as indicated by recent high-impact studies in breast carcinoma (34). Gene expression arrays in thyroid carcinoma have revealed differential expression of genes in papillary carcinoma as compared with follicular thyroid carcinoma and normal thyroid tissue, including genes so far not associated with thyroid carcinoma (35; 36). These approaches will without doubt provide new insights into thyroid tumor biology and thereby new candidates for therapy.

Conclusion (2)

The clinical and therapeutic dilemmas as well as the intriguing biological features of differentiated thyroid carcinoma offer unique challenges for both clinicians and basic researchers. Although thyroid carcinoma research will profit from insights gained in other fields of cancer, in reverse, thyroid carcinoma research has contributed importantly to the understanding of processes of dedifferentiation and malignant transformation. Given the low prevalence, coordination and coupling of research efforts are vital.

Summary

- Although the primary therapy with near-total thyroidectomy and radioiodide ablation therapy in combination with biological characteristics results in an overall good prognosis for differentiated thyroid carcinoma, therapeutic options for patients with advanced disease are limited.
- Research strategies are aimed at:
 - improving the susceptibility of differentiated thyroid carcinoma for radioiodide therapy and
 - the identification of other , often non-thyroid specific, therapeutic targets.

- Improving radioiodide therapy centers around the understanding of the pathophysiology of the human sodium iodide symporter (NIS). Approaches to enhance NIS expression or function can be divided in:
 - genetic therapies and
 - pharmacological therapies. These include redifferentiation therapy, demethylation inducing agents and histone-deacetylase inhibitors.
- Other therapeutic targets parallel developments in general oncology. Noteworthy are the intervention in tumor induced neovascularization, the introduction of tyrosine-kinase inhibitors and the evolving role of PPAR-g agonists.
- An interesting development is the therapeutic targeting of somatostatin receptors in differentiated thyroid carcinoma with radionuclide-labeled somatostatin analogues.
- Conventional palliative therapeutic options are limited to radiotherapy. Selective embolization of bone metastases offers an additional option.
- Thyroid carcinoma research has provided important insights into tumor biology in general. The advent of high-throughput genomic and proteomic techniques will offer new knowledge on processes of thyroid dedifferentiation and thereby novel candidates for therapeutic approaches.

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The thyroid and the preterm infant.

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Introduction

Thyroid hormone is known to regulate neurodevelopment, probably from early fetal life onwards (1). Thyroid hormone deficiency can cause long term morbidity in terms of behaviour, locomotor ability, cognition and hearing ability, if the onset is early in development (2).

Since the introduction of neonatal screening for congenital hypothyroidism in the 1970's it became clear that preterm infants have lower plasma concentrations of (free)T4 and (free)T3 than full term infants of the same postnatal age and this has raised an ongoing discussion on the need for thyroid hormone supplementation in preterm infants in order to improve clinical and neurodevelopmental outcome.

The thyroid state of the preterm infant can be viewed as a subtle form of hypothyroidism or hypothyroxinemia. As in maternal hypothyroxinemia during the first half of pregnancy (3), a low supply of thyroid hormone to the fetus could be harmful for brain maturation during its early development (4), and this phenomenon must be studied carefully not only in a descriptive manner, but also in the light of a possible need for thyroid hormone supplementation.

Fetal thyroid state

By 7 weeks of gestation, before the onset of fetal thyroid functioning, thyroid hormone has been demonstrated in fetal fluids, including serum, FT4 concentrations being at least one third of the mother's FT4 concentration (5). Also thyroid hormone receptors and the deiodinating enzymes are present in human fetal cerebral cortex by that time (1). Nuclear thyroid hormone receptors occupied by bioactive T3 have been found in human brain and lung tissue in the 9th week of fetal life. Although, the hypothalamus and pituitary start to synthesize hormones by 12 weeks, significant thyroid hormone production does not occur before the 20th week of gestation (6). Before mid-gestation, materno fetal transfer of T4 is therefore pivotal for the fetal thyroid hormone status.

Fetal thyroid function and the hypothalamic-pituitary-thyroid axis continue to mature throughout pregnancy; serum levels of TT4, FT4, thyroglobulin, and TSH increase until the end of pregnancy (7). Serum levels of TT4 increase from about 5 nmol/l at 12 weeks gestation to about 120 nmol/l at term, while the increase of serum TT3 is much less: from 0.5 nmol/l at 12 weeks to about 1.5 nmol/l at term (5,7). Serum FT4 in cord blood seems to increase from about 5 pmol/l at 12 weeks gestation to about 20 pmol/l at term (5,7).

During fetal life, the concentration of TT3 is tightly controlled in the tissues. The already abundantly present T4 is preferably converted by type III deiodinase to rT3, which is present in high concentrations during fetal life and only decreases in the last weeks, while T3 is readily converted to diiodothyronine. Also, sulfatation by hepatic sulfotransferase enzymes to the inactive

sulfated metabolites T4 sulfate, T3 sulfate, and rT3 sulfate is an inactivating metabolic pathway in fetal life (8).

Presumably, when increases in local intracellular T3 concentrations are needed for thyroid hormone-dependent maturational processes, local type II deiodinase increases, converting T4 to T3, whereas type III deiodinase activity decreases, favoring intracellular T3 accumulation. In this respect, the plasma T4 concentration is far more important than the plasma T3 concentration. The local concentration of thyroid hormone receptors and possibly mechanisms regulating T4 uptake in the cells also play a role in this ontogenetically programmed production and action of T3 (1).

The described regulatory mechanisms are also important in protection against thyroid dysfunction. Thus, also in human fetal brain, like in the rat brain, type II deiodinase, was found to increase in response to plasma T4 decrease (9), but the onset of this regulatory mechanism was only found at mid-gestation.

Postnatal function in the preterm infant

After preterm birth, TT4 and TT3 levels remain lower than in term born infants during the first weeks (10). There is an obtunded TSH peak immediately after birth, while it remains below 20 mU/l, being the cut-off point for congenital hypothyroidism, in the period of low TT4. This period during which total and free T4 (and T3) levels are low is generally referred to as transient hypothyroxinemia of the preterm infant.

Table 1. Factors that influence (very) preterm thyroid function

Immaturity of the hypothalamic-pituitary-thyroid axis

Immature thyroidal capacity to concentrate iodine and synthesize and iodinate thyroglobulin

Increase of thyroid hormone needs for example for thermogenesis, heart function, skeletal muscles etc

Sudden interruption of materno-fetal transfer of T4

Immaturity of thyroid hormone metabolism, causing low T3 and high rT3 and sulfated iodothyronines

Effects of neonatal disease (non-thyroidal illness)

Insufficient iodine supply

Iodine excess (iodine containing antiseptics and radio opaque agents)

Table1

In infants of less than 30 weeks gestational age, TT4 concentrations are about 60 nmol/l in the first week of life (11), while in term infants TT4 concentrations are generally 4 times higher. Postnatal free thyroid hormone concentrations are also lower the earlier in gestation the infant is born. FT4 concentrations are about 2-fold lower in very preterm infants as compared with term infants of 1 week of age. (12, see also the figure).

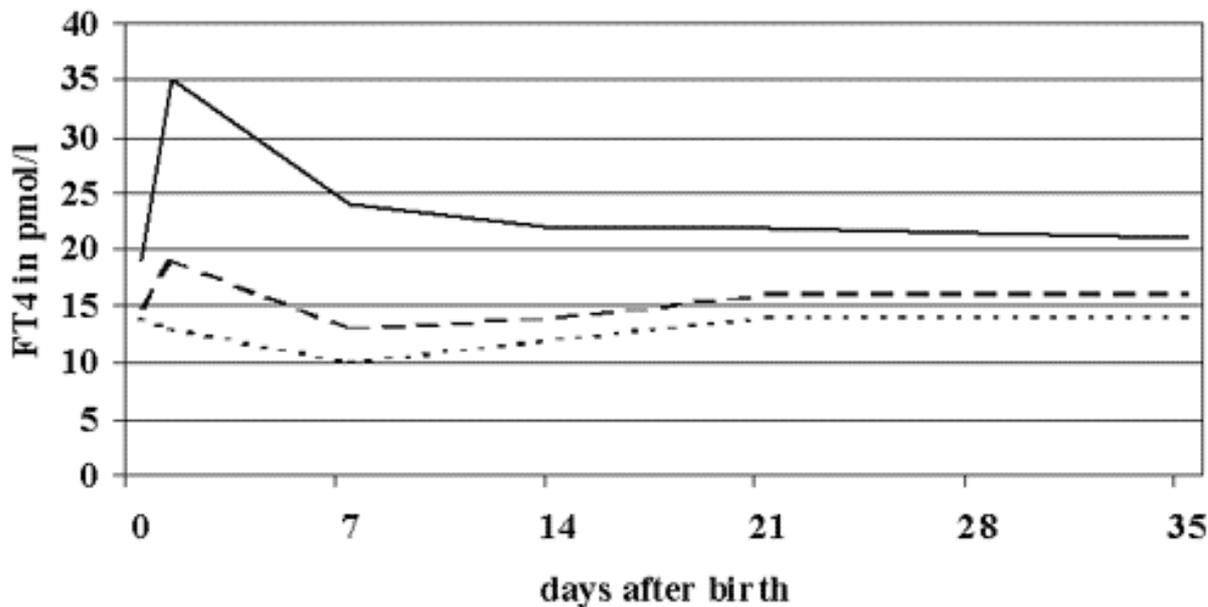


Figure:

Mean Free T4 concentrations in the first 5 weeks after birth, as measured by a two-step RIA in infants of 25-28 weeks gestation (.....), infants of 28-30 weeks gestation (-----) and of term infants as estimated from literature (->), (ref. 12,14,25)

The average of five FT4 measurements drawn between day 3 and 28 in infants <30 weeks gestation, by a two-step RIA, was found to be between 10.1 and 21.1 pmol/l (13). TSH has a variable time course, but comes down to about 2-4 mU/l by 4 weeks after birth (14). The postnatal time course of T3 in preterm infants misses the sharp peak after birth and only slowly rises to term values in the course of 6-8 weeks (10,14).

Consequences of transient hypothyroxinemia

Preterm infants are at risk for neurodevelopmental impairments. The more preterm the infant is born, the higher the risk of neurological impairments. These concern speech, language, behaviour and learning, and in serious cases overt mental retardation may occur. The neuromotor deficiencies vary from clumsiness to disabling cerebral palsy. Visual and hearing

impairments are also frequent.

Four studies (Table 2) show an association between low thyroid hormone levels in the first weeks of life and worse developmental outcome.

Table 2. Retrospective cohort studies on the association between neonatal plasma (F)T4 and T3 levels in preterm infants and later development.

<u>Source</u>	<u>Findings</u>
Lucas A, et al. 1988, 1996 (15,16) N=236	Low neonatal TT3 is associated with lower IQ both at 18 months and 8 years of age
Meijer WJ et al 1992; den Ouden AL et al 1996 (4,17) n=563	TT4 at day 7 of life is associated with developmental delay at 2 years of age and school problems and minor neurological dysfunction at 9 years of age
Reuss et al 1996	A TT4 at <day 7 of life of >2.6 SD below the test mean is associated with an increased risk of disabling cerebral(18) N=463 palsy and a 7 points IQ reduction
Van Wassenaer et al 2002 (13) N=75	Low FT4 during first 4 weeks of life is associated with worse neurodevelopmental outcome at 2 and 5 years

Table2

Of course they do not provide evidence that preterms infants should be treated with thyroid hormone. Low thyroid hormone levels are also associated with higher mortality and more respiratory disease (13,14,19), a higher incidence of cerebral hemorrhage (19) and ischaemic lesions (20).

Only randomized clinical trials, testing the effect of thyroid hormone treatment in preterm infants, can untangle the complicated relationships between thyroid hormone levels, gestational age, morbidity and neurodevelopment.

Studies with thyroid hormone administration in preterm infants

Between 1997 and 2003 four randomized, double-blind trials were published (21,22, 24,25), see Table 3, with different treatment protocols and endpoints.

Table 3. Summary of four randomized double-blind T4 or T3 treatment studies in infants <32 weeks gestational age.

study	Intervention	Endpoint	no. (T vs co) at assessment of endpoint gestational age (Ga)	main results
Vanhole, et al 1997 (21)	T4, daily i.v. bolus, 20 mg/kg, d1-14	Endocrine and Clinical (also neuro-development at 7 mo	17 versus 17 Ga <31 wk	No difference in clinical outcome and development
Van Wassenaer et al, 1997 Briet et al, 2001(22,23)	T4, daily bolus, first 2-3 wks iv, later orally; 8 mg/kg, d 1-42	Neuro-development at 24 mo; and outcome at 5.7 yr	82 versus 75 Ga <30 wk	No difference in total groups. Subgroup analyses: at 2 and 5yrs better outcome with T4, if Ga <27-29 wks
Smith et al, 2000 (24)	T4, bolus, start iv: 10 mg/kg; then orally: 20 mg/kg, d 2-21	Chronic lung disease Need for supplemental oxygen at day 28	29 versus 18 Ga <32 wk	No effect

Biswas, et al 2003 (25)	T3, continuous iv, 6 mg/kg/d plus hydrocortisone 1 mg/kg/d; d 1-7	Death or ventilator dependence at d7	125 versus 128 Ga <30 wk	No difference in adverse outcome at d7
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Three of the studies (21,24,25) chose short term clinical outcome as primary end point, in one of them T3 was administered instead of T4 (25). In none of the studies mortality or morbidity was significantly influenced by thyroid hormone treatment. In only one study neurodevelopmental outcome was chosen as primary end point (22,23); children were assessed five times between corrected term age and 5.7 years. Neurodevelopmental outcome was similar in both groups at all time points. However, both at two years and at 5.7 years, post-hoc subgroup analyses revealed a gestational age-dependent effect of T4. T4-treated infants of <29 weeks gestation had a better neurodevelopmental outcome, but for T4-treated infants of 29-30 weeks the reverse was true (23). The latter possible harmful effect of T4 was not related to high FT4 concentrations (13). Taken together, none of these studies provides evidence for the need of thyroid hormone supplementation in very preterm infants and therefore the current advice is to not supplement low thyroid hormone concentrations in these infants, unless accompanied by elevated TSH (26,27) .

Conclusions and Recommendations for further studies

Until now, a low T4 with TSH of less than 20 mU/L has been used in the definition of transient hypothyroxinemia, with cut-off values for TT4 that vary between the different authors from 40 to 100 nmol/l. TBG concentrations are also low, however, and therefore FT4 may even be high, when TT4 is in the low range. In our material, we found that TT4 concentrations of <60 nmol/l are accompanied by FT4 concentrations between 5.2 and 16.6 pmol/l with 60% of FT4 values below 10 pmol/l (unpublished observation). It is therefore necessary to include the FT4 concentration in the definition of transient hypothyroxinemia. The normal range for FT4 concentrations in preterm infants should be established, but this can only be done if developmental outcome of these infants is part of these studies. Normal ranges of FT4 should be known for any specific type of assay, as FT4 is reported to be higher by dialysis method than by other methods (28). Because of the strong association with gestational age and birthweight, normal values should be established per gestational age or birthweight group .

Using different treatment protocols in each of the randomized controlled trials, researchers have not been able to demonstrate a positive effect of T4 and/or T3 treatment on clinical outcome. Presumably, improvement of clinical outcome should not be the aim of studies but rather of neuro-developmental outcome when thyroid hormone supplementation studies are designed. Our own study is the only study of this type (22). The results of the post-hoc subgroup analyses of our study seem to show that T4 supplementation may be beneficial in infants of less than 28-29 weeks of gestation (22,23)

Therefore, a new randomised controlled trial with T4 in a selected patient group of infants of <29 weeks gestation, who also have a FT4 measurement in the low range during the first 3 days of life, appears to be a logic next step. Whether the thyroid status of the mother contributes to that of her child(ren) is a question that also has not been answered so far .

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NON-NUCLEAR ACTIONS OF THYROID HORMONES: the case of T₂

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Introduction

Thyroid hormones (THs) exert a multiplicity of effects. Among these are crucial effects on development, differentiation and metabolism. The first two are particularly relevant in the early stages of development, and a deficit in THs in the neonatal period has serious consequences, such as mental retardation and growth disturbance. However, notwithstanding the knowledge of this wide spectrum of activity, the publication of an enormous number of reports and a long list of hypotheses, the mechanism by which THs exert their diverse actions has not finally been established. In particular, with regard to the stimulatory effects of THs on metabolism we can distinguish three historical periods during which some major hypotheses regarding their mechanisms of action have been developed and have attracted the attention of investigators in the field. The first period was from the early 1950s to the middle 1960s. At this time, the most intriguing hypothesis put forward was "the uncoupling hypothesis", which suggested that THs stimulated metabolic rate by acting at the mitochondrial level by uncoupling the electron transport chain from ATP synthesis (1,2). Mainly because of the large doses employed and also because of the non-reproducibility of the observations "in vivo", this hypothesis was subsequently discarded on the grounds that it was not physiologically relevant.

Then, at the beginning of the 1960s the results obtained by Tata et al. (3-5) provided a basis for the most widely recognized action of THs. They showed that the stimulation of metabolic rate induced by a single injection of T₃ took about 2-3 days to achieve a maximal effect and that the effect was blocked by simultaneous administration of the DNA-transcription suppressor actinomycin D (AD). These results for the first time clearly pointed to the nucleus as the cellular site of action of THs. At the beginning of the 1970s, Oppenheimer et al. (6) were the first to describe the presence of specific nuclear binding sites with a high affinity and low capacity for T₃ in rat liver and kidney. Such sites were subsequently found in other tissues and cell cultures (7). In 1986, two groups (8,9) reported the identification of the cellular proto-oncogene c-erbA, which encodes the high-affinity thyroid hormone receptor (TR). Now, it is well established that most of the physiological effects of T₃ within cells are exerted at the level of transcription, via interactions with specific TRs belonging to the superfamily of nuclear hormone receptors (10-12). TRs are transcription factors that modulate transcription by binding to thyroid-hormone response-elements (TREs). The genes for TRs each express alternative receptor isoforms, including TRb1 (widely expressed), TRb2 (expressed in cochlea, retina and pituitary), TRb3 (expressed in lung, kidney and in an osteosarcoma cell-line), TRa1 (widely expressed) and TRa2 (a C-terminal splice variant that does not bind T₃) (for further details, see Refs. 11-13).

Non-nuclear actions of THs

For a long time, the predominant view was that the actions of THs are initiated exclusively by an interaction of T_3 with TRs. In recent years (end 1980s-beginning 1990s), however, an increasing number of effects have been described for which a nuclear genomic-dependence can be excluded, leading to the possibility of distinguishing between nuclear and extranuclear effects (due to the presence of a mitochondrial genome, the terms "nuclear" and "extranuclear" are preferable to "genomic" and "non-genomic"). Because of the dogmatic nature of the oft-repeated statement that thyroid hormones act via nuclear receptors, it is difficult to convince many people that they may not necessarily always do so (14). In fact, several extranuclear effects have been described at the level of: 1) the plasma membrane; 2) the cytoskeleton; 3) the sarcoplasmic reticulum and the endoplasmic reticulum; 4) the cytoplasm; 5) mitochondria; and 6) contractile elements in vascular smooth muscle cells. The mechanisms responsible for these extranuclear effects may involve: a) a direct interaction of THs with effector-cell proteins; b) cell-surface receptors coupled to G proteins; c) activation of protein kinase; d) promotion of protein trafficking; e) protein polymerization; f) a change in intracellular calcium concentration; g) ionic exchange. All these aspects have been extensively covered in two reviews (15,16) and will not be further discussed here.

What are the characteristics that should allow us to distinguish between the nuclear and extranuclear effects of THs? Unlike the nuclear effects, the extranuclear ones: i) are independent both of the nuclear receptors for THs and of protein synthesis; ii) may be mediated by signal-transducing pathways; iii) have a short latency to onset (minutes or few hours, even if some nuclear effects have a short latency: see Spot 14 (17); iiiii) may involve various iodothyronines. Concerning the last point, until some years ago it was a common assumption that T_3 was the active hormone (following its formation by deiodination of the precursor T_4). A growing body of evidence, however, has led to a revision of that opinion: it seems that at least four iodothyronines exist, and that these all have significant (although not identical) biological activities. These are: L-thyroxine (T_4), triiodo-L-thyronine (T_3), reverse T_3 (rT_3) and 3,5-diiodo-L-thyronine (T_2).

The case of T_2

T_2 is particularly intriguing because its effects on metabolism seem to be mostly extranuclear. In recent years, in fact, a growing volume of evidence has accumulated to indicate that T_2 , a putative product of the inactivating deiodination pathway in T_3 metabolism, could be of biological relevance. In 1989, Horst et al. (18), who studied the effects of several iodothyronines on the oxygen consumption of perfused livers, showed that T_2 , like T_3 and T_4 , was able to rapidly stimulate hepatic oxygen uptake. While the effects induced by T_3 and T_4 were abolished by inhibiting hepatic deiodinase activity, those induced by T_2 were unaffected by such inhibition. These results stimulated our group and others to focus more deeply on a putative physiological role for T_2 . Several investigators, indeed, have demonstrated a rapid effect of T_2 on mitochondrial respiration both in vitro and after its in vivo administration (18-27). The clearest demonstration of an effect of T_2 on energy metabolism comes from "in vivo" studies (28-33). In one of these (29), the effects of a single injection of T_2 were measured on resting metabolic rate (RMR; oxygen consumption at rest, in thermoneutrality and in the post-absorptive state). This study employed an animal model in which all three known types of deiodinase enzymes were inhibited and the rats made

hypothyroid, effects produced by administration of propylthiouracil (PTU) and iopanoic acid (IOP) ("P+I" in Fig.1). Under such conditions it was shown that T_2 , as well as T_3 , is able to enhance the RMR of hypothyroid rats, although their effects differed in terms of both time course and dependency on protein synthesis. Injection of T_3 stimulates RMR through a nuclear-mediated pathway: indeed, its latency to maximal effect is 2-3 days and the effect is completely blocked by simultaneous administration of actinomycin D. In contrast, T_2 stimulates RMR more rapidly, the effect reaching peak on day one after the injection and being insensitive to actinomycin D (see Fig.1).

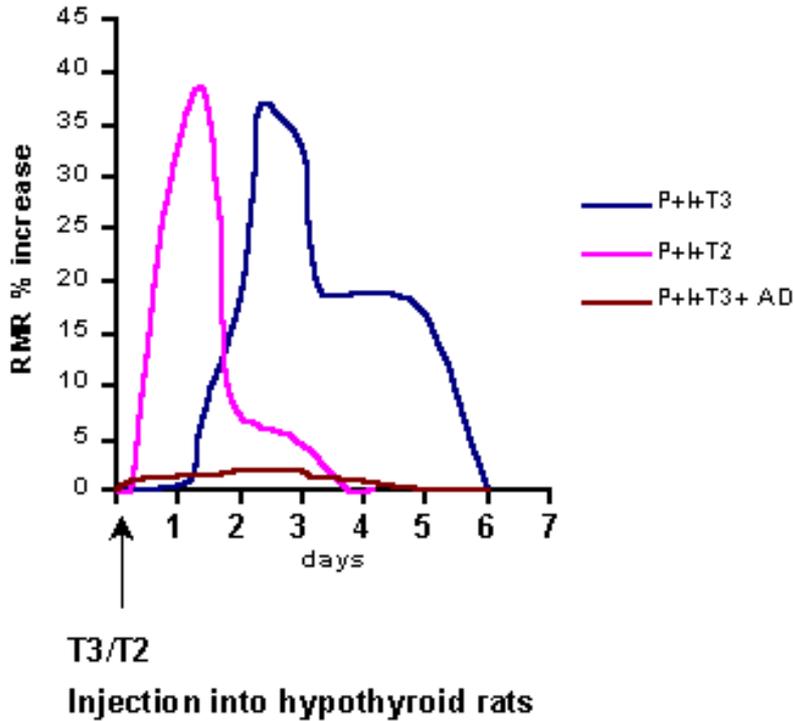


Fig.1 For explanations see text

The effect of T_2 is most likely due to a direct interaction of this iodothyronine with mitochondria. The major difficulty encountered in studying the effect of T_2 in vivo is that injection of T_2 into euthyroid rats results in a slight, insignificant change in RMR. A rapid metabolic degradation of T_2 , differences in the metabolic status of the animals or the need to be formed from a precursor such as T_3 could be some of the reasons for this inefficacy of T_2 when it is injected into euthyroid rats. How is T_2 formed? Is T_3 a possible precursor from which T_2 may be formed intracellularly (even if an enzyme that converts T_3 into 3,5- T_2 has not yet been discovered, at least from in vitro studies)? The best way to answer these questions is to inject T_3 into euthyroid rats and to compare the time course of the variations in RMR (either in the presence of or absence of actinomycin D) with the time course of the changes in the serum and hepatic levels of T_2 . If some of the effects on RMR that follow the administration of T_3 rely on its transformation into T_2 , then in the curves expressing the time course of the change in RMR and the time course of the increases in the serum and tissue levels of T_2 , the initial rising phases should be of similar steepness (only if T_2 acts instantaneously). In a recent study, designed to test this hypothesis, was observed that an acute injection of T_3 had an evident effect on RMR earlier in euthyroid rats ("N" in Fig.2) than in rats made hypothyroid by administration of PTU plus IOP (see above) thus indicating that the effects observed following the administration of T_3 to

euthyroid rats are not entirely due to T_3 itself (34). In fact, following administration of T_3 the maximal increase in RMR occurred 2-3 days after the injection in hypothyroid rats but after only 25 h from the injection in euthyroid ones. The patterns of response induced by T_3 in euthyroid rats and by T_2 in hypothyroid rats were temporally similar (compare Fig.1 with Fig.2) suggesting that at least part of the early effect of T_3 in euthyroid animals might be due to T_2 . In addition, the peak observed on day one after T_3 injection into euthyroid rats was markedly reduced when inhibitors of deiodinases were simultaneously injected (see Fig.2).

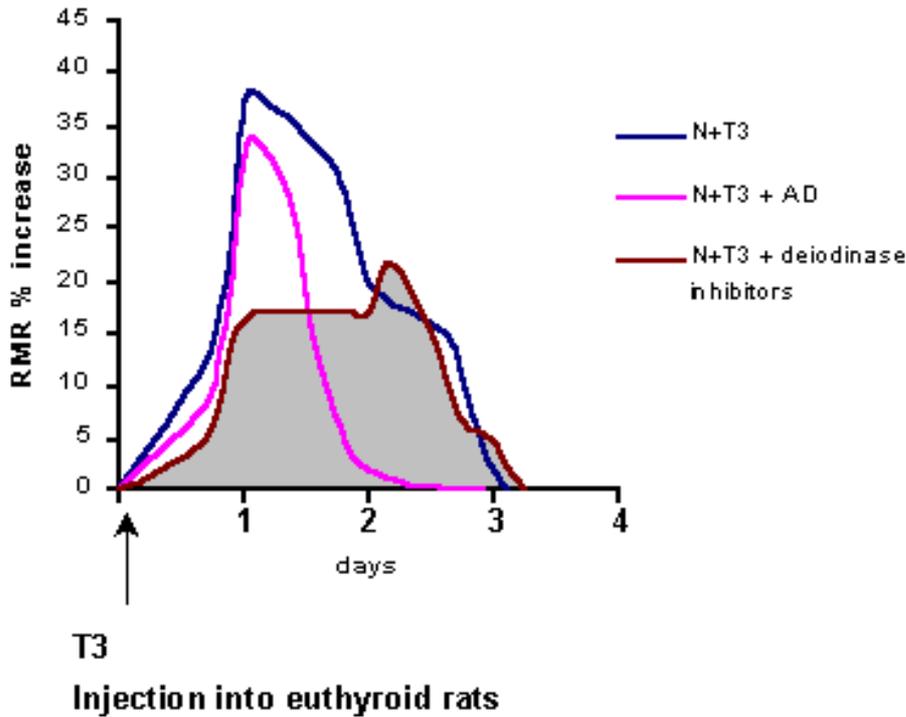


Fig.2 For explanations see text

Moreover, the serum and hepatic levels of T_2 were increased at 12-24 h after T_3 injection and peaked on day one, coincident with the peak in the actinomycin D-insensitive metabolism. Collectively, these data promote confidence in the physiological nature of the effects exerted by T_2 , even if further studies are needed to confirm this idea.

As stated before, T_3 is also able to stimulate metabolism, although its effect occurs through a different (nuclear-mediated) mechanism. The recently discovered uncoupling-protein homologues may play a major role in the T_3 -mediated effect (for review, see 35) as their expressions and uncoupling activities in skeletal muscle are regulated by T_3 and increase in line with the increase in RMR (36).

Conclusion

On the basis of the available evidence, we believe that the coexistence of nuclear and extranuclear mechanisms of action for THs action may adequately explain the multiplicity of effects exerted by iodothyronines (including the short-term and the long-term ones).

As a last consideration, we should like to outline some problems related to studies of the effects of THs. Some of the controversies surrounding the mechanism of action of THs may be, at least to some extent, a consequence both of the actual TH used and of the thyroid state of the animals. Indeed, discrepant results and opinions may derive from the use of :1) acute vs. chronic treatment, 2) smaller vs. larger doses of the hormones, 3) different iodothyronines, and/or 4) different animal models (created using different ways of inducing hypothyroidism).

Among the points cited above, number 4 is of particular relevance. In fact, the clarification of the biochemical properties of deiodinase enzymes has revealed that surgical and chemical thyroidectomy result in very different animal models of hypothyroidism, characterized by different TH serum levels and different effect on deiodinase enzymes. In addition, different animal models of hypothyroidism are obtained when different drugs are used to induce chemical thyroidectomy. When methimazole is used, there is an inhibition of the synthesis of TH, while the activities of the three types of deiodinase enzymes respond in the same way as they do to surgical thyroidectomy [type I deiodinase (present in liver and kidney) decreases; type II deiodinase (present in brain and brown adipose tissue) increases; type III deiodinase (present in brain, skin and placenta) is differently affected depending on the tissue examined). When PTU is used alone, there is an inhibition of the synthesis of TH and a concomitant strong inhibition of type I deiodinase. When IOP is used alone there is no influence on the synthesis of TH but a significant change in the peripheral metabolism of TH resulting from the inhibition of all three types of deiodinase enzymes. All this suggests that considerable caution needs to be exercised when comparing results obtained from different animal models.

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ROLE OF TYPE 3 DEIODINASE IN THYROID HORMONE METABOLISM.

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Introduction

A large number of thyroid hormone (TH)-dependent effects are mediated via nuclear TH receptors. These receptors bind to TH-response-elements on specific genes and thereby activate or inactivate gene transcription. The affinity of the known TH receptors for 3,3',5-triiodothyronine (T_3) largely exceeds their affinity for thyroxine (T_4). With T_4 being the main secretion product of the thyroid gland, it is obvious that the extrathyroidal deiodination of T_4 to T_3 plays an important role in TH activity and this process has been studied for long. However, with the more detailed characterisation and the cloning of three types of deiodinating enzymes, first in mammals and later in almost all vertebrate classes, it became clear that the availability of T_3 in extrathyroidal tissues is regulated not only by changes in T_3 producing deiodinases but also in T_3 degrading deiodinases. The T_3 inactivating type 3 deiodinase plays a crucial role in this process.

Some characteristics of the three iodothyronine deiodinases

Type 1 deiodinase (D1) catalyses both outer ring deiodination (ORD) and inner ring deiodination (IRD). It can therefore produce T_3 via ORD (activating pathway) and degrade T_3 or divert T_4 metabolism towards 3,3',5'-triiodothyronine (rT_3) via IRD (both deactivating pathways). The two other deiodinases are selective enzymes. Type 2 deiodinase (D2) only catalyses ORD and is considered an activating enzyme, while type 3 deiodinase (D3) is a purely inactivating enzyme via catalysis of IRD. Although the abundance and the tissue distribution may vary, D2 and D3 isoforms have been found in all vertebrate classes. Amphibians as well as a great number of fish species lack D1. Several criteria have been described allowing to differentiate between the three deiodinases (1-3). One of them, the K_M (Michaelis-Menten) constant, can help to understand why even low amounts of D3 (and D2) can have a substantial impact on TH metabolism. The K_M values of D1 for T_4 and T_3 are in the low micromolar range while the K_M values of D3 and D2 for T_3 and/or T_4 lay in the low nanomolar range. This means that, except maybe for the thyroid gland, even the total intracellular TH concentrations (free + protein-bound) are far below the levels where D1 can work most efficiently, while this is not the case for D2 and D3. Therefore comparison of the absolute amount of D1 versus D2 or D3 present in a tissue is not necessarily representative for their relative contribution to TH deiodination *in vivo*. Another point that may be important is the fact that several studies in mammals and birds have reported short half-lives for D2 and D3. This is true not only for the proteins but also for their mRNAs with values ranging from 20 min to 3 h (4-7). For D1 mRNA and protein a longer half-life of more than 8 to 12 h was measured (5,7). As a consequence, acute regulation of D2 and D3 activity is not only possible via posttranslational mechanisms, but can also easily be achieved via changes in gene transcription rate. Since D3, be it in low amounts, is present in most vertebrate

tissues and can be regulated in a tissue-specific way, it is a good candidate for the moment-to-moment fine-tuning of intracellular T_3 availability.

Role of D3 during development

In general, the expression of D3 is higher in embryonic, fetal or larval tissues compared to adult tissues. This allows D3 to play a substantial role in the developmental regulation of T_3 concentrations, both in circulation and in specific tissues. The studies of Galton and coworkers (8-10) and Borges and colleagues (11) in metamorphosing frogs and developing chickens were among the first to emphasize the opposite changes in IRD activities and plasma T_3 levels. They suggested that the increase in T_3 during metamorphosis or towards hatching was not only dependent on an increased T_3 production but also on a decreased T_3 breakdown. The key role of D3 was further demonstrated in chicken when it was shown that the increase in plasma T_3 during the last days of incubation and the first days posthatch was strongly negatively correlated with hepatic D3 activity, while no significant correlation was found with hepatic D1 activity (12) (Fig 1). Ontogenic studies of D1 and D3 activity in human liver suggested that hepatic D3 might be equally important in the regulation of plasma T_3 in the human fetus (13,14). Strong evidence for the importance of a decrease in D3 for normal development came from the finding that overexpression of D3 in *Xenopus* tadpoles inhibits normal metamorphosis (15). Tissue-specific and coordinated changes in D2 and D3 expression seem essential to ensure the correct intracellular T_3 levels at each specific stage of metamorphosis (16). During mammalian development, the high levels of D3 present in the placenta are thought to be important to inactivate part of the maternal T_4 and T_3 supply and protect the fetal tissues from an excess exposure to T_3 . The fact that D3 is abundantly expressed in rat and human endometrium suggests that D3 may also regulate local T_3 concentrations in the uterus during the process of implantation (17,18). Clear tissue-specific changes in D3 expression are also found in the fetal and early posthatch rat (19).

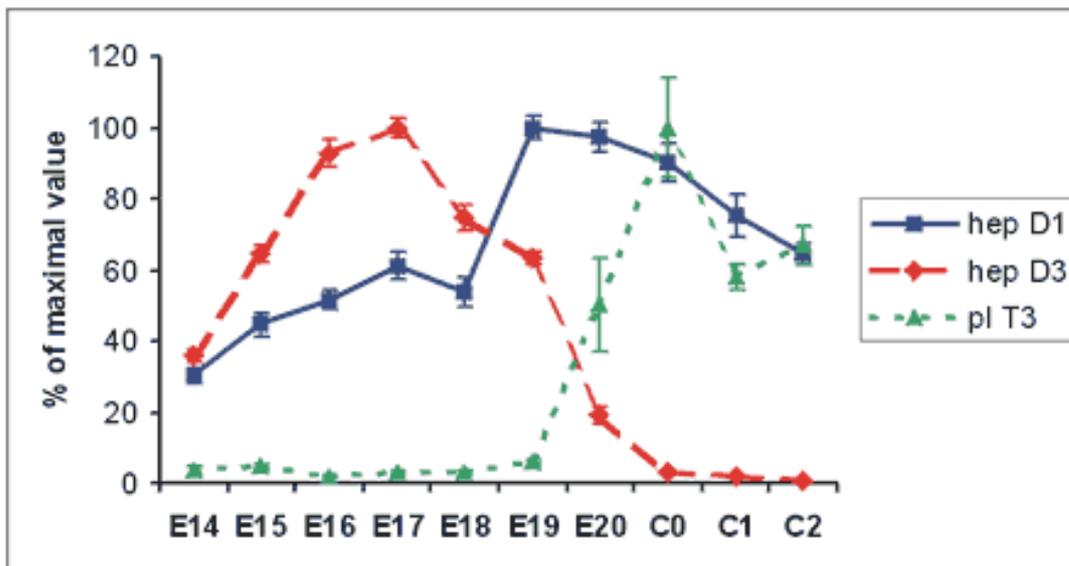


Fig 1: Relative changes in plasma T_3 and hepatic D1 and D3 activities in developing chicken embryos (E14-20) and newly hatched chicks (C0-2) (results taken from ref 12).

Hormonal regulation of D3

The D3 gene is known to be upregulated by T_3 (20), in agreement with its role in eliminating an eventual excess of T_3 . Several other hormones can change D3 expression and often change D3 activity in an acute way. Growth hormone (GH) treatment has been shown for long to increase circulating T_3 in several mammalian, avian and fish species. In vitro deiodination tests showed a higher recovery of T_3 following incubation of liver homogenates with T_4 and it was logically concluded that GH stimulates the conversion of T_4 into T_3 . This process was more recently studied in detail in the embryonic chicken liver making use of the knowledge that both D1 and D3 are present in liver and using isoform-specific in vitro tests. It was found that a single injection of GH had no effect on hepatic D1 activity but strongly decreased D3 activity within 2 h after injection (Fig 2). Although this remains to be investigated in other vertebrates, the rise in plasma T_3 in the chicken can be explained by a decreased hepatic T_3 breakdown by D3 (21). A similar decrease in hepatic D3 has been shown to play a role in the glucocorticoid-induced increase in plasma T_3 observed in embryonic chickens (22) (Fig 2) and in larval amphibians (23). The fact that adult hepatic D3 levels are manifold lower may help explain why glucocorticoid treatment in posthatch chickens not only decreases T_4 (as is the case in chicken embryos) but also decreases T_3 , as it does in postnatal mammals. Studies at the molecular level have shown that the effect of GH and glucocorticoids in chicken take place directly at the level of gene transcription (7). In *Xenopus* tadpole too, GH downregulates hepatic D3 expression while it is upregulated in tail tissue (24). This clearly illustrates the tissue-specificity of hormonal effects on D3 expression, confirming findings in other species (7, 22, 23).

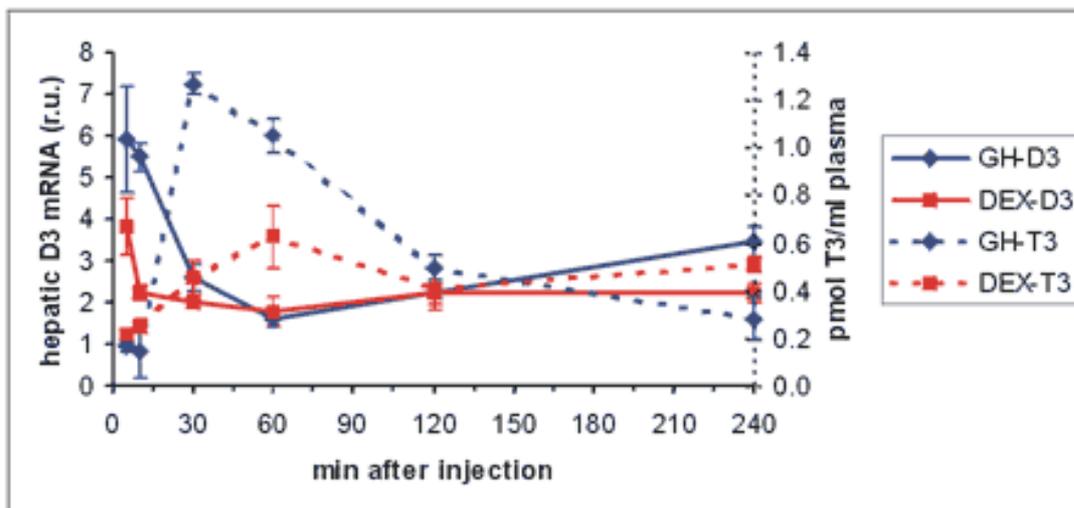


Fig 2: Acute changes in hepatic D3 expression following a single injection of growth hormone or dexamethasone in 18-d-old chicken embryos (results taken from ref 33).

Changes in D3 during food restriction and illness

While the importance of D3 in TH metabolism during early development is generally accepted by now, its role in adult life is

easily underestimated. One of the main reasons is undoubtedly the fact that the amount of D3 present in most adult tissues is very low or even non-existing. However, the number of reports on changes in D3 expression, mainly upregulation, in mature animals and men continues to grow. Food restriction decreases circulating T_3 concentrations in all vertebrates. In rat, this could be easily explained since fasting induces a decrease in plasma T_4 as well as in hepatic D1 activity. In chicken, short term (1-2 d) fasting does not lower plasma T_4 while T_3 is decreased. When the time course of changes in hepatic D1 and D3 activity were followed in fasted chickens, it was found that D1 remained fairly stable, while D3 was increased within 4 h after food removal, concomitant with the decrease in plasma T_3 (25). Refeeding reduced D3 back to basal levels within 2 h (26). These rapid changes in D3 expression were specific for liver. Since this organ is the main regulator of circulating T_3 levels, the rapid increase in D3 could serve as an energy saving mechanism by reducing T_3 supply to peripheral tissues. When rats were subjected to only partial food restriction, avoiding the fasting related hypothyroidism, it could be shown that in this species too, hepatic D3 increased more than 3-fold, while hepatic D1 and plasma T_4 remained stable (27). The expression of D3 is also increased under some pathological conditions. During experimentally induced cardiac dysfunction in rat, D3 activity was several times increased in hypertrophic right ventricular tissue compared to non-hypertrophic left ventricle. The increase was highest in animals in which hypertrophy progressed to heart failure and this may contribute to a local hypothyroid state (28). Increased D3 expression has also been reported in human tumours, such as pituitary tumours (29), hemangiomas (30) and a hepatic vascular tumour (31). In the latter case, the decrease in circulating T_3 and T_4 as well as the increase in rT_3 illustrates that even in adults, increased hepatic D3 expression can severely affect thyroid status. Recently, induction of D3 expression has also been shown in liver and skeletal muscle of critically ill patients (32). Together with the lowered hepatic D1 activity, this probably contributes to the decreased plasma T_3 and increased plasma rT_3 levels.

Conclusion

Although early studies on TH deiodination mainly focussed on D1 and D2 activities to estimate T_3 production, it has become clear that the T_4 and T_3 inactivating D3 deserves full attention as well. High amounts of D3 are present in many tissues during the early stages of ontogeny. The tissue-specific changes in D3 expression during development are proof for its essential role in regulating the correct local T_3 supply at the appropriate developmental stage. The fact that D3 expression is acutely controlled by several hormones that are also known to be important for normal development, adds to this evidence. The role of D3 is, however, not restricted to early life. The mostly low to even zero levels in adult tissues can be manifold increased in adverse conditions such as food restriction and illness. The effects of this increase may remain local or may affect T_3 supply to the whole body.

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Physiological Roles of the Iodothyronine Deiodinases

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Introduction:

The concept that iodothyronine deiodination might play an important role in thyroid hormone (TH) action began in 1951 when Gross and Pitt-Rivers identified the 5'-monodeiodinated derivative of thyroxine (T₄), 3,5,3'-triiodothyronine (T₃), in human plasma (1) and rat thyroid (2). They demonstrated that it was more potent than T₄ in several bioassays (3), a finding that led them to propose that T₃ was the active TH and T₄ was its precursor.

However evidence to support this hypothesis was not immediately forthcoming. Although it was clear that T₄ could be converted to T₃ in human tissues *in vitro* (4), attempts to identify [¹³¹I]T₃ in human plasma following injection of [¹³¹I]T₄ yielded equivocal results (5, 6). Furthermore, while there were some exceptions (7-9), most studies in animals did not substantiate the view that plasma T₃ was derived by the peripheral deiodination of T₄ (10). It was not until 1970, when the development of binding-displacement techniques permitted the measurement of very low levels of stable TH in plasma (11), that T₃ was demonstrated unequivocally in the plasma of athyreotic subjects following administration of T₄ (12). This was followed by a plethora of studies in both man and animals strongly suggesting that a majority of the T₃ in plasma is produced by peripheral deiodination of T₄ (13). Accumulation of evidence, which led ultimately to the recognition of T₃ as the hormone primarily responsible for TH action, started shortly thereafter with the identification and characterization of the nuclear receptors (14), and the discovery that they were occupied primarily with T₃ (15).

Characterization of the Deiodinases:

In the last twenty-five years, much effort has been expended by many investigators to characterize the systems responsible for deiodinating the TH. Conversion of T₄ to T₃ by 5'-deiodination (5'D) has been demonstrated in many mammalian tissues and in tissues of other vertebrates (16-21). It soon became apparent that there were major differences in the characteristics of the process among tissues, and further studies using kinetic analyses and specific inhibitors revealed that there are two separate enzymes in mammals capable of catalyzing 5'D (18, 22): type 1 (D1), and type 2 (D2). A notable finding was that in mammals these enzymes respond differently to changes in thyroid status (16, 18). Meanwhile significant amounts of 3,5,3'-triiodothyronine (rT₃), a metabolite with minimal thyromimetic activity, had been documented in plasma, and the evidence suggested that it was derived primarily from inner-ring or 5-deiodination of T₄ (23). It was found that D1 can catalyze both 5'D and 5D of iodothyronines depending on the sulfation state of the substrate. T₄-sulfate and T₃-sulfate undergo preferential 5D, whereas the non-conjugated iodothyronines T₄ and rT₃, undergo preferential 5'D (24, 25). However, a third deiodinase, the type 3 (D3), which catalyzes only the 5D of T₄ and T₃ thereby inactivating both hormones, has been characterized and its existence as a separate enzyme clearly established (26, 27).

Cloning of the deiodinases:

The existence of three separate deiodinases was established unequivocally by the cloning and characterization of their cDNAs. In 1991, *Berry et al.* reported the cloning of a cDNA for the rat D1 enzyme and demonstrated that it coded for a protein that contained a selenocysteine at its active site (28). Subsequently they characterized a functional selenocysteine insertion sequence in its 3'-untranslated region (29). D1 cDNAs have now been isolated from human (30), dog (31), mouse (32), chicken (33) and fish (34, 35).

In 1993, Wang and Brown reported the cloning of a cDNA, XL-15, from a subset of *X. laevis* genes that are upregulated by T_3 . It was subsequently shown to code for an amphibian D3 (36). D3 cDNAs have since been isolated from rat (37), *R. catesbeiana* (38), human (39), chicken (33) and tilapia (40) were isolated.

Finally, an amphibian D2 cDNA was cloned using a PCR strategy, primers based on the conserved regions of the known D1 and D2 cDNAs, and *R. catesbeiana* hind-limb tissue (41). This cDNA was then used to isolate its homologue in rat (42). D2 cDNAs have also been isolated from human (43), chicken (44) and fish (45, 46).

All these D1, D2 and D3 cDNAs code for selenoproteins in which the selenocysteine is essential for efficient deiodination of iodothyronines.

Physiological roles of the deiodinases:

There is considerable evidence to suggest that the deiodinases play a critical role in regulating plasma and intracellular levels of T_3 in peripheral tissues, both during development and in adult forms. Since the enzymes are differentially expressed in tissues (17, 18, 47) and in some cases in cell types within a tissue or organ (48, 49), the intracellular level of T_3 presumably varies among cells according to the complement of D1, D2 and D3 activity present.

Because the activities of the deiodinases are themselves subject to regulation by a variety of factors, the most notable of which is thyroid status per se, they also appear to provide some protection for some tissues against internal and environmental challenges that threaten thyroid hormone economy. Evidence also strongly suggests that the deiodinases play a critical role in coordinating growth and differentiation in developing species.

As will be discussed below these concepts of deiodinase function are based in large part on a body of compelling but mostly indirect evidence obtained in a variety of vertebrate species.

The roles of the deiodinases in development of lower vertebrates:

Lower vertebrate species, including fish, amphibia and birds, have been widely used as models for the study of thyroid hormone action, and evidence concerning the importance of the deiodinases in TH-dependent developmental processes is accumulating. In fish, the thyroid is important for many processes including the transformation from the larval and the juvenile phase (often termed metamorphosis) that generally occurs in teleost fish, and the parr-smolt transformation that takes place when young salmon move from fresh water to seawater (50, 51). As indicated above, all three deiodinases have been identified in fish. However, although their tissue profiles have been documented in some species and at some stages,

information regarding their ontogeny in this class of vertebrates is too limited at present to permit any conclusions to be drawn regarding their individual roles in regulating plasma or intracellular levels of T_3 .

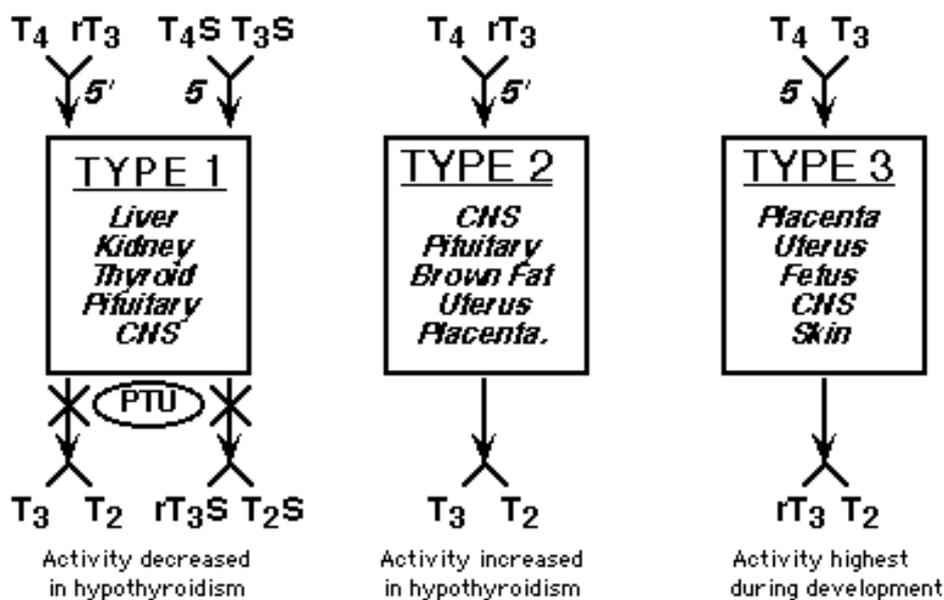
In contrast, studies in avian and amphibian species have been very fruitful. TH is essential in birds for yolk sac retraction, functional maturation of the lungs, pipping and hatching (52). In chick embryos, the plasma T_4 level starts to rise at embryonic (E) day 15 reaching a maximum just prior to hatching. However, the plasma T_3 level is minimal until E20 when it exhibits a sudden marked increase around the time of pipping (21). Ontogenic studies of hepatic D1 and D3 in chickens have revealed that D3 activity reaches a peak at E17 and then decreases markedly by the time of hatching, while D1 activity exhibits a more modest increase just prior to hatching (21, 53). These findings are consistent with the view that the sudden rise in plasma T_3 prior to hatching is achieved by a decrease in its breakdown by D3 and an increase in its generation from T_4 by D1.

The amphibian is a very valuable model since TH is essential for metamorphosis and development ceases completely in its absence. Relevant to the assessment of individual deiodinase function, the model offers a number of advantages. Thus the larvae are free swimming, several end-points of TH action can be readily measured (54) and it expresses only one 5'deiodinase, the D2 (55). A notable feature of amphibian metamorphosis is that, in spite of being exposed to the same plasma level of T_4 at any given time, not all tissues metamorphose at the same time. The hindlimb develops in prometamorphosis while the tail does not resorb until climax. Since both tissues express T_3 receptors prior to climax the potential of both tissues to respond to TH exists in both phases. The answer lies at least in part to the differential expression of D2 and D3 in tadpole tissues. During prometamorphosis, D2 is expressed in the hindlimb but not in the tail. During climax, by which time the hindlimb has developed, D2 is expressed in the tail while expression in hindlimb is minimal (56). These findings suggest that metamorphosis of individual tissues is dependent at least in part on the expression of D2 which is required for local generation of T_3 from circulating T_4 . Direct evidence for this view is provided by the finding that when tadpoles are treated with methimazole to block thyroid function, and iopanoic acid to inhibit D2 activity, the arrested hindlimb growth could be rescued with T_3 but not with T_4 (56).

The role of deiodinases in mammals:

It is generally accepted that D1-expressing tissues, which include liver, kidney and thyroid, generate T_3 from T_4 primarily for export to plasma, while D2-expressing tissues such as brain, pituitary and brown adipose tissue (BAT), generate T_3 from T_4 primarily for local use. This concept arose from a study in which the proportions of the receptor-bound T_3 derived from plasma and from locally generated T_3 were determined in a number of tissues (57, 58). The D3, which is expressed primarily in placenta, uterus, fetal and neonatal tissues, likely serves to protect tissues from exposure to inappropriate levels of TH (17).

Classification of the deiodinases and their tissue distribution in mammals.



There is good indirect evidence to support these concepts. For example, in hypothyroidism, fetal and neonatal rats can maintain T_3 homeostasis in the brain to a greater degree than would be expected from changes in the plasma T_4 levels, probably due in part to the accompanying increase in the level of D2 activity in the brain (59). A second example concerns the D3. Pregnancy is associated with the expression of D3 activity at very high levels, first in the decidual tissue surrounding the blastocyst, and later in placenta, the epithelial cells of the uterine wall and in the fetus per se. It is postulated that these high levels of D3 activity protect the fetus from exposure to maternal levels of T_3 and T_4 which are much too high for normal fetal development (48). However, another possibility is that the rT_3 generated from T_4 by the D3 has some physiological function of its own. It is present at much higher concentration in the fetus than in the adult (60, 61) and, although it is a relatively inert iodothyronine with respect to the genomic actions of TH, it is known to have some physiological activity (62). These concepts of the functions of the deiodinases are basically working hypotheses supported by abundant indirect evidence, but awaiting direct proof. A major factor limiting the study of the physiological roles of the individual deiodinases is the lack of suitable specific inhibitors. Thus studies are currently being carried out in mice made deficient in a single deiodinase by targeted disruption of the relevant gene. Knock-out mice models have been created in our laboratories for all three deiodinases and all are viable and capable of producing live offspring. The D2-deficient mouse (D2KO) has been further characterized. It appears healthy, reproduction is unimpaired and growth is only transiently slowed. However, hearing is impaired (63) and, although the D2KO mouse can survive in the cold, thermoregulatory processes in BAT are impaired (64). Moreover, although the plasma T_3 level is normal, plasma levels of both T_4 and TSH are elevated and further studies have confirmed that the mouse exhibits a phenotype of pituitary resistance to T_4 (65). Since the pituitary and BAT are two major sites of D2 expression, and D2 activity rises dramatically in the mouse cochlea just before the onset of hearing (66), these findings provide some direct evidence for the importance of D2 in the local generation of T_3 from T_4 in these three tissues.

However, a third major site of D2 expression is the central nervous system (CNS) and the available data indicate that most of the receptor-bound T_3 is generated locally, presumably by the D2 (58). Thus one would anticipate that, in the absence of D2, the mice would exhibit problems in locomotion and impairment in learning and memory skills similar to those observed in

congenitally hypothyroid mice (67). However, none of these functions is impaired in the D2KO mouse (Galton, unpublished data). These findings suggest that at least some parts of the developing CNS in the mouse are not dependent on the presence of D2 to furnish the necessary intracellular levels of T_3 required for thyroid hormone-dependent development. Further studies regarding CNS development are in progress.

Future directions:

With models of D1, D2 and D3-deficient mice now in hand, it should be possible to determine unequivocally the roles of the three enzymes. In addition, by making double and triple deiodinase-deficient mice, assuming they are viable or can be rescued with appropriate treatment, we hope to seek answers to other intriguing questions. How essential to life are the deiodinases as a group? Does T_4 have inherent physiological activity? In other words, in the absence of all 5'D activity, can T_4 given in sufficient amounts, act like T_3 ? Does T_4 have some other hitherto unknown effects? Only one thing is certain: we will be in for some surprises.

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TSH RECEPTOR STRUCTURE-FUNCTION RELATIONSHIP

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Introduction

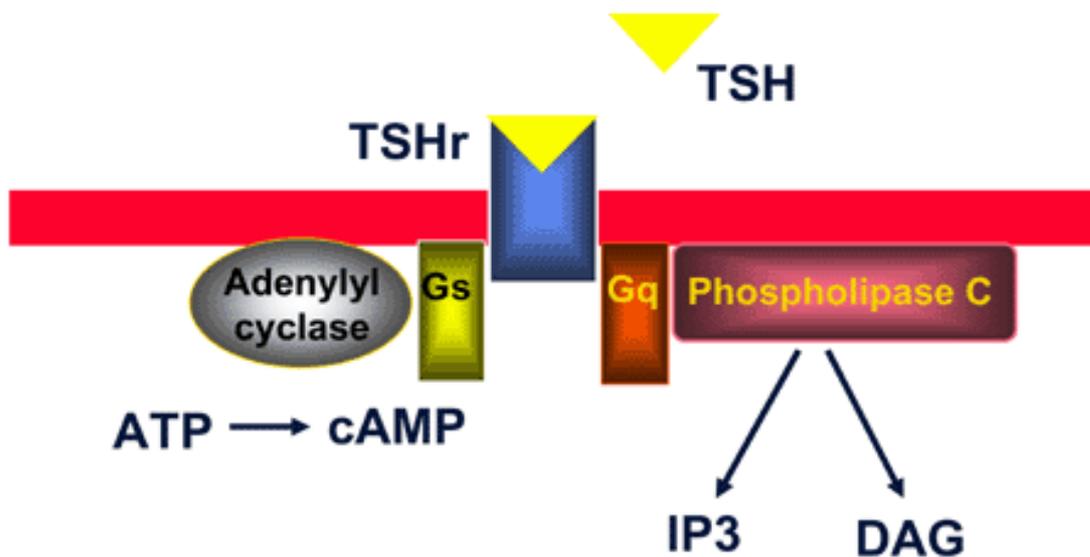
The thyrotropin receptor (TSHr) is a key protein in the control of thyroid function and a major thyroid autoantigen (1). After cloning the complementary DNA (cDNA) of the TSHr considerable progress in elucidating the structure and function of the TSHr has been made. Analysis of recombinant TSHr proteins expressed in prokaryotic and eukaryotic systems has indicated that post-translational processing is important for the formation of active receptor. Studies of TSHr glycosylation have shown that a mature form of the receptor containing mainly complex-type sugar residues is principally involved in TSH and TSHr autoantibody (TRAb) binding. Sulfation of the TSHr is also required for efficient recognition and activation of the receptor by TSH. The processing of the TSHr peptide chain into two subunits observed with native receptor has been confirmed using recombinant TSHr. However, the binding site(s) for TSH and TRAb on the TSHr have not been well characterized. The discovery of naturally occurring amino acid mutations of the TSHr confirm the complexity of the hormone and autoantibody binding sites.

TSHr STRUCTURE

The TSH, LH/CG and FSH receptors belong to a subfamily of G protein-coupled receptors and their primary structure, as deduced from their cDNA, predicts the existence of seven segments with hydrophathy (in common with all the other G protein-coupled receptors) compatible with transmembrane segments (1, 2). The glycoprotein receptor subfamily (TSH, LH/CG, FSH) share common characteristics that distinguish them from the other G protein-coupled receptors. They contain a signal peptide (20 amino acids for the TSH receptor) and they have a long extracellular aminoterminal domain (398 amino acids for the TSH receptor) with the loose repetition of a motif of 25 residues rich in leucine (1, 2), with six potential N-linked glycosylation sites. Similar leucine-rich motifs are also found in a number of widely different proteins (3, 4) which confer the ability to interact with other proteins.

From site-directed mutagenesis studies it seems that the binding specificity and the effector properties of the glycoprotein receptors are encoded in separate domains of the protein (1); the extracellular N-terminal domain mediates the binding specificities and the portion with the seven transmembrane domains display the effector properties triggering G-protein activation. When aligned, the three glycoprotein receptors show stronger conservation in the transmembrane domains (70% homology) than in the extracellular domain (40% homology). A peculiarity of the TSH receptor is a 51 residue insert at the hinge between the extracellular and the first transmembrane segment with no counterpart in the FSH or LH/CG receptors. A first model of the three dimensional structure of the thyrotropin receptor has been recently proposed (5) based on the analogy with another leucine-rich repeats protein, the ribonuclease inhibitor, which has been crystallized.

PHYSIOLOGY OF THE TSHr



In human thyroid TSH activates both the cAMP and the phospholipase C-diacylglycerol regulatory cascades, although the latter effect requires concentrations of hormones 5 to 10 times higher than the former (1, 6, 7). This activation is mediated by the TSH receptor and it involves the exchange of GDP by GTP and the consequent dissociation of G in its subunit alpha and beta-gamma; the former, to which GTP is bound stimulates the effector enzymes a) adenylyl-cyclase which generates cyclic AMP (cAMP) from ATP and is important for growth and differentiation and b) phospholipase C cascade which stimulates the production of inositol 1,4,5-triphosphate (IP3) and diacylglycerol, which are important for iodination and hormone synthesis (1, 6, 7).

Modifications, over or under production of the natural ligand (TSH), or the presence of agonists or antagonists of the natural ligand (example antibodies or drugs) or alterations of the intrinsic mechanism of the receptor activation may result in diseases.

RECOMBINANT THYROTROPIN RECEPTOR

The TSHr is present in very low numbers on the surface of thyrocytes, which has made the receptor difficult to clone and, even after the cDNA sequence had been published, difficult to produce in high numbers and to purify (8). Various methods of expressing the recombinant TSHr in different systems to obtain larger quantities of the protein for purification and production of both polyclonal and monoclonal antibodies have been investigated. Low levels expression of some G-protein receptors have been obtained in Escherichia Coli. However, the full length TSH receptor has not been successfully expressed in this system. Large amounts of TSH extracellular domain in prokaryotic systems can be produced but most protein is present in inclusion bodies and the denaturated non-glycosylated product does not bind TSH (8). Despite extensive studies neither the full-length nor the extracellular domain of the TSHr has been successfully expressed in Yeast (8). A third system using the baculovirus system failed to produce full-length or the extracellular part of the TSH receptor or a low yield was obtained (8). Eukaryotic expression systems such as Chinese hamster ovary (CHO) cells, 293 human embryonal kidney cells, L cells, and a transformed myeloma cell line SP65, have been used to produce stably transfected cell lines expressing the TSHr (8). In each case the recombinant receptor produced is expressed on the cell surface, is functional for hormone (TSH) binding, is coupled to cAMP, is highly glycosylated and is able to bind TSH receptor antibody (TRAb). The amount of receptor produced in CHO stably transfected with the cDNA of the human TSHr or in transient expression in COS cells is not sufficient for purification because the trypsin treatment necessary to detach cell causes proteolytic cleavage, even if an adaptation of CHO cells grown in suspension has been reported. Until very recently, constructs encoding the complete extracellular amino-terminal domain (ECD) alone did not yield bioactive material capable of binding TSH with high affinity. Costagliola et al. (9) created a chimeric cDNA construct encoding the ECD of the TSHr fused to the signal for addition of glycosylphosphatidylinositol from the Thy-1 gene which directs efficient expression of the ECD at the plasma membrane of transfected CHO cells (9). Treatment of these transfected cells with a specific phospholipase C released a soluble 80 kDa molecule which neutralizes the antibodies from Graves' patients. Whereas it does not bind TSH when released from the cells after incubation with phospholipase C in free form, the soluble ECD displays clear TSH binding activity when it is released as a complex with a monoclonal antibody recognizing a conformational epitope of the ECD (9). These observations together with those from Osuga et al (10) showed that the complete ECD of the TSHr require additional signal sequences to be correctly targeted to the plasma membrane in a native form. In the holoreceptor, the signaling is probably carried out by the serpentine portion of the receptor itself. It is likely that the Glycosylphosphatidylinositol-anchoring peptide (9) provide adequate substitutes for this targeting, allowing a significant proportion of the ECD molecules to undergo normal glycosylation and maturation, during the journey through the membrane system of the cell. The soluble ectodomain could be released from the cells by treatment with a GPI-phospholipase C and purified to apparent homogeneity by chromatography (11). This soluble ectodomain purified in a functionally competent conformation allows direct studies of its interaction with TSH and autoantibodies and open the way to structural studies.

TSH RECEPTOR AS A TARGET OF AUTOIMMUNITY

To increase our understanding of the processes involved in the pathogenesis of autoimmune thyroid disease it is important to understand the structure of the TSHr and especially the sites of interaction between the receptor, TSH and TRAb. TSH receptor antibodies can be classified as: a) TBII which inhibits the binding of TSH to the receptor b) TSAb which stimulates cAMP production and are responsible for growth and hyperfunction of thyrocytes characteristics of Graves' disease and c) TBAb which inhibit TSH mediated cAMP accumulation and are the cause of some cases of hypothyroidism in Hashimoto's thyroiditis and idiopathic myxedema.

There have been several reports using chimeras of full-length TSHr with segments of the LH-CG receptor extracellular domain exchanged for the corresponding regions in the TSHr extracellular domain expressed in CHO cells. The reports have concluded that the binding sites for TSH and TRAb are not identical but appear to overlap and cover most of the length of the extracellular domain of the receptor (8).

Another approach to studying the binding sites of the TSHr has involved the use of synthetic peptides corresponding to regions of the TSHr extracellular domain and the effects of these peptides on stimulation of cAMP production by TSH and TRAb has been investigated. Polyclonal antibodies have been raised to synthetic TSHr peptides in both rabbit and chicken while bacterially expressed fusion proteins of the TSHr extracellular domain and the TSHr extracellular domain expressed in the baculo virus system have also been used to immunize rabbits. There are conflicting results as to whether antibodies do or do not inhibit TSH binding to the TSHr. Some of these studies reported that the binding sites for TRAb with TSH antagonistic activity were at the C-terminal segment of the TSHr extracellular domain whereas TRAb with TSH agonistic activity bound to the N-terminal part of the TSHr extracellular domain (8).

The production of human monoclonal antibodies to the TSHr has proved to be very difficult (8). Isolation of Epstein-Barr virus transformed, IgG expressing B cell lines from patients with autoimmune thyroid disease with TSH agonist and TSH antagonist activity has been reported but these preparations do not inhibit TSH binding to TSHr. To obtain a true TSHr stimulating monoclonal antibody, several animal models of Graves' disease have been generated in recent years. Murine monoclonal TSHr antibodies generated with these models have been shown to recognize the native conformation of the TSHr, but all have been without thyroid stimulating activities. Recently Ando et al (12) isolated a TSHr-stimulating monoclonal antibody that had a marked thyroid-stimulating activity at nanogram concentrations. This antibody recognized a conformational epitope. By using genetic immunization Costagliola et al (13) were able to produce a monoclonal antibody with thyroid stimulating activity and surprisingly this antibody was very effective in detecting the purified ectodomain in hTSHr on Western Blot. The mere existence of monoclonal antibodies directed against the TSHr and capable of activating it tells us that there is no need for cooperation of multiple immunoglobulins with different recognition specificities to achieve stimulation of the receptor in Graves' disease. The question, however, remains whether recognition of different epitopes, overlapping or not, would similarly result in receptor activation. Further experiments will be needed to determine the relation if any between the epitope identified here and those of autoantibodies from Graves' patients. If there is structural relation between them, monoclonal antibodies may constitute tools allowing development of in vitro binding assays capable of differentiating autoantibodies with TSAb from those simply displaying TBII activity.

POST-TRANSLATIONAL MODIFICATIONS OF THE TSHr GENE

Glycosylation

The extracellular domain of the hTSHr contains six potential N-linked glycosylation sites and has been shown to be heavily glycosylated with approximately 35 kDA of carbohydrate residues contributing to the overall molecular weight when expressed in CHO cells (1). However when expressed in E Coli the TSHr is unglycosylated and has been found to be incapable of both high affinity TSH binding and autoantibody binding (8).

The full-length TSHr expressed in CHO cells has been shown to consist of two species of full-length receptor, one of approximately 100 kDA and the other approximately 120 kDA. Pulse labeling of L cells expressing the TSHr showed that the 100 kDA product was produced first and was shown to be the precursor for the upper band of 120 kDA. Some of the upper band, mature receptor, then appeared to be cleaved into two subunits. The upper band contained complex-type carbohydrate residues with a high content of sialic acid. The lower band contained predominantly high mannose type carbohydrates (8).

Sulfation

Sulfation of tyrosines is a late post-transcriptional modification taking place in the trans-Golgi network and affecting a wide spectrum of membrane or secreted proteins. Recently, sulfation of tyrosine residues of the N-terminal extension of three GPCRs belonging to the chemokine or chemoattractant receptor family has been demonstrated. In CCR5 tyrosine sulfation was required for high affinity recognition of the receptor by its natural agonist. Similarly to the situation described recently in CCR5, Costagliola et al. (14) demonstrated that the TSHr, as it is present at the cell surface, is sulfated on tyrosines in a motif located downstream of the C-terminal cysteine cluster. Sulfation of one of the two tyrosines in the motif is mandatory for high affinity binding of TSH and activation of the receptor. Site directed mutagenesis experiments indicate that the motif,

which is conserved in all members of the glycoprotein hormone receptor family, seems to play a similar role in the LH or FSH receptors.

TSH RECEPTOR MUTATIONS

G protein-coupled receptor naturally occurring mutations can be cause of diseases. Depending on the nature of the mutation (somatic, germline), and on its localization in the protein, and in the case of dominant diseases differences in genetic background as well as environmental factors, can be responsible for different phenotypes.

TSHr gain-of-function mutations

Any molecular lesion leading to constitutive activity of the cAMP cascade (TSH receptor, G protein, cyclase, protein kinase) could be responsible for the growth and hyperfunction typical of thyroid adenoma. After somatic mutations impairing GTPase activity of Gs-alpha had been found in some of these benign tumors it was logical to study the TSH receptor gene. In the first study from the group of Vassart (15, 16), nine out of eleven tissues studied were shown to harbor an activating TSHr mutation. Other studies have confirmed this observation, describing mutations in other residues (17, 18). All the mutations found are heterozygous, as expected from gain-of-function mutations with dominant effect, and confined to the adenomatous tissue.

Recently we (19) reported that similarly to solitary toxic thyroid adenoma, activating TSHr mutations are present in single hyperfunctioning nodules (either adenomas or hyperplastic nodules) within toxic multinodular goiters in which nonfunctioning nodules also coexist.

TSHr loss-of-function mutations

Mutations that inactivate the thyrotropin receptor protein can cause thyrotropin resistance, resulting in either hypothyroidism or euthyroidism depending on the completeness of the defect (18). When transfected in COS cells the mutated thyrotropin receptors showed no or a reduced biological activity (18).

STRUCTURE-FUNCTION RELATIONSHIP OF THE TSHr, AS DEDUCED FROM THE STUDY OF ACTIVATING AND INACTIVATING MUTATIONS

The majority of activating mutations of the TSHr gene have been studied by transient expression in COS cells. When mutant receptors are transiently expressed from recombinant constructs in COS cells the result is a constitutively stimulation of cAMP accumulation (18).

Interestingly these experiments clearly show that the wild type TSH receptor displays easily measurable constitutive activity (18). When comparing all G protein-coupled receptors not all show significant basal activity. For example while the TSH receptor shows a measurable constitutive activity when expressed in COS cells the LH/CG receptor displays little constitutive activity if any (18). In agreement with a current model for G-protein coupled receptor activation, this observation suggests that the unliganded TSH receptor would be less constrained than others when in the inactivate state (18). This model has two important physiopathological consequences: 1) a minor structural alteration caused by a mutation determines a destabilization which is responsible for a phenotype and the diversity of mutations able of increasing the constitutive activity of the TSHr is surprisingly high with respect to other receptors 2) the existence of a basal tonic activity opens the possibility of regulating them negatively by so called inverse agonists (20).

Some mutants (two with modified residues in the extracellular loops, one in the third intracellular loop and one in the sixth transmembrane segment) activate also the phospholipase C-dependent cascade (18). The different mutant receptors show a different level of expression when transfected in COS cells in identical conditions; to compare their efficacy we derived a specific constitutive activity (basal cAMP/receptor number); some mutants, though expressed at low levels at the surface, cause strong stimulation of the cAMP cascade (I486F, T632I, C672Y). Besides, for many mutants a higher affinity for the ligand is observed. Most mutants respond to stimulation by TSH by further increasing both cAMP and inositolphosphate accumulation, but the magnitude is highly variable, some mutations behaving as if they were fully activated (e.g. mutant I486F for cAMP) or displaying very little stimulability (e.g. mutation C672Y for inositol-phosphates). Other mutants (N670S for example) clearly show a dissociation in the ability of the receptor to respond to bTSH for the Gs-alpha and Gq-alpha dependent regulatory cascades, favoring the idea of the existence of multiple active conformations of the TSH receptor, with differential capabilities to couple to Gs-alpha and Gq-alpha.

A current favored model for GPCR activation holds it that a structural constraint is responsible for the maintenance of unliganded receptors in the inactive state. This model was elaborated from the observation that a variety of aminoacids substitutions, first in the third intracellular loop of adrenergic receptors, then in transmembrane helices of other receptors could activate them in the absence of agonists. A series of experimental observations suggest that the extracellular domain of the TSHr could contribute in keeping its serpentine portion inactive. 1. Aminoacid substitution in the first (Ile 486) and second extracellular loops (Ile 568) are amongst the strongest activating mutations identified. 2. The TSH receptor can be

activated by a limited proteolytic treatment by trypsin which removes an epitope (residues 354-359) of the extracellular domain. 3. The group of Kosugi (21) has demonstrated significant increase in constitutive activity of a deleted mutant lacking residues 339-367. These observations are compatible with a model in which the unliganded inactive conformation of the receptor would be stabilized by interactions between the extracellular N-terminus and the extracellular loops. Ruptures of these interactions would activate the serpentine portion while increasing the affinity of the extracellular domain for TSH binding. According to the model, unliganded receptors would exist as an equilibrium between a closed inactive conformation and an open active conformation lacking the interaction between the loops and the N.-terminal domain (22). The concentration of the latter would be responsible for the constitutive activity of the wtTSHr. Binding of TSH to the extracellular domain would activate the receptor by stabilizing the open conformation. The model does not exclude that an interaction of TSH with the extracellular loops contributes to the stabilization of the active conformation of the serpentine portion as suggested by some experiments with LH receptor (22).

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RECENT DEVELOPMENTS IN THE DIAGNOSIS AND THERAPY OF DIFFERENTIATED THYROID CARCINOMA (I).

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Introduction

Despite the low incidence and favorable prognosis, non-medullary thyroid carcinoma presents important diagnostic and therapeutic challenges. In this first part, new developments in initial diagnosis and therapy will be reviewed. In the second part, to be published in a forthcoming issue, recent research on therapeutic targets for recurrent and metastatic disease will be discussed.

Initial Diagnosis

Fine Needle Aspiration (FNA) is the procedure of choice in patients presenting with thyroid enlargement, with the exception of patients with suppressed thyrotropin levels. The sensitivity of FNA is 90-95%, but the specificity is lower, 60-80%, as FNA cannot distinguish between benign and malignant follicular lesions (1). The frequency of follicular thyroid carcinoma in hemithyroidectomies performed after 'suspicious' FNA is only 20-30%, in other words: 70-80% of the patients with suspicious FNA will undergo unnecessary thyroid surgery (2). Furthermore, even when the nodule appears to be malignant, a second 'completion' thyroidectomy will be performed in most patients.

Many attempts have been undertaken to identify molecular markers for diagnosis and prognosis. Candidate markers can be distinguished in 3 groups: thyroid specific proteins that lose expression during thyroid dedifferentiation (including thyroid peroxidase (TPO)), chromosomal rearrangements specific for thyroid carcinoma and markers associated with malignant transformation in general, like oncogene activation and proteins involved in cell cycle regulation and apoptosis, cellular attachment, extracellular matrix and angiogenesis.

The most promising markers to improve the diagnostic accuracy of FNA to date are TPO and galectin-3. In the process of dedifferentiation, TPO appears to be the first protein with diminished expression. Clinically, this leads to decreased organification of iodine, which may have consequences for radioiodine therapy. In studies originally initiated by De Micco et al, a cut-off value of 80% of thyroid epithelial cells staining positive with the anti-TPO antibody MoAb47 has been found to have superior sensitivity (100%) and specificity (up to 99%) for follicular carcinoma in surgical specimens (3). Later studies, mostly from the same center, confirmed the diagnostic value of TPO immunostaining in FNA. In the distinction between follicular adenoma and carcinoma, sensitivity is reported around 100%, specificity varying from 61-99% (4; 5).

Recently, studies have been published on the diagnostic properties of galectin-3 immunostaining. The galectins are carbohydrate binding proteins involved in cell adhesion, cell growth and cell death. The diagnostic potential of galectin-3 was suggested in surgical samples (6). Subsequent small studies confirmed the diagnostic potential in FNA, sensitivities and specificity ranging from 82-100% and 88-100% respectively (7; 8). In a large multicenter study, FNA staining with galectin-3 had a sensitivity and specificity of 88% resp. 91% in a retrospective series, and 100% and 94% in a prospective series (9). However, in a recent publication, galectin-3 was found in 45% of adenomas and 17% of multinodular goiters (10).

Nevertheless, these studies are promising: as in the present practice the specificity of FNA for follicular carcinoma within the subgroup of 'follicular proliferation' is zero (all patients are operated), any increase in specificity will decrease the number of surgical procedures. A study combining TPO and galectin-3 staining may be particularly worthwhile, but has not been published to date.

Other molecular markers that have been investigated include telomerase activity. Telomerase is an enzyme that adds nucleotides to telomeres, DNA sequences at the ends of chromosomes that enhance chromosomal stability. Assessment of telomerase in thyroid FNA reveals telomerase in 14-38% of follicular adenoma and in 75% of follicular carcinoma (11; 12), indicating that these assays alone have insufficient diagnostic properties as compared with TPO and galectin-3.

The observation of genetic rearrangements in thyroid carcinoma has offered new perspectives for diagnostic procedures. The detection of a PPAR γ /PAX8 rearrangement in 75% of follicular carcinoma's and not in papillary carcinoma and follicular adenomas (13), suggested that this rearrangement could be used as a diagnostic tool. However, in a recent study (14) the rearrangement was observed in 13% of follicular adenomas, which weakens the diagnostic value of this marker.

Where the above mentioned studies all followed a candidate-target approach, the recent introduction of high-yield genomic and proteomic techniques has provided enormous perspectives to identify diagnostic and prognostic factors in disease, as indicated by recent high-impact studies in breast carcinoma (15). Gene expression arrays in thyroid carcinoma have revealed differential expression of genes in papillary carcinoma as compared with follicular thyroid carcinoma and normal thyroid tissue, including genes so far not associated with thyroid carcinoma (16; 17).

Although so far no high-throughput studies have been published discriminating benign and malignant follicular thyroid tumors, it is to be expected that these techniques will lead to novel candidate markers.

Initial therapy

Initial therapy in thyroid carcinoma involves near-total thyroidectomy followed by radioiodine ablation therapy in almost all cases. Although it can be argued that total thyroidectomy and radioiodine ablation are safe and have minimal side effects, the disadvantages of decennia-long TSH suppressive thyroxin substitution are gaining attention, not in the least from patient focus groups. Therefore, it would be worthwhile if at diagnosis, the extent of therapy could be tailored according to a more differentiated risk profile.

In contrast with other tumors, where additional immunohistochemical or molecular markers are included in the routine pathological diagnostics, the histopathological diagnosis of thyroid carcinoma is still based on histomorphological criteria. Prognostic factors are largely based on classic histology, TNM staging and epidemiological data. Even specific markers of thyroid endocrine function are not included in routine pathological examination, whereas for instance the expression of TPO and the human sodium iodide symporter have important implications for later radioiodine therapies (18).

In recent years, many studies have been published about potential prognostic molecular markers. Examples of markers associated with unfavorable prognosis include genetic alterations (19), factors associated with cellular proliferation (20), cellular adhesion, extracellular matrix and the cytoskeleton (21; 22), immunological markers (23) and angiogenesis (24). In a tissue-array study in Hürthle cell carcinoma, high expression of Ki-67, a cellular proliferation marker, was evident only in the clinically aggressive Hürthle cell carcinomas and was associated with significantly reduced survival. In contrast, positivity for the apoptotic protein Bcl-2 was associated with improved survival (25). As mentioned before, high throughput analyses may reveal prognostic markers: In a study by Chen et al. cDNA arrays revealed differentially expressed genes associated with metastasis of follicular thyroid cancer (26).

In conclusion, large scale studies are necessary in this low prevalent malignancy to confirm the diagnostic validity of diagnostic and prognostic factors identified so far, and to apply the expensive but high-yield innovative investigational tools.

Follow-up

Thyroxin suppression therapy

Virtually all patients who have undergone total thyroidectomy will receive thyroxin suppressive therapy. Although the rationale for this therapy is evident, it is not clear if all patients benefit from suppressed TSH levels, to what extent TSH should be suppressed and for how long. In two recent studies, it is concluded that in patients without evidence of active disease, TSH can be kept within the normal range (27; 28). In both studies however, the relation between TSH levels and prognostic markers was not studied. In a retrospective study, a longer relapse-free survival was found in patients with a consistently suppressed TSH than in patients with TSH > 1 mU/L (29). In a large study, with a median follow-up of 5 years, the level of TSH suppression (undetectable vs. normal) was a significant prognostic factor in high-risk papillary carcinoma only (30). However, until more definite answers from large scale analyses in USA cohorts, currently underway, become available, most physicians leave patients with thyroid carcinoma on TSH suppressive therapy while knowing that this may not be necessary in a majority of patients.

Diagnostics during follow-up

Although most follow-up protocols are based on measurements of serum Tg concentrations and radioiodine scintigraphy, no consensus exists on the indication, the frequency, the threshold values and the necessity of thyroid hormone withdrawal. The lack of consensus is related to technical aspects (for Tg reviewed in (31)), but more importantly, the absence of well designed clinical studies, with clear 'gold standards' for active disease. Overall, sensitivity and specificity of Tg measurements for active disease in patients after thyroid remnant ablation do not exceed ~90%. Sensitivity of radioiodine scintigraphy depend greatly on the 'gold standard' for disease, which is poorly defined in a surprising number of studies, but is generally around 75%.

New serological markers

Because of the limitations of Tg, novel serological markers have been searched for. Of interest is the demonstration of Tg mRNA in peripheral blood, which indicates the presence of circulating Tg producing cells (e.g. thyroid cancer cells). In a number of studies, Tg mRNA alone did not have sufficient diagnostic power to discriminate between patients with active tumor and thyroid remnants (32) or thyroid carcinoma and healthy volunteers (33). However, the combination of Tg and Tg mRNA allowed the identification of all patients with active disease in another study (34).

Interestingly, RT-PCR can also be applied to detect cells that produce other thyroid specific proteins. In a study on TPO (35), RT-PCR correlated significantly with metastatic disease.

Recombinant human TSH (rhTSH)

Because withdrawal from thyroid hormone therapy has obvious disadvantages, the value of rhTSH for diagnosis of recurrent thyroid carcinoma has been studied in several investigations. However, methodological imperfections prevent definite conclusions.

In 2 studies high concordance rates for radioiodine scintigraphy (36) and Tg (37) were found between withdrawal and rhTSH. In (36), the combination of Tg and scintigraphy during rhTSH detected all metastases. However, in this investigation, the study parameters (Tg and scintigraphy) were at the same time used as criteria for active disease. In (37), only radioiodine scintigraphy was used to define active disease.

In the following studies, no comparison was made between rhTSH and thyroxin withdrawal. In one study in 99 patients, rhTSH stimulation elevated Tg in 21 patients. Ultrasound of the neck revealed tumor in 6 of these patients but also in 2 patients without Tg elevation. Radioiodine scintigraphy was negative in all patients, indicating that radioiodine scintigraphy misses active tumor recurrences and is less sensitive than ultrasound for neck recurrences. Although Tg measurements after rhTSH are more sensitive than radioiodine for recurrent tumor, tumor in the neck can be present even in Tg negative patients (38).

In another study in 300 patients without evidence of disease, Tg was elevated in 18% after rhTSH (39), but no comparison was made with conventional withdrawal, and no 'gold standard' for active disease was defined. In a retrospective analysis in 366 patients, evidence for active disease was found in 76% of patients with Tg > 2 ug/liter after rhTSH and in 13% of those whose stimulated Tg was 2 ug/liter or less. No comparison with conventional withdrawal was made, whereas criteria for active disease were mainly based on WBS (40).

In an analysis of 107 patients, Tg measurements after rhTSH had a sensitivity of 100% and a specificity of 91% for tumor (cut-off 2 ug/l). Sensitivity of Tg during thyroxin (cut off value 0.5 ug/l) was only 45% and sensitivity of WBS after rhTSH was only 27%! (41).

It has also been investigated whether rhTSH may be sufficient for thyroid remnant ablation. In a study in 162 patients, it was found that thyroid remnant ablation was less successful in patients on thyroxin substitution who received rhTSH (54% successful ablation) than during conventional hypothyroid state (84% success rate) (42).

Other radiological imaging procedures

Ultrasound

The favorable results for ultrasound as found in (38) were also observed in a recent study: 494 patients underwent serum Tg measurements after thyroxin withdrawal, radioiodine scintigraphy and neck ultrasound. Neck recurrences were detected in 10% patients. Serum Tg had a sensitivity 57%, radioiodine scintigraphy a sensitivity 45.1%, whereas ultrasound had a sensitivity 94.1%. This interesting study confirms the role of ultrasonography in the surveillance of thyroid cancer (43).

Somatostatin Receptor Scintigraphy (SRS)

The fact that non-medullary thyroid cancer cells express somatostatin receptors (SSTR3 and SSTR5) lead to the investigation of SRS imaging and therapy in this disease. In 2 studies (44; 45), SRS had a moderate sensitivity for disease detection in metastatic thyroid carcinoma, but revealed lesions that otherwise would be undetectable by either radiological or FDG-PET imaging.

In addition, the use of somatostatin analogues can provide new therapeutic options, which will be reviewed in part 2.

18-F Fluorodeoxyglucose-positron emission tomography (FDG-PET)

The diagnostic accuracy of FDG-PET in patients suspected of recurrent thyroid carcinoma was analyzed in a systematic review of the recent literature (46). Although all 14 studies analyzed claimed a positive role, methodological problems were numerous. The most consistent data were found on the ability of FDG-PET to provide an anatomical substrate in patients with elevated serum Tg and negative radioiodine scans. FDG-PET during TSH stimulation may be more sensitive than during suppressive therapy (47).

Conclusion (1)

Important imperfections in the initial diagnosis, extent of initial therapy and diagnostic follow-up are present in thyroid carcinoma. These imperfections may be obscured by the overall favorable prognosis of thyroid carcinoma, but nevertheless result in a considerable burden for patients and the health care system in general. The new diagnostic developments as reviewed may offer opportunities to improve clinical protocols. However, clinical (and fundamental) research in this low-prevalent malignancy is often too fragmented and limited in study-design to allow definite answers. Instead, more research protocols should be initiated in multi-center settings. The important clinical and financial implications of the diagnostic dilemma's described should be able to attract support from health care organizations beyond clinical endocrinology.

Key-notes

Initial diagnosis

- >> Primary diagnosis of differentiated thyroid carcinoma is still hampered by the inability to distinguish between benign and malignant follicular lesions at FNA.
Recent studies suggest a role, among others, for TPO, galectin-3 and telomerase as markers to improve the specificity of FNA.
The recently described chromosomal rearrangement PPAR α /PAX-8 in follicular carcinoma, that was considered a candidate diagnostic marker, has also been detected in benign thyroid disease.
- >> Routine histopathological diagnosis is almost exclusively based on histomorphological criteria. The identification of molecular prognostic markers would enable to differentiate the extent of initial therapy as well as the follow-up regimen according to the risk-profile.
The introduction of high-throughput techniques may be expected to reveal novel diagnostic and prognostic markers, which is supported by recent studies in thyroid carcinoma.

Follow-up

- >> In the recent literature, controversies exist on the level, the duration and the patient selection for TSH suppressive therapy
- >> Imperfections in the routine diagnostic procedures during follow-up have led to the search for alternative markers and techniques:
 - Thyroglobulin measurements and radioiodine scintigraphy after recombinant human TSH may be comparable with thyroxin withdrawal.
 - Ultrasound appears to be superior to radioiodine scintigraphy in the detection of neck recurrences.
 - Reports on novel markers as Tg mRNA have not been very promising.
 - The value of somatostatin receptor scintigraphy and positron emission tomography is the ability to detect lesions that could not have been detected by radioiodine imaging.

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THE CONSORTIUM OF FACTORS REGULATING THYROID GROWTH AND FUNCTION

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Introduction

Hyperplasia of the thyroid gland is common and involves the coordinated growth of the vasculature (angiogenesis) and epithelial cell growth (folliculogenesis). In patients with multinodular goiters, blood tests usually show no elevation of circulating TSH levels and the patients are euthyroid. The role of TSH, which is the classical thyroid cell mitogen, is therefore uncertain. The vast majority of the nodules found are nonfunctioning and benign. What then is the stimulus for the frequent lumps in the thyroid, why are the nodules nonfunctional and why is the thyroid gland seemingly more susceptible to hyperplasia than other endocrine tissues? This brief treatise will explore putative interrelationships of TSH and non-TSH growth factors, their receptors, estrogen and thyroid hormones in human thyroid. First some of the growth factors will be considered.

SOME OF THE PLAYERS

Fibroblast growth factors. Thyroid follicular cells are known to express several angiogenic factors. These include FGF-1 and FGF-2 and probably other members of this large family (1, 2) . FGFs can stimulate not only the growth of the follicular cells, but also that of the cells of the mesenchyme (fibroblasts), the pericytes and the smooth muscle cells surrounding the vasculature and the endothelial cells (3). FGF inhibits thyroid cell iodide metabolism in FRTL5 cells (4), consistent with the non-functional property of many nodules. However, we found inconsistent effects of FGF on the growth and function in human thyroid cells (5) in vitro suggesting that there may be means to control FGF receptor activation. What stimulates FGF production and which cells are the source? In other tissues cAMP (6), estrogen (7), protein kinase C (PKC) activation (8) and thyroid hormones (9) have all been shown to directly stimulate FGF production.

Insulin-like growth factors. Insulin-like growth factors (IGF-I and IGF-II) are synthesised by thyroid cells (10). Like FGFs, IGFs exert effects on many tissues due to the ubiquitous expression of the IGF-I receptor. For both these growth factors, therefore, controls exist on the availability of the ligands and their receptors. Unlike the FGFs, the IGFs are essential for TSH effects on thyroid growth and function (5). Overexpression is therefore unlikely to inhibit thyroid function.

Angiopoietins. The angiopoietins, Ang1 and Ang2, bind with similar affinity to the endothelial cell receptor tyrosine kinase known as Tie-2. Although not potent mitogens for endothelial cells, Angs synergise with vascular endothelial growth factor (VEGF) in tube formation. Ang1 functions as an agonist for Tie-2, resulting in phosphorylation of its tyrosine kinase domain, whereas Ang-2 appears to act predominantly as competitive antagonist. Knockout studies have shown the essential role of the Tie-2 receptor in angiogenesis (11). Another receptor Tie-1, does not bind Angs but associates with tyrosine phosphatases, Shp signalling proteins and Tie-2 (80% Tie-2 complexed to Tie-1) (12). Tie-1 is not phosphorylated by Tie-2 and could conceivably act in a dominant negative capacity. In knockout studies Tie-1 has been shown to be important in the control of fluid exchange across capillaries. In addition to their role in regulating Tie-2 signalling, the angiopoietins bind to integrins and other cell adhesion molecules suggesting that they can modulate cell-cell adhesion (13). We recently showed that thyroid follicular cells synthesise and secrete Ang1 and Ang2. Even more surprisingly we found that human thyroid follicular cells and the clonal rat thyroid cell line FRTL5 express the Tie-2 receptor. Tie-2 expression is increased in the rat model of iodide deficiency and goitrogen treatment but it is also elevated in humans in multinodular goiter and thyroid cancer suggesting regulation independent of TSH (14). Whether follicular cell Tie-2 is a functional receptor exerting mitogenic effects through actions on PI3K (15) or whether follicular Tie-2 acts to sequester angiopoietins to assist/prevent angiogenesis is not yet known. The receptor expressed on follicular cells is however frequently fragmented (14) which may indicate that regulation of function could occur by modification such as proteolysis

Vascular endothelial growth factors. These growth factors are synthesized by many cell types and like the FGFs with whom they cooperate, are frequently increased in tumorigenesis. Their receptors are restricted to endothelial cells. However, there is one report indicating that FRTL5 cells may express this receptor (16).

Growth factor receptors. In addition to regulation of receptor expression, (precedents for which exist in the literature), more subtle ways of controlling receptor activation exist. For the IGFs there are IGF binding proteins (IGFBPs). For FGFs (23 now characterised) and their receptors (5 now recognised) several ways for controlling activation may be important. These include the selective expression of gangliosides which modulate FGF:FGFR interaction (17) or tyrosine phosphatases that selectively inhibit specific signal transducers that act down stream of receptor tyrosine kinases or members of the Sprouty family which control the Ras/MAPK signaling pathway (18). Soluble extracellular domains of FGFR, Tie-2 and VEGFR1 exist and these can act to sequester available ligand and prevent binding to active receptor. FGFbps exist which are thought to promote FGF binding to its receptor (19).

Antiangiogenic factors. Endostatin and angiostatin are both fragments of larger proteins produced by proteolysis. Control of their production is regulated by a protease cascade and by availability of their precursors, collagen XVIII or XV and plasminogen (20). Both these factors inhibit angiogenic factors and thus enter into the equation for controlling angiogenesis and thus goitrogenesis. Thrombospondin which is a product of thyroid follicular cells is also antiangiogenic (3) and its synthesis is decreased by TSH and increased by PKC activation in porcine thyroid cells (21).

REGULATORY MECHANISMS

Regulation by TSH/Cyclic AMP. TSH is a candidate factor to increase FGF-2 production and FGFR expression due to its stimulatory effects on cAMP production. In rats treated with low iodine diet and methimazole, FGF-2 was elevated within 2 weeks consistent with an effect of TSH although in this model the concurrent reduction in thyroid hormone levels and the effects of methimazole, should not be ignored (22). TSH was found to increase IGF-I expression (23) and inhibit the expression of IGFBPs by follicular cells (5). The IGFBPs may enhance or inhibit the effects of the IGFs or may have independent effects. In human thyroid cells in culture, we found that TSH/cAMP increased Tie-2 expression markedly (14). There were small changes in Ang1 mRNA with TSH but this was not confirmed at the protein level. VEGF was shown in a rat goiter model and in primary cultures of human cells to be positively regulated by TSH (24).

Regulation by Estrogen Women are known to experience more thyroid disorders than men (4:1) and obviously estrogen is implicated in this increased incidence. Twenty five percent of women who had been pregnant had thyroid nodules detectable by ultrasonography compared with 9.4% in those who had not (25). Estrogen promotes endothelial cell proliferation and survival in the vasculature. Could estrogen also increase thyroidal production of angiogenic factors permitting endothelial cell growth to support follicular cell growth? Estrogen was shown to increase the release of FGF-2 via a mechanism involving PKC (8). Estrogen is reported to regulate Ang and Tie-2 expression (26). Estrogen affects circulating IGF-I levels (27) but the effect on follicular cell expression is unknown and may depend on the estrogen receptor (ER) isoform expressed (α or β). The regulation of IGFBP expression by estrogen may similarly be determined by the ER isoform expressed. Cells expressing ER α show increased expression of VEGF with estrogen treatment. The converse is true for those expressing ER β . Which ER isoforms the cells comprising the thyroid express is not yet known. Determination of ER status may yield useful insights into the often conflicting roles of ER isoforms.

Regulation by Protein kinase C activation. Activation of PKC is a common signalling pathway evoked by many receptors coupled to tyrosine kinases. Is the stimulus for the increase in growth factor expression in multinodular goiter then indirect and due to the effect of another circulating growth factor and are the growth factors themselves capable of promulgating their own production? IGFBP expression is markedly increased by PKC activation by growth factors (5) which is likely to inhibit IGF effects on the cells comprising the thyroid. This may be a means to limit mitogenic stimulation through this axis and to inhibit thyroid function since IGF is a requirement for TSH stimulation of thyroid function

Regulation by T3 and T4. The role of thyroid hormones in regulating goiter size is largely unexplored. Goiters in Graves' disease are highly vascular and do not achieve the size of those in iodide deficiency. A distinction between the 2 conditions is that in Graves' disease, hyperthyroidism coexists with TSH receptor stimulation through autoantibodies whereas in iodide deficiency, hypothyroidism coexists with elevations in TSH. The size and the pathological appearance of the glands differs in the 2 conditions; yet both are thought to be due to TSH receptor activation (29). Could thyroid hormone levels modulate goiter? In endothelial cells thyroid hormones are known to increase FGF expression. In Graves' disease, does the high level of thyroid hormones increase FGF expression allowing vascular growth but is follicular cell growth promoted under these conditions of hyperthyroidism and hyperstimulation of function? Intrathyroidal levels of free thyroid hormones are hard to estimate. Although there is a large reservoir of thyroid hormone within the matrix of thyroglobulin, the actual amount available to bind to thyroid hormone receptors will depend on the thyroidal production of T3 and T4 which depends on the level of TSH stimulation and on the level of iodide. A thyroid hormone analog which lacks the chronotropic effects of thyroid hormones per se increases angiogenesis in heart (30). This was an effect specific to the vasculature since there was no change in heart mass only in the number of terminal arterioles, length and density. These effects were preceded by increases in FGF-2, Angs, VEGF and Tie-2. For IGFs and IGFBPs. As for estrogen, most studies have concentrated on the effects on hepatic expression of IGFs and IGFBPs. Whether thyroidal expression is regulated by the prevailing intrathyroidal levels of thyroid hormones is not yet known. These studies will however be difficult to undertake. Primary cultures continue to secrete thyroid hormones from their stores of iodinated thyroglobulin for several weeks in culture and the thyroid cell line model, FRTL5, does not synthesise IGFBP-3, the IGFBP most markedly regulated in primary cultures of human thyrocytes (5).

Regulation by Hypoxia. The most well-reported regulator of Ang and Tie-2 expression is hypoxia and HIF (hypoxia inducible factor) (11). Hypoxia would result if there were inadequate blood supply to the follicular cells. Such localised hypoxia may explain the patchy expression of Tie-2 and Ang mRNA we observed in the rat model of goitrogenesis and in multinodular goiter. Additionally HIF may regulate the expression of other angiogenic factors e.g thrombospondin is decreased. Hypoxia may be responsible for the fibrosis found in goiters where there is asynchrony between angiogenesis and folliculogenesis (29) Summaries of what is known and what is not known of the regulation of growth factor and growth factor receptor expression in thyroid cells are shown in Tables 1 and 2 respectively

Table 1. REGULATION OF HUMAN THYROID PRODUCTION OF GROWTH FACTORS

	FGFs	Angs	IGF	VEGF	Antiangiogenic
TSH	?↑	-	↑	↑	?↓
E2	?↑	?+/-	?+/-	?+/-	?↓
PKC	?↑	?	?	?↑	?↑
T3/T4	?↑	?↑	?↑	?↑	?

Table 2. REGULATION OF HUMAN THYROID GROWTH FACTOR RECEPTORS

	FGFR	TIE-2	TSHR	IGFBPs	VEGFR
TSH	?↑	↑	?↓	↓	↑
E2	?↑	?↑	?	?↑	?↑
PKC	?↑	?	?↓	↑	↑
T3/T4	?↑	?↑	?	?	?↑

?↑ denotes that the regulation in the human thyroid is not known but in other systems, regulation is as shown

Conclusions

Thyroid growth is well served by a plethora of angiogenic/follicular cell growth factors known to be secreted by follicular cells and other cells comprising the thyroid. Some of these growth factors inhibit thyroid function which may account for the nonfunctional nodules. Cells in the thyroid are likely to be susceptible to the effects of estrogen and thyroid hormone which, in other cell types, act to increase angiogenic growth factor production. To offset this proangiogenic push is the production of antiangiogenic factors such as thrombospondin, endostatin and angiostatin. Against this lively background iodide levels fluctuate, thyroid hormone levels fluctuate and TSH levels respond which perturbs this balancing act. Add to this the contribution of dendritic cells (see previous Hot Thyroidology, Lam-Tse and Drexhage) and perhaps it is not so surprising that many of us develop lumpy thyroids!

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AMIODARONE AND THYROID HORMONE METABOLISM

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Abstract

Amiodarone, an iodinated antiarrhythmic drug has complex effects on thyroid hormone metabolism. Main effects are due to the inhibition of deiodinase activity, which lead to variations in serum thyroid hormone concentrations; besides these functional effects, amiodarone may directly interact with thyroid hormone receptors, taking part in the hypothyroid-like condition in the peripheral tissues; moreover, amiodarone and the iodide excess may directly damage the follicular thyroid cells through apoptosis and necrosis, causing leakage of preformed thyroid hormone.

Introduction

Amiodarone is an iodine-rich drug widely used for the management of ventricular arrhythmias, paroxysmal supraventricular tachycardia, and atrial fibrillation and flutter (1). However, its effectiveness on the heart is counterbalanced by its side effects on other organs and tissues (2). The latter include complex effects on the thyroid, ranging from abnormalities of thyroid function tests to overt thyroid dysfunction, either thyrotoxicosis or hypothyroidism (3-5).

This editorial will focus on the complex effects of amiodarone on thyroid hormone metabolism.

Pharmacology of amiodarone

Amiodarone is a benzofuranic derivative whose structural formula closely resembles that of thyroxine (T₄). It contains approximately 37% iodine by weight. Because approximately 10% of the molecule is deiodinated daily, and the maintenance daily dose of the drug ranges from 200 to 600 mg, approximately 7-21 mg iodide is made available each day. If one considers that the optimal daily iodine intake is considered to be 150-200 microg (6), amiodarone treatment releases 50-100 fold excess iodine daily. Furthermore, it is slowly released from several tissues. This explains why, after amiodarone withdrawal, the drug and its metabolites remain available for a long period, the half-lives (mean±SD) being 40±10 days for amiodarone and 57±27 days for desethylamiodarone (DEA) (7). Amiodarone is metabolized through different pathways, the most important being dealkylation which leads to formation of DEA, the main metabolite of amiodarone (8).

Effects of amiodarone on the hypothalamic-pituitary-thyroid function tests

In peripheral tissues, particularly the liver, amiodarone inhibits type I 5'-deiodinase (5'-D) activity, which removes an atom of iodine from the outer ring of T₄ to generate T₃ and from the outer ring of rT₃ to produce 3,3'-diiodothyronine (T₂) (9-11). This inhibition of 5'-D activity may persist for several months after amiodarone withdrawal (2-4). Apparently, amiodarone does not affect the distribution and fractional removal of T₃ from the plasma pool (12). In addition, the drug inhibits thyroid hormone entry into peripheral tissues (13). Both mechanisms contribute to the increased serum T₄ concentration and the decreased serum T₃ concentration in euthyroid subjects given long-term amiodarone therapy (2-5). Serum T₄ concentrations are often at the upper limit of the normal range, but may be increased, especially in patients receiving higher daily doses of the drug (14). The decrease in serum T₃, due to decreased production from T₄, and the concomitant increase in serum rT₃ concentrations, due to decreased clearance, are often found as early as two weeks after institution of amiodarone therapy (15,16). The increase in serum rT₃ levels is usually far greater than the decrease in serum T₃ concentrations (14, 17, 18). Indeed, serum T₃ concentrations often remain within the low normal range.

Amiodarone administration is also associated with dose- and time-dependent changes in serum thyrotropin (TSH) concentration. With a daily dose of 200-400 mg of the drug, serum TSH levels are usually normal, although an increased TSH response to intravenous TSH-releasing hormone (TRH) administration is frequently observed (3). With higher doses of the drug, an increase in serum TSH concentration may occur during the early months of treatment, but this is generally followed by a return to normal (15, 16). These changes in serum TSH concentration are believed to be related to the variations of serum thyroid hormone levels; amiodarone may also directly affect TSH synthesis and secretion at the pituitary level (19). The increased serum TSH concentration may also result from the inhibition of type II 5'-D, which converts T₄ to T₃ in the pituitary, by either amiodarone or desethylamiodarone (20). Indeed, after a loading dose of amiodarone by intravenous infusion, TSH is the first hormone to undergo significant variations, even during the first day of therapy (21). During long-term amiodarone therapy, clinically euthyroid patients may show modest increases or decreases in serum TSH concentration, possibly reflecting episodes of subclinical hypo- or hyperthyroidism, respectively.

The above description of changes in thyroid function tests occurring during chronic amiodarone therapy underscore the important concept that for a correct laboratory-based interpretation of thyroid function, amiodarone-treated subjects should be referred to appropriate reference values, which are somehow different from normal values of subjects not treated with amiodarone and take into considerations the expected variations caused by amiodarone even in euthyroid subjects.

Table 1. Effects of amiodarone on thyroid hormone (TH) metabolism

Mechanism of Action	Effect
Inhibition of Type I 5'-deiodinase	Increased T4
inhibition of TH entry into cells	Decreased T3
	Increased rT3
Inhibition of Type II 5'-deiodinase	Increased TSH*
Inhibition of TH entry into cells	Decreased peripheral T3 production
Thyroid cytotoxicity	Leakage of preformed TH
Interaction with TH receptors	Decreased transcription of TH-responsive genes
	Tissue hypothyroid-like condition

TH: thyroid hormone

* with high doses of amiodarone (>400 mg/day) during the short-term therapy; in long-standing treated patients, phases with slightly decreased or increased serum TSH concentrations may occur.

Cytotoxicity of amiodarone on the thyroid

Besides the above functional effects, mostly due to the influence of amiodarone on thyroid function-related enzymatic activities, amiodarone and its metabolites also have direct effects on the thyroid: cytotoxicity occurs at a lower molar concentration in freshly prepared human thyroid follicles than in rat FRTL-5 cells, probably due to the lack of organification functions in the latter (22). Methimazole, which inhibits iodide organification, significantly reduces the cytotoxic effects of amiodarone in human thyroid follicles (22), suggesting that amiodarone-related thyroid cytotoxicity is mainly due to a direct effect of the drug on thyroid cells. However, excess iodide released from amiodarone may contribute to its toxic action. DEA, the main amiodarone metabolite, has intrathyroidal concentration higher than that of the parental drug (23), and is even more cytotoxic for thyroid cells than amiodarone (24) and its. Ultrastructural changes indicative of thyroid cytotoxicity, including marked distortion of thyroid architecture, apoptosis, necrosis, inclusion bodies, lipofuscinogenesis, macrophage infiltration, and markedly dilated endoplasmic reticulum have been observed in the rat (25). Similar changes have been reported in small series of patients with amiodarone-induced destructive thyroiditis (26, 27). These tissue changes seem to be related to the length of Amiodarone treatment, since in dogs, thyroid subcellular changes were not observed after a single, high-dose intravenous amiodarone injection, while they became apparent after multiple injections for one week (25). Both Amiodarone and DEA displayed a dose-dependent toxicity in TAD-2 and HeLa cells, although DEA was more effective. Amiodarone induced cytochrome c release from mitochondria, triggering apoptosis through an iodine-independent mechanism not involving the generation of free radicals (28, 29).

Interaction of amiodarone and its metabolites with thyroid hormone receptors

Amiodarone may also induce a hypothyroid-like condition at the tissue level. This is partly related to a reduction in the number of catecholamine receptors (30) and to a decrease in the effect of T3 on β -adrenoceptors (31). There is evidence that thyroid hormone is required for the effect of amiodarone on β -adrenoceptors, and that amiodarone does not exert a direct action on beta-adrenoceptors.

Amiodarone causes a decreased transcription of the T3-responsive gene encoding for the low density lipoprotein receptor in liver (32, 33) and of growth hormone in pituitary (34). These antagonistic effects might be related to a down-regulation of thyroid hormone receptors (TR) caused by amiodarone. Myocardial nuclear T3 receptor maximum binding was reduced to a similar degree in hypothyroid and amiodarone-treated rats (35). TR α 1 and TR β 1 subtypes were down-regulated in amiodarone-treated mice in a dose-dependent manner. DEA, but not amiodarone, has been reported to affect the binding of T3 to chicken TR α 1, but not to TR β 1 expressed in *E. coli* (36, 37), probably not taking contact with the T3 binding pocket (38). This would be in keeping with the noncompetitive feature of the DEA inhibition on T3 binding to TR β 1. Recently it was observed that in NIH3T3 cells DEA (but not amiodarone) behaves as a weak thyroid hormone agonist using both TR α 1 and TR β 1, and antagonizes the effect of T3 only when present in large excess (39). Bakker et al. have also reported that DEA has a thyroid hormone agonist effect in transfection assays (40). Thus, DEA, the main amiodarone metabolite, might act both as thyroid hormone agonist and antagonist, possibly depending upon TR isotype and coactivators expression in different tissues. The high tissue levels reached by the drug during chronic amiodarone treatment might explain the prevailing antagonist effect and the "hypothyroid-like" situation observed in tissue such as the heart and liver (10).

Effect of amiodarone on thyroid autoimmunity

The relationship between amiodarone and thyroid autoimmunity is a matter of argument. In a prospective study of 37 patients randomly assigned to either placebo or amiodarone treatment after myocardial infarction, anti-thyroid peroxidase antibodies de novo transiently detected in the serum of 6 out of 13 (55%) amiodarone-treated patients (41). These results were not, however, confirmed in several subsequent prospective or cross-sectional studies (42-47). Thus, most studies indicate that it is unlikely that thyroid autoantibodies appear in subjects who have negative tests prior to treatment. In susceptible individuals amiodarone may precipitate thyroid function of a preexisting autoimmune chronic thyroiditis, as it happens in amiodarone-induced hypothyroidism (AIH).

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HOW SERIOUS IS IODINE DEFICIENCY IN EUROPE ?

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Introduction

The disorders induced by iodine deficiency (Iodine Deficiency Disorders, IDD) belong to the history of Europe as all countries, including the Scandinavian countries with the exception of Iceland, have been exposed in the past to this medical and socioeconomic scourge. And yet, only limited attention has been paid to IDD in Europe, probably because of the impact of the outstanding efficient program of salt iodization in Switzerland (1) and also perhaps because legislations on iodized salt became available in many additional countries. The exhaustive review on IDD in the world, including in Europe, published in 1960 by WHO (2) was followed in the late 1980's by a report by the European Thyroid Association which clearly indicated that, with the exception of the Scandinavian countries, Austria and Switzerland, most of the European countries or at least certain areas of these countries were still affected, especially in the Southern part of the continent (3). A next crucial evaluation of IDD in Europe took place during the international workshop entitled « Iodine deficiency in Europe : a continuing concern » held in Brussels in 1992 (4), during which one representative from each European country summarized the latest IDD data from his country, including the preventive measures.

In 1997, a follow-up meeting entitled « Elimination of Iodine Deficiency Disorders (IDD) in Central and Eastern Europe, the Commonwealth of Independent States and the Baltic States » was organized in Munich (5). This meeting emphasized the severity of the problem in many parts of Eastern Europe, including recurrence of goiter and occasionally of endemic cretinism in some countries such as Russia after interruption of former programs of salt iodization.

The objective of the present paper is to provide updated information on the status of iodine nutrition in the European region. This report is based on an extensive report on the status of iodine nutrition in Western and Central Europe (6), on the preliminary results of an ETA-ICCIDD Satellite meeting to the 28th Annual Meeting of the ETA held in Göteborg in October 2002 (7) and on an exhaustive report on the IDD status in the countries of the Commonwealth of Independent States (CIS) and Central Asia (8).

1. IDD in Western and Central Europe

The review by Delange (6) has been based mostly on the compilation of publications in peer reviewed journals and occasionally on information kindly provided as personal communications by prominent personalities in the field of IDD in the different European countries. This paper also provided updated information on the regulations governing the use of iodized salt and market shares of iodized household salt. For this part, the information has been largely collected by Mr. Bernard Moinier, Secretary of the European Salt Producers Association, ESPA, and by Professor Hans Bürgi. This review paper provided essentially clinical data collected during the past 10 years on the prevalence of goiter and on urinary iodine concentrations. Thus, it evaluated the present status of iodine nutrition. It concluded that a country could be considered as iodine sufficient if, based on a national survey, the prevalence of goiter in the country was below 5 % and the median urinary iodine was within the normal range, i.e. between 100 and 200 µg/L (9). This report was unable to state that iodine deficiency had been eliminated in a given country as the criteria proposed by WHO, UNICEF and ICCIDD for reaching this conclusion are more exhaustive, including a proportion of households using adequately iodized salt above 90 %, a frequency of urine samples with an iodine concentration of 100 µg/L iodine lower than 50% and the fulfilment of at least 8 of 10 programmatic indicators dealing essentially with the administrative and political aspects of the organization of the programs at country level (9).

Epidemiology

Western and Central Europe include 32 countries, plus Andora, San Marino and Lichtenstein for which no data are available. National surveys on the status of iodine nutrition have been conducted during the past 10 years in 17 of these 32 countries. The outcome of these surveys in terms of prevalence of goiter and urinary iodine concentrations are detailed in the recent review of the region (6). They cannot be reported in details here. The global outcome of this European review in terms of status of iodine nutrition is summarized in the Table :

Table. Status of iodine nutrition in Western and Central Europe in early 2003, based on urinary iodine concentrations.

Sufficient	Likely sufficient	Deficient	Likely deficient
Austria	Greece	Belgium	Albania
Bulgaria	Poland	Bosnia	
Croatia	Portugal	Denmark	
Cyprus (North)	Serbia	France	
Czech Republic	United Kingdom	Hungary	
Finland		Ireland	
Germany		Italy	
Iceland		Luxemburg	
Macedonia		Romania	
The Netherlands		Slovenia	
Norway		Spain	
Slovak Republic		Turkey	
Sweden			
<u>Switzerland</u>			

Iodine sufficiency was unquestionably reached in 14 countries and probably reached in 5 additional countries, namely Greece, Poland, Portugal, Serbia, and the United Kingdom. Iodine deficiency, varying from mild to severe, persisted in an additional 12 countries and no data were available from the last European country, namely Albania, which is most probably affected.

Some countries deserve particular consideration : in Germany, a national survey of 3065 school-aged children performed in 2000 reported a national median of 148 µg/L indicating iodine sufficiency. However, iodine deficiency continues in some areas with median urinary iodine of 88µg/L. In Poland, the latest published national survey conducted in 1999 showed a mean urinary iodine of 96 µg/L indicating an almost complete correction of iodine deficiency which might have been achieved since. However, the national program could be in danger if the national authorities interrupt their support to the national program. Iodine sufficiency has been reached in Serbia but not in Montenegro. Portugal used to be affected in several areas but is probably almost close to iodine sufficiency. United Kingdom is often considered as iodine sufficient but recent national data are missing and at least pockets of iodine deficiency persist, for example in Scotland (10). National surveys performed in 1999 in Bosnia and Herzegovina showed a median urinary iodine of 77.6 µg/L while the figure was 127 µg/L in the Republika Srpska (7).

Public health consequences.

The state of mild to severe iodine deficiency persisting in many European countries has important public health consequences on all age groups but especially during pregnancy, in the neonates and young infants, with impairment of the intellectual development as the most significant consequence (6).

In adults, the frequency of simple goiter is elevated and the cost of therapy of thyroid problems resulting from iodine deficiency is enormous. For example, in Germany, endemic iodine-deficiency goiter causes economic costs of approximately one billion US\$ or Euros per year (11). Elevated thyroidal uptake due to iodine deficiency aggravates the risk of thyroid irradiation and the development of thyroid cancer in case of a nuclear accident. Thyroid function is frequently altered during pregnancy with a progressive decline in serum free T4 and consequently an elevation of serum TSH resulting in the development of goiter in about 10 % of pregnant women. The alterations are still more marked in neonates than in their mothers and in Europe, as in other parts of the world, the results of neonatal thyroid screening for a congenital hypothyroidism can be used as a sensitive tool for monitoring iodine deficiency and its control. Another consequence of longstanding iodine deficiency in the adult is the development of hyperthyroidism, especially in the elderly with multinodular autonomous goiters. The evidence of this side effect of iodine deficiency has been the main reason why a country such as Denmark initiated an efficient program of salt iodization while it was the last European country in which salt iodization was forbidden up to 1999.

A key issue is that clinically euthyroid schoolchildren born and raised in moderately iodine deficient regions of Europe exhibit subtle or even overt neuropsychointellectual deficits when compared to iodine-sufficient controls living in otherwise identical

ethnic, demographic, nutritional and socioeconomic populations. These deficits are of the same nature, although less marked, than those found in schoolchildren in areas with severe iodine deficiency and endemic mental retardation. As already indicated, the most important and frequent alterations of thyroid function due to iodine deficiency in Europe occur in neonates and very young infants with a high frequency of transient hyperTSHemia and primary hypothyroidism. The hypersensitivity of neonates to the effects of iodine deficiency is their low iodine content of the thyroid with an extremely fast turnover rate of intrathyroidal iodine.

Prevention and therapy.

Seventeen of the 32 countries in the region have a legislation on iodized salt but which is implemented in only 11 of them. The level of salt iodization recommended varies from 5 to 70 ppm and the figures for the market share of iodized packed salt sold to the households vary from 1 % in Portugal to at least 90 % in Austria, Bulgaria, Croatia, The Czech Republic, Finland, Macedonia and Poland.

2. IDD in Eastern Europe, Central Asia and the Baltic States

Doctor Gerasimov recently produced an extensive report with comprehensive bibliography on the IDD status, control program and salt iodization in 15 countries of Eastern Europe and Central Asia, including 12 countries of the Commonwealth of the Independent States (CIS) and the three Baltic States (8).

In the past 5 years, significant information has been collected on the extent of iodine deficiency in the region. National and sub-national IDD surveys were conducted in Armenia, Azerbaijan, Belarus and Uzbekistan. The quite impressive outcome of this survey is that, with the exception of Armenia where iodine deficiency appears as currently under control with a median urinary iodine above 100 µg/L, but with persisting goiter prevalence up to 30 %, iodine deficiency persists in all other countries, varying from mild to severe ; generally mild in the Baltic countries, especially Estonia and Lithuania up to mostly severe in Tajikistan where a survey performed in 1999 reported a prevalence of goiter varying from 33 to 90 %. Russia never had a national IDD survey on its enormous territory but several regional assessments conducted from 1998 to 2001 concluded that iodine deficiency persists in most of the administrative regions. An IDD survey performed in Ukraine in 2000 indicated that significant iodine deficiency was present not only in the Northern area close to the Tchernobyl nuclear station but also nationwide.

The iodized salt production was extremely limited in almost all countries in the region until 1997. Since then, significant efforts by the salt industry, with international support, have made iodized salt now available in all countries, and production is scaling up. For example, Russia increased its iodized salt production from 10,000 ton in 1997 to 120,000 ton in 2001. Most countries in the region adopted harmonized levels of salt iodization at 40 ±15 ppm and shifted from potassium iodide to the more stable potassium iodate.

Discussion and conclusion

This review underlines major improvement of iodine nutrition in Europe, as compared to the situation described in details in 1993 for Western and Central Europe and in 1997 for Eastern Europe, Central Asia and the Baltic States.

In Western and Central Europe, the 1993 report (4) indicated that only 5 countries had reached iodine sufficiency, namely Switzerland, Austria, Norway, Finland and Sweden. The present figure is 14 countries plus 5 additional countries which have almost reached the goal. In 1999, WHO, UNICEF and ICCIDD reported that 18 countries in Western and Central Europe were still affected by iodine deficiency (12). But data from the literature indicate that three additional countries were also affected, namely Denmark, France and Ireland (6), which makes a total of 21 affected countries in this part of the continent. This figure has now been decreased to 12 countries plus Albania for which no firm data are available but where iodine deficiency is very likely.

In Eastern Europe, the countries have made substantial progress in evaluation of IDD status and in expanded production, supply and use of iodized salt.

However, the goal of sustainable elimination of IDD has not yet been reached, especially in Eastern Europe where only Armenia, and to a lesser extend Turkmenistan, are close to virtual elimination of iodine deficiency (8). In 1999, Europe was the less efficient region in the world in terms of access to iodized salt at the household level in iodine deficient countries (12). In spite of the progress achieved since, further efforts have to be developed in order to ensure the recommended daily intake of iodine for all ages in all inhabitants of Europe, i.e. 90 µg/day from 0 to 59 months, 120 µg/day between 6 and 12 years, 100 µg/day in adolescents and adults and 200 µg/day in pregnant and lactating women (9). This has to be achieved principally through implementation of efficient programs of salt iodization without undue concern to the possible side effects of the increase of iodine intake (13,14).

The main impact of iodine deficiency is on pregnant and lactating women and young infants due to role of maternal, fetal and neonatal hypothyroxinemia in the development of brain damage resulting in irreversible mental retardation (15-17). As a reply to the famous editorial by Peter Laurberg in 1994 (18): « Iodine intake. What are we aiming at ? », the reply is clearly that the correction aims not only at increased access to properly iodized salt and normalization of urinary iodine but mostly at the

correction of the thyroid function during the critical period of brain development and, consequently, at the prevention of brain damage (19). Iodine deficiency remains the leading cause of potentially preventable mental retardation in childhood (14). The iodine nutrition of Western and Central Europe differs in several ways from that in other parts of the world (7). Most Western European countries have iodized salt available but in about half its use is only voluntary. As in the United States and Canada, most dietary salt comes from processed food, so the amount of salt added at table and cooking at home is a relatively minor component of salt intake. Therefore, table salt is a less important source of iodine nutrition than in developing countries and the iodization of salt for the baker and for the food industry are particularly important. National responsibility for iodine nutrition and its prophylaxis is much weaker in most Western European countries than in Eastern Europe and in developing countries. The laws and practices relating to iodized salt vary widely among the countries of Western and Central Europe and additional efforts to educate the government, the citizens and even the health professionals have to be markedly increased.

In conclusion, more than half of the people in Western and Central Europe and a large majority of the people in Eastern Europe and Central Asia still live in conditions of iodine deficiency. In Western Europe, in contrast to developing countries, governmental programs to deal with iodine nutrition are weak or non-existent. Consequently, much of the responsibility for optimal iodine nutrition must be shouldered by others, especially thyroidologists, academic institutions and the salt industry. In Eastern Europe where national programs are much stronger, additional efforts are needed especially in the field of quality control in monitoring the programs. In Europe as a whole, as long as USI (the Universal salt Iodisation efforts launched in 1992) is not systematically implemented, special attention has to be devoted to the protection of the two main target groups to the effects of iodine deficiency, i.e. pregnant and nursing women, neonates and young infants. If iodine deficient, these age groups should be supplemented with physiological quantities of iodine for example by including iodine to the multivitamins prepared for them or by using iodized oil. Moreover, the iodine content of formula milk should be increased in Europe to the presently recommended level of 10 µg/dl milk for fullterms and 20 µg/dl for preterms (4). The elimination of iodine deficiency is within reach and would constitute an unprecedented public health success in the field of non-communicable diseases. Additional efforts have to be developed in Europe in order to reach the goal and, in this part of the world, thyroidologists and their scientific societies should play a leading role in this direction.

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DENDRITIC CELLS IN THYROID AUTO-IMMUNE DISEASE

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Introduction

In the early 1980s it was reported that thyrocytes aberrantly express MHC class II molecules in Hashimoto and Graves' disease (normal thyrocytes do not) and that these thyrocytes are able to stimulate T cells in vitro. This led to the hypothesis that an aberrant expression of MHC class II molecules on thyrocytes is the basic abnormality in thyroid autoimmunity and that an immune response to thyroid auto-antigens is initiated by an erroneous local presentation of such antigens to intra-thyroidal accumulated T cells (1).

Another mechanism of initiation of the thyroid autoimmune reaction was also proposed (2). A class of antigen presenting cells (APC), the so-called dendritic cells (DC), was reported to be present in the thyroids of Hashimoto and Graves' patients. DC are known to be the most potent professional APC of the immune system. Therefore these cells are the most likely candidates for the initiation of the thyroid autoimmune response.

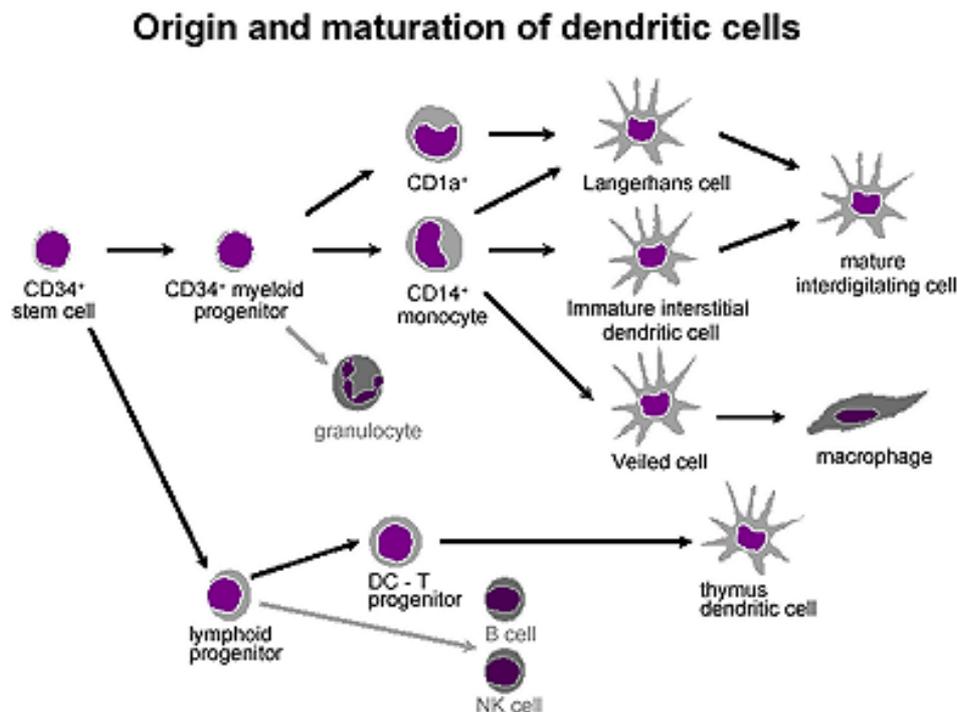
Up to the early 1990's a plethora of studies has been concentrating on the first hypothesis. However progress in immunology over the last 10 years point to the DC as one of the most important APC in the initiation of autoimmune reactions. There is perhaps a small role for thyrocytes aberrantly expressing immune molecules in later phases of the thyroid autoimmune reaction.

Dendritic cells and the immune response.

DC are the most potent APC of the immune system and are critically involved in the initiation of primary immune responses, the generation of T cell dependent auto-antibody formation, graft rejection and auto-immune diseases (3). DC are present in the interstitium of all tissues (except the brain) and in lymphatic tissues. DC in lymphatic tissues are characterized by a strong expression of MHC class II molecules and other essential co-stimulatory molecules (CD80, CD86, CD40, etc) essential to initiate a proliferation response of naïve T cells.

Heterogeneity of DC.

DC form an enormously heterogeneous group of APC with different lineage backgrounds (so-called lymphoid versus myeloid lineage), precursors and various stages of differentiation and maturation (figure 1).



A scheme of origin and maturation of dendritic cells

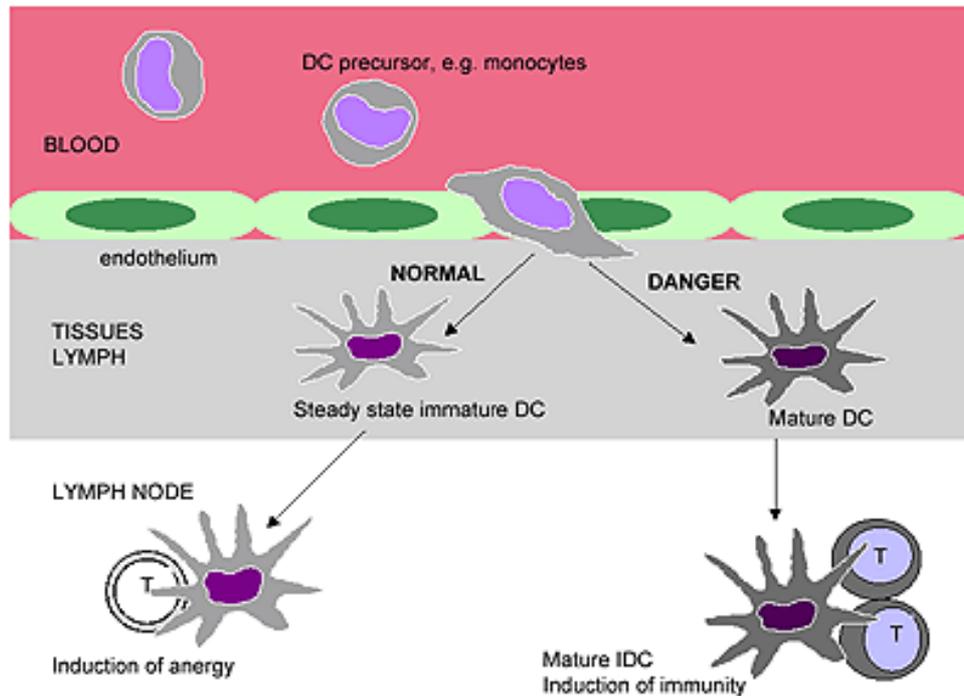
The "lymphoid" DC originate from pre T cells in the thymus and predominantly populate the thymic cortico-medullary junction where the cells are instrumental in the deletion of erroneously created auto-reactive T cells. The "myeloid" DC originate from a special CD34+ precursor in the peripheral blood (giving rise to epidermal S100+ Langerhans cells) or from CD14+ circulating monocytes. The monocyte-derived DC are closely linked to other classes of APC, such as the veiled macrophages (figure 1).

Immature and mature DC.

The presently generally held paradigm (4) in immunology is that DC present in the interstitium of non-lymphatic tissues are in an immature state, suitable for their sentinel function. The immature cells express various molecules for the uptake of foreign and damaged material (mannose receptors, Toll receptors), and have a high endocytotic capability enabling to capture and process antigens. These immature DC have a limited potency to stimulate T cells.

Mature DC and the initiation of immune and inflammatory responses.

In response to a local inflammatory stimulus (the so-called danger signals), such as endotoxin (LPS), TNF α and bacteria, interstitial DC undergo maturation (figure 2). During maturation the cells migrate via the lymph to the T cell areas of the draining lymph nodes.



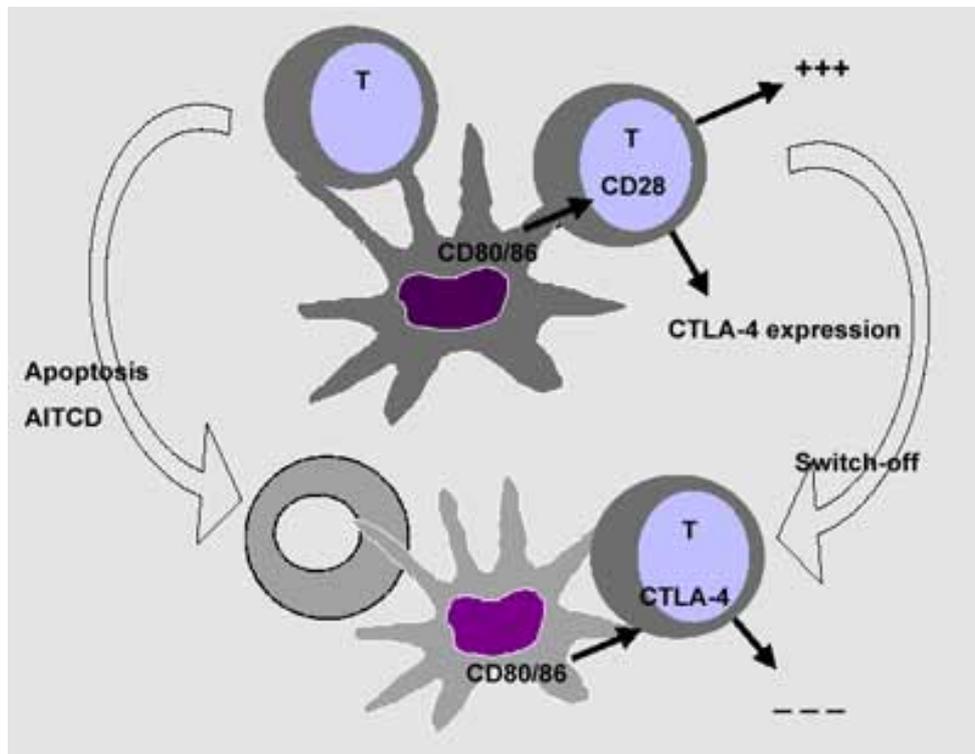
DC precursors, such as the monocyte, continuously migrate through the epithelium via the tissues to the draining lymph nodes. Various molecules, such as adhesion molecules (integrins), interleukins and chemokines are instrumental in this traffick. In a non-danger ("steady state") environment DC stay immature and induce after migration T cell anergy in the draining lymph node. However when DC encounter danger signals in the tissues, e.g. microbial agents or necrotic cells, they mature and are able to induce proliferation and activation of naïve T cells in the draining lymph node.

The matured DC have lost their antigen-capturing capacity, but have acquired a strong potency to stimulate the proliferation of naïve antigen-specific T cells, by directing the antigen-loaden MHC molecules to the cell membrane and up-regulating their co-stimulatory molecules. Thus, mature, inflammatory DC are the initiators of immune responses (figure 2).

DC and tolerance.

The last years the idea has gained acceptance that DC are also prime inducers of tolerance (5). With regard to central tolerance, there are strong indications that thymic DC express f.i. thyroglobulin (Tg) and can therefore act as the prime deletors of Tg-reactive T cells created in the thymus. However this deletion process is far from complete: low, but sufficient Tg-reactive T cells escape to the periphery. Peripheral tolerance mechanisms should keep these circulating auto-reactive T cells under control.

When interstitial, immature DC are not triggered by "danger" signals and stay under "steady state" conditions, there is nevertheless a continuous travel of such immature DC (carrying auto-antigens) to the draining lymph nodes (7, figure 2). Such DC lack sufficient co-stimulatory molecules and are able to induce anergy in circulating auto-reactive T cells (5). Whether these DC are capable of inducing deletion of auto-reactive T cells is presently a matter of debate and research (6). On the other hand when mature DC in the lymph node give strong signals not to naïve auto-reactive T cells, but to T cells that have recently expanded in multiple proliferation rounds, the latter T cells stop to proliferate and go into apoptosis (the so-called Activation Induced T cell Death, AITCD, figure 3).



A scheme of the co-stimulatory signals in the induction of activation and in the silencing of effector T cells. Mature DC with a high expression of the co-stimulatory molecules CD80 and CD86 stimulate proliferation of naïve T cells via triggering of CD28 on the T cells. After such activation the T cells respond by an up-regulation of CTLA-4, which is a molecule able to down-regulate the functions of T cells. In addition, after various rounds of proliferation T cells become vulnerable to apoptosis. The same co-stimulatory signals given by the mature DC now induce a silencing of the activated T cells or induce apoptosis (AITCD) in these T cells.

Hence there are various ways in which DC are indispensable for tolerance induction and ending auto-reactive T cell reactions.

Dendritic cells (DC) and the physiology of the normal thyroid.

DC and the normal thyroid.

DC are present in low number in normal thyroids composing 2-3% of the interstitial cell population (8, 9, 10). There are indications that such DC are able to proliferate (11), which means that not all of the thyroid DC need to be recently immigrated from the blood stream. Malignancies of thyroid DC have been described (thyroid Langerhans cell histiocytosis) (12).

Thyroidal DC are often in close contact with thyrocytes. The thyroidal DC are in a clear immature state and often show monocyte marker characteristics (13). It is thought that soluble factors produced by TSH-stimulated thyrocytes, such as GM-CSF, TGF β , IL-6 and osteoprotegerin, keep intra-thyroidal DC in their immature state (11, 14, 36). TNF α on the other hand induces maturation of immature thyroidal DC (13). Also thyroid hormones and related iodinated compounds are known to influence the maturation of immature DC and the differentiation of related APC (15, 16).

DC as thyroid regulators.

DC are capable to regulate the growth of thyroid follicles in vitro. Simons et al (17) showed that the co-culture of isolated thyroid or splenic DC with thyroid follicles resulted in intense interactions of the DC with the thyroid follicles and a dampening of TSH-induced proliferation of thyrocytes composing the follicles. The thyroid hormone release from the follicles was suppressed to a limited extent. Cytokines (IL-1 and IL-6) secreted by the DC, and not adhesive interactions are important in this regulatory function of DC (17).

Interestingly spleen DC and pituitary folliculo-stellate cells (which are in part pituitary interstitial DC) express functional TSH-receptors (18, 19). Upon stimulation with TSH, spleen DC up-regulate their c-AMP, phagocytic capability and production of IL-1 and IL-12 (18). If such TSH-receptor expression and cytokine production also occurs at the level of the thyroidal DC this would imply that the T4-TSH feed back mechanism does not only targets thyrocytes, but also thyroid DC via which thyrocyte growth can be regulated.

DC and iodine deficient goiters.

In view of the above-reviewed evidence for a thyrocyte regulatory role of DC, it is note-worthy that DC accumulate and form homotypic clusters in iodine deficient goiters both in man as well as in an animal model (20, 21). The accumulation of the DC might be taken as a sign that the cells are instrumental in a growth regulation of the iodine-deficient goiter, yet direct evidence for this has not been given. The homotypic cluster formation of DC is a sign of maturation of the cells (22), and indeed during the formation of iodine-deficient goiter there is an activation of Tg-specific T and B cells (21).

Dendritic cells (DC) and the autoimmune diseased thyroid.

Early accumulation and clustering of DC in the thyroids of animal models of autoimmune thyroiditis.

A small increase in the number of DC and a homotypic clustering of the cells in the interstitium of the thyroid is one of the first

signs of a developing thyroid autoimmune reaction in the bio breeding diabetes prone (BB-DP) rat, an important animal model of spontaneously developing autoimmune thyroid disease (8). These signs precede the T cell expansion and the production of auto-antibodies in the thyroid draining lymph nodes and the actual infiltration of the rat thyroid with lymphocytes. At this early phase of the autoimmune reaction BB-DP thyrocytes are negative for MHC class II (8). In another autoimmune thyroiditis rat model, the neonatal thymectomized Buffalo Strain rat and in the non obese diabetic (NOD) mouse (a mouse model of spontaneous autoimmune thyroiditis), similar findings have been reported (23, 24). After isolation of BB-DP thyrocytes from these "early phase" thyroids, the cells were very poor stimulators of T cell expansion in vitro. In contrast, isolated thyrocytes were excellent in this function equaling splenic T cells (13). These arguments provide, at least in the animal models, sufficient proof to reject the idea that an aberrant expression of MHC class II molecules is a prime event in thyroid auto-immunity initiating the process.

With regard to the human, thyrocytes are not capable of expressing co-stimulatory molecules to a noteworthy extent (25, 26), there is however some evidence that they might in Hashimoto's thyroiditis (27). In addition, while the co-culture of thyrocytes and T cells alone, resulted in a relatively weak T cell proliferation, addition of low numbers of monocytes or APC to the culture led to a clear enhancement of the T cell proliferative response (27). These observations point in the direction that also in the human professional APC play a prime role in the induction of autoimmunity.

Thyroid factors instrumental in the early attraction of DC to the thyroid of animal models of autoimmune thyroiditis. In the majority of the NOD strains the incidence of autoimmune thyroiditis is in general very low, but it varies from colony to colony (24). Certain dietary iodine regimens, however, have a triggering effect on the development of autoimmune thyroiditis in the low-incidence NOD strains. In such strains an iodine-induced necrosis of thyrocytes is a clear factor leading to an intra-thyroidal accumulation of various inflammatory cells, amongst which the DC. This early inflammatory influx is followed by a reaction of the draining lymph nodes and an initiation of an auto-immune thyroiditis (24).

In the BB-DP rat and obese strain (OS) chicken (both other animal models of autoimmune thyroiditis) there are however no signs of an early iodine-induced necrosis of thyrocytes attracting the DC in an inflammatory reaction. Interestingly intrinsic disturbances in the growth and the differentiation of thyrocytes have been shown in both models, in the BB-DP rat leading to a high incidence of ultimobranchial cysts and an altered production of IL-6 by thyrocytes (15, 29). Whether such alterations do lead to a higher influx of DC is not known.

Late accumulation of DC in the thyroid of the BB-DP rat.

After the appearance of Tg autoantibodies in the circulation, T and B cells start to infiltrate the thyroid and at the same time as this infiltration there is a remarkable sharp increase in the number of thyroid DC (8). The T cell, B cells and DC accumulating in the thyroid do not form destructive infiltrates, but are organized as peripheral lymphatic tissue (8, 31). The diapedesis of lymphocytes from the bloodstream to this tissue is facilitated through the formation of specialized "high endothelial venules" (HEV) (30). Intra-thyroid lymphatic tissue probably serves a further expansion of auto-reactive T cells and a further production of Tg auto-antibodies. Thyrocytes adjacent to areas of intra-thyroid lymphatic tissue start to express MHC-class II molecules (8, 29, 30), probably as a consequence of the cytokines produced in the intra-thyroid lymphatic tissue. Perhaps such MHC class-II positive thyrocytes, but more likely auto-antigen specific B cells (32) play a role as APC in this late phase of autoimmune reactivity.

DC in the thyroids of patients with Graves' disease and Hashimoto's disease.

Thyroids of patients (predominantly Graves' goiters) are available for microscopical investigation only in a late phase of the disease, when one has decided to a surgical intervention. The glands show areas of intra-thyroid lymphatic tissue and areas in which the thyroid follicles are largely intact with significantly elevated numbers of perifollicularly located DC (9, 33, 34).

Such perifollicular DC have an immature phenotype (33).

Phenotypically mature DC are predominantly present in the larger areas of intra-thyroid lymphatic tissue and co-localized with activated CD4+ T cells (33). In such areas HEVs are present with an up-regulated integrin expression on their endothelial cells (30, 35).

Abnormal DC differentiation in spontaneous animal models of thyroid autoimmune disease. A role in defective tolerance induction?

Since DC are critically involved in the initiation of the autoimmune process (see above), it is important to note that there is accumulating evidence that the differentiation of DC from precursors is abnormal in the BB-DP rat and the NOD mouse. In the BB-DP rat precursors were more abundant than DC in the very early infiltrates of the thyroid (13), and lymph node and spleen DC were in a relatively immature (interstitial) state (37, 38).

In the NOD mouse, studies have concentrated on the in vitro development of DC from bone-marrow precursors. This development was found to be hampered leading to a low yield of DC with a low grade of maturation and a low capability to stimulate T cells (39). However opposite findings have been made (40) and differences in results are probably dependent on the culture conditions used (Leenen, personal communication). Functional studies on interstitial DC are lacking in the NOD mouse. Spleen and lymph node DC of the NOD mouse however have a normal state of maturation and are perfectly capable of stimulating T cells (41).

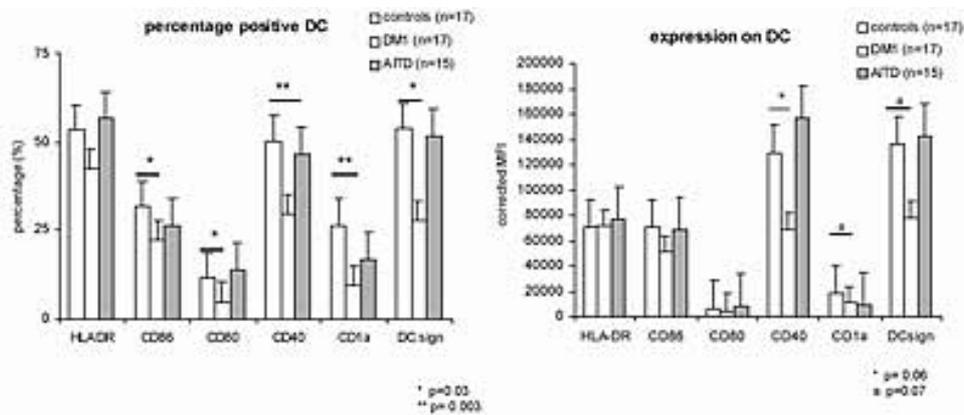
In fact there is an excessive proliferation reaction of the NOD T cells in vitro, when stimulated with DC. This is most likely due to a defect other than the DC maturation defects in the immune system of this animal, but a defect in the mechanisms of apoptosis of T cells leading to a hampered AITCD (41).

Although it is not clear if and how differentiation defects of DC in the animal models play a role in their defective ability to mount tolerance to auto-antigens, there are a few indications that they might. The immature lymph node and spleen DC of the BB-DP rat were in particular less capable of expanding an important suppressor T cell population of the rat, the so-called RT6+ T cells (37), while in the NOD mouse, transfers of in vitro matured DC prevented the development of type 1 diabetes

in this animal (42).

An abnormal DC differentiation in type 1 diabetic patients, but not in thyroid autoimmune patients.

In analogy to the data on differentiation and maturation defects in the animal models there are reports accumulating on similar defects in type 1 diabetic patients. When monocytes of type 1 diabetics (figure 4) or pre-diabetics (43) are stimulated in vitro by GM-CSF and IL-4 to differentiate into immature DC, it appeared that the differentiation of such immature DC is hampered.



The percentages immature DC positive for the indicated surface molecules (left graph) and the expression level of these molecules per immature DC (right graph) are shown. DC of healthy controls (white bars, n=17), type 1 diabetic patients (DM1) (dotted bars, n=17) and autoimmune thyroiditis patients (AITD) (grey bars, n=15) were generated by culturing monocytes with GM-CSF and IL-4 during 6 days. The percentages DC positive for CD86, CD80, CD40, CD1a and DC-sign are significantly reduced in DM1, but not in AITD. The expression levels for CD40, CD1a and DC-sign on DC of DM1, again not of AITD, are decreased.

We have confirmed these findings and also showed that this is not the case for monocytes of type 2 diabetic patients or patients with auto-immune thyroiditis (to be published).

However, defects other than differentiation defects of monocyte-derived DC are noticeable in the monocytes of patients with autoimmune thyroiditis, such as an altered expression of integrin molecules, a hampered ability to arrange the actomyosin cytoskeleton after chemotactic stimulation, and a lower potency to differentiate into a population of APC other than the classical DC, namely the motile veiled macrophages (44, 45). Although this suggests that adhesive, motile and migratory functions of monocytes are hampered in thyroid autoimmune disease, it is not known if and how such defects have any effect on the apparent hampered tolerance for thyroid auto-antigens in these patients.

Future prospects: DC as putative tools in the treatment of autoimmune diseases.

Although the role of the above-described defective APC function is far from clear in the development of type 1 diabetes and autoimmune thyroiditis, it is clear that DC form a potent group of cells to modulate immune responses. DC vaccination protocols are presently under design to elicit strong immune reactions (46) including autoimmune reactions to eradicate tumors, also thyroid malignancies (47). Such vaccination protocols aim at constructing DC potent to elicit strong effector immune responses. The present state of the art points to mature DC expressing important tumor antigens and producing IL-12 as the most likely candidates to perform this job.

Since DC are also involved in tolerance induction, it is not a far-fetched idea to construct DC to induce or restore tolerance. Very immature steady state DC, expressing important auto-antigens and producing IL-10, are thought to be able to perform this job (5). However the differentiation and maturation defects of DC in the BB-DP rat and the NOD mouse suggest that there is no shortage of such very immature DC in these animals. The disease preventing effects of the transfer of artificially matured DC in the NOD mouse point in another direction, namely that mature DC are more critical to elicit or restore tolerance in conditions of endocrine autoimmunity (48, 49). Such DC might be critical for a deletion of auto-reactive T cells in the periphery via AITCD.

Conclusions.

- DC form a heterogeneous group of APC with different lineage backgrounds (lymphoid versus myeloid lineage), different precursors and various stages of differentiation and maturation.
- DC are the conductors of the T cell orchestra and regulate both immune activation and tolerance induction.
- Interstitial thyroid DC regulate the growth and hormone production of thyrocytes.
- DC and not aberrantly MHC-class II expressing thyrocytes are the cells initiating thyroid autoimmune reactivity.
- The differentiation of immature and mature DC from precursors is abnormal in the animal models of spontaneously developing endocrine autoimmune disease, but consequences are not clear for the defective state of tolerance in these animals.
- The differentiation of immature and mature DC from precursors is normal in patients with autoimmune thyroiditis, apart

when co-occurring with type 1 diabetes in the setting of an APS type 3.

- DC are good candidates for a novel form of treatment for auto-immune thyroiditis, i.e. vaccinations to induce tolerance.

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THYROID HORMONE SULFATION

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1 Introduction

Cytosolic sulfotransferases catalyze the sulfation of the hydroxyl group of different exogenous and endogenous compounds. These enzymes use 3'-phosphoadenosine-5'-phosphosulfate as sulfate donor. Apart from its function as a detoxification mechanism for exogenous chemicals, sulfation plays an important role in hormone metabolism, among which the metabolism of thyroid hormone. Sulfated iodothyronines do not bind the thyroid hormone receptors, yet can be subject to deiodination (Fig. 1) (1). Especially this interaction between the sulfation and deiodination pathways is intriguing. Deiodination is catalyzed by three different deiodinases (D1-3). The prohormone T4 is converted by D1 or D2-catalyzed outer ring deiodination to 3,3',5-triiodothyronine (T3); T4 and T3 are inactivated by D1 or D3-catalyzed inner ring deiodination to rT3 and 3,3'-T2 respectively. Neither D2 nor D3 are capable of deiodinating sulfated iodothyronines. Sulfation also blocks the outer ring deiodination of T4 by D1. However, it strongly facilitates the inner ring deiodination of T4 and T3 by D1 (1). Therefore, the main function of sulfation is to induce the irreversible degradation of thyroid hormone.

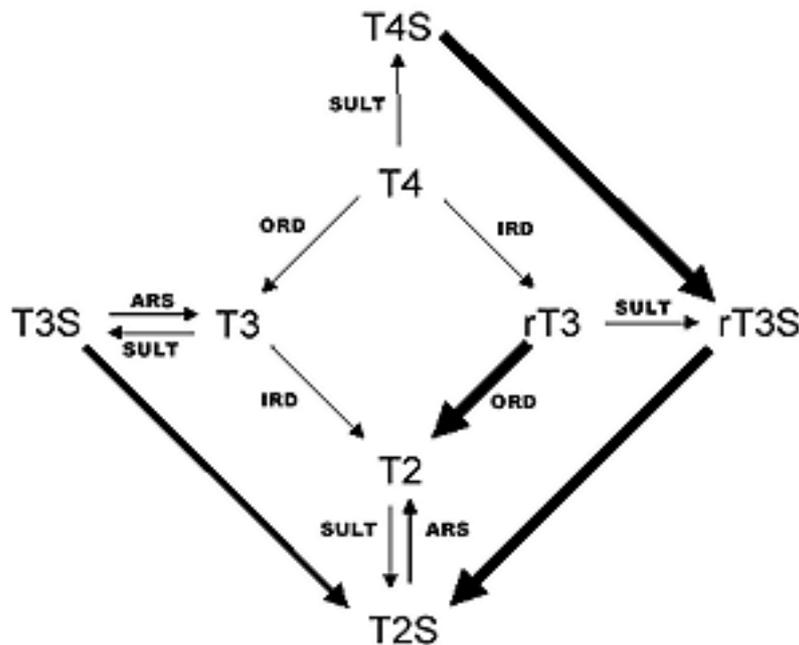


Fig. 1 Interaction between sulfation and D1-catalyzed deiodination of iodothyronines (courtesy of Dr. M.W.H. Coughtrie)

2.1 Which sulfotransferases are involved in iodothyronine sulfation?

All cytosolic sulfotransferases are members of a large gene superfamily. They are located in different tissues such as liver, kidney and brain, have a molecular weight of about 33 kDa, and exist predominantly as homodimers (2). The sulfotransferases, which show overlapping substrate specificities, are classified on the basis of amino acid sequence homology. Three subfamilies have been identified: the SULT1 family of phenol sulfotransferases, the SULT2 family of hydroxysteroid sulfotransferases and the SULT4 family of sulfotransferase-like proteins, for which no enzyme activities have yet been reported (3,4).

All members of the the SULT1 family, i.e. SULT1A1-3, 1B1, 1C2, 1C4 and 1E1, catalyze the sulfation of iodothyronines (5-8). 3,3'-T2 is the preferred substrate for SULT1A1-3, 1B1 and 1C4. These enzymes catalyze T3 and rT3 sulfation less efficiently and show only little catalytic activity towards T4 sulfation. SULT1E1 equally prefers 3,3'-T2 and rT3 over T3 and T4 (i.e. 3,3'-T2~rT3>T3~T4). Figure 2 compares the sulfation of the different iodothyronines by purified human SULT1A1, 1A3, 1B1 and 1E1. Whereas 3,3'-T2 is a better substrate for SULT1A1 than for the other enzymes, SULT1E1 is the most effective in catalyzing rT3 sulfation (Fig. 2) (6). Crude cytosols of SULT1C2-expressing cells show a substrate preference for T4>T3>rT3>3,3'-T2. This apparent preference of SULT1C2 for T4 is interesting, but has not yet been confirmed in purified SULT1C2¹.

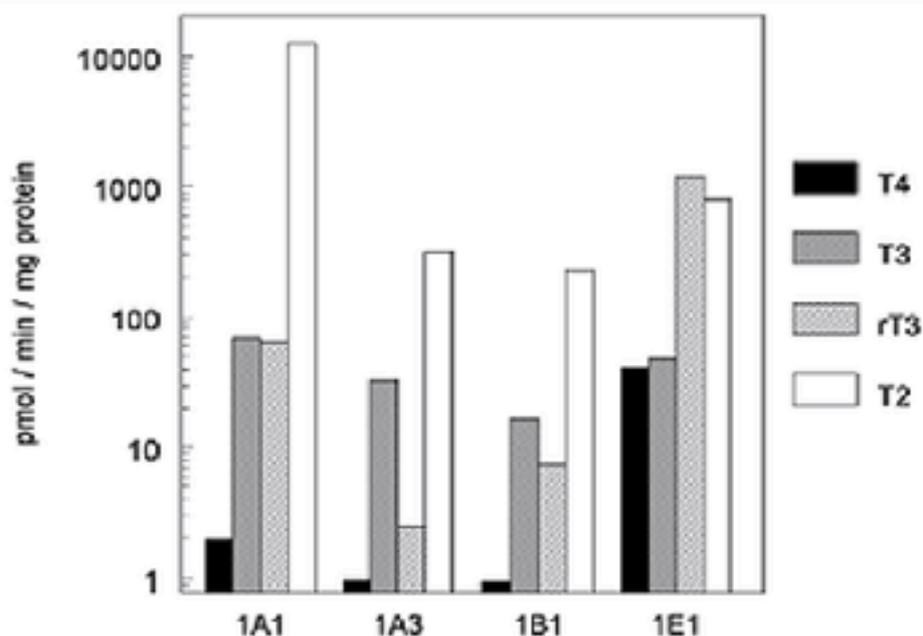


Fig. 2 Sulfation of iodothyronines by purified human sulfotransferases (adapted from Kester et al. (6))

It is remarkable that SULT1E1 (estrogen sulfotransferase) also catalyzes iodothyronine sulfation, but it should be noted that the estrogens estrone (E1) and estradiol (E2) are clearly the preferred substrates for this enzyme. Apparent K_m values of E1 and E2 for SULT1E1 are around 5 nM, whereas apparent K_m values of 3,3'-T2 and rT3 are in the low micromolar range (6). Nevertheless, given the facile sulfation of iodothyronines by SULT1E1, in addition to its role in reversible estrogen inactivation, a physiological role for the enzyme in thyroid hormone metabolism cannot be excluded. For example, high iodothyronine sulfate concentrations are present in amniotic fluid and fetal serum (9-11). Since SULT1E1 is expressed in the uterus (12), these pools of inactive thyroid hormone may be partly derived from iodothyronine sulfation by SULT1E1 in the uterus. Furthermore, SULT1E1 may contribute to iodothyronine sulfation in the choroid plexus. Namely, in this brain region, in which SULT1A1 and 1E1 are expressed, both 3,3'-T2 and rT3 are very efficiently sulfated. This facile rT3 sulfation may indicate a tissue-specific contribution of SULT1E1 to iodothyronine sulfation. Richard et al. studied SULT1A1 and 1A3 expression in fetal liver, lung and brain. They showed strong correlations between SULT1A1 expression and 3,3'-T2 sulfation in the different tissues (13). In agreement with this, we showed a strong correlation of the substrate specificities of hepatic and renal sulfotransferase activities with that of SULT1A1, suggesting a prominent role of SULT1A1 in iodothyronine sulfation in the liver and kidney (5). Still, also other sulfotransferases contribute to iodothyronine sulfation. When purified SULT1A1, 1A3, 1B1 and 1E1 are compared, the receptor-active T3 appears to be sulfated at similar

rates by the different enzymes (Fig. 2) (6). Further kinetic analysis of the purified enzymes (e.g. of SUL1C2) and cellular localization of the enzymes is necessary to elucidate their relative importance in different tissues.

2.2 Potent inhibition of SUL1E1 by hydroxylated PHAHs

Polyhalogenated aromatic hydrocarbons (PHAHs) such as polychlorinated biphenyls (PCBs) are known for their endocrine-disrupting effects. Part of these effects may be mediated by the hydroxylated metabolites of these compounds. Intriguingly, various PCB-OHs and other PHAH-OHs very potently inhibit SUL1E1 (14,15). Possibly, the estrogenic effects of PHAH-OHs can be partly explained by the increased E2 bioavailability through the inhibition of SUL1E1-catalyzed E2-inactivation, rather than by agonistic estrogen receptor-binding (14-16). Since SUL1E1 also efficiently catalyzes the sulfation of iodothyronines, inhibition of SUL1E1 may also have a thyroid hormone-disrupting effect. The significance in vivo of inhibition of SUL1E1 in estrogen- and thyroid hormone-disruption by hydroxylated PHAHs remains to be investigated.

3.1 Importance of D1 in T3S clearance

As a result of the very rapid inner ring deiodination of T4S and T3S and outer ring deiodination of rT3S, the plasma concentration of these sulfated iodothyronines are very low in the normal adult (9-11,17). However, in patients with non-thyroidal illness and in hypothyroid patients, in which D1 activity is known to be impaired, T3S levels are increased (9). Increased serum T3S levels have also been reported in healthy subjects treated with iopanoic acid (IOP), which is a X-ray contrast agent which also inhibits D1 (9). In addition, T3S levels were elevated in rats which were administered IOP or other D1 inhibitors such as propylthiouracil (PTU), or when they were fed a selenium-deficient diet (18,19). These data demonstrate that in the adult T3S may accumulate if its deiodination by D1 is inhibited.

3.2 Sulfation of thyroid hormone during fetal development

As reported in section 2.1, high concentrations of different iodothyronine sulfates have been observed in human fetal serum and amniotic fluid (9-11). Based on studies in rats, these high iodothyronine sulfate levels were believed to be due to low hepatic D1 expression until after birth. However, although D1 activity in rat fetal liver is low, increasing just before birth (20), significant D1 activity is already present in the human liver at the end of the first trimester (21). Little is known about hepatic transporters of thyroid hormone conjugates in the developing fetus; absent or low expression of these transporters would be an alternative explanation for the high iodothyronine sulfate levels in the human fetus.

Although in normal adult serum 3,3'-T2S is undetectable by radioimmunoassay, significant 3,3'-T2S immunoreactivity has been found in plasma and urine of pregnant women (22-23). Intriguingly, HPLC analysis demonstrated that this immunoreactivity did not represent 3,3'-T2S, but was caused by a cross-reacting agent, called compound W. The structure of this compound has not been identified so far. Since maternal compound W is positively correlated with fetal fT4, whereas no correlation was found between maternal compound W and maternal fT4, compound W may represent a thyroid hormone metabolite of fetal origin (24). If so, measuring maternal compound W would be an interesting tool to evaluate fetal thyroid state.

Different sulfotransferases are involved in producing iodothyronine sulfates (Section 2.1). It has been suggested that the sulfates in the fetal circulation represent a reservoir of inactive thyroid hormone, from which active hormone is recovered when required, by the action of sulfatases present in different tissues (Fig. 1) (1). Significant iodothyronine sulfate hydrolysis has been observed in tissues such as liver, kidney, brain and placenta (25,26). So far, arylsulfatase C (ARSC) is the only member of the arylsulfatase family which is known to catalyze iodothyronine sulfate hydrolysis (26). We characterized iodothyronine sulfatase activities of human ARSC and of human placenta and liver, and demonstrated that ARSC may be largely responsible for placental iodothyronine sulfate hydrolysis. However, also other, still unidentified arylsulfatases contribute to iodothyronine sulfate hydrolysis in the liver (26). Further investigations are necessary to more clearly understand the importance of the different iodothyronine sulfotransferases and sulfatases in iodothyronine sulfation and desulfation in different tissues, to further elucidate the role of iodothyronine sulfation/desulfation in the regulation of thyroid hormone bioavailability in the developing fetus.

¹ M.H.A. Kester, M.W.H. Coughtrie and T.J. Visser, unpublished observations

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REPRODUCTIVE FUNCTION IN PATIENTS WITH THYROID DISEASES

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INTRODUCTION

The reproductive system has been regarded as relatively resistant to the effects of thyroid dysfunction. This view has been challenged by recent evidence, though most of the consequences are minor and reversible. However, the reproductive sequelae of thyroid disease are by no means trivial, particularly as the prevalence of thyroid dysfunction is high in the general population.

REPRODUCTIVE EFFECTS OF THYROID DYSFUNCTION IN MALES

Sex steroid metabolism

Thyrotoxicosis increases, and hypothyroidism reduces the concentration of serum SHBG (1). Total concentration of serum testosterone may alter accordingly, although free testosterone is usually normal (2). In some men with thyrotoxicosis oestrogen production is increased (3). Basal serum gonadotrophin concentrations are usually normal in adult males with thyroid dysfunction, but increased sensitivity of gonadotrophin secretion to GnRH has been described in thyrotoxic patients (4), and the reverse in hypothyroidism (5). In rare cases of severe prolonged primary hypothyroidism, pituitary hyperplasia can cause hypopituitarism (6). Hypogonadism may also be associated with hyperprolactinaemia caused by hypothyroidism (7). These changes are reversible when euthyroidism is achieved (8).

Effects of thyroid dysfunction in early life

Maternal hypothyroidism during pregnancy, cretinism and congenital hypothyroidism are not associated with abnormal development of the male reproductive tract (9). When adequately treated, boys with congenital hypothyroidism progress through puberty normally (10). Untreated hypothyroidism in early childhood can result in delay in sexual maturation, which can be reversed by thyroid hormone therapy (11). Severe juvenile hypothyroidism may rarely be associated with precocious pseudopuberty (9).

Spermatogenesis and fertility

Hyperthyroidism

Defective spermatogenesis was reported anecdotally in thyrotoxic patients several decades ago (12, 13). Abalovich et al. (14) found that of 21 patients with hyperthyroidism, 43% had a low total sperm count, and the majority had sperm motility problems. In a recent detailed prospective study, 23 thyrotoxic males and 15 healthy controls were assessed (15). Semen volume of thyrotoxic patients was normal. A non-significant trend towards low sperm density, and low percentage normal sperm morphology was noted in thyrotoxic subjects. Sperm motility was significantly lower in thyrotoxic males than in controls. Following treatment of thyrotoxicosis, sperm density and motility improved but sperm morphology remained unchanged.

Hypothyroidism

Hypothyroidism is associated with decreased libido or impotence (16). A small study of 5 men with primary hypothyroidism demonstrated normal sperm counts, but loss of sperm motility in some cases (17). Testicular biopsies of 6 adult males with onset of hypothyroidism in early life, revealed histological abnormalities in all patients (18). Testicular atrophy has also been reported in hypothyroid men (16). A prospective study of 10 adult patients demonstrated that short-term hypothyroidism does not cause seminal abnormalities sufficiently severe to impair male fertility (19).

The use of radioiodine in the management of hyperthyroidism and thyroid cancer in male patients of reproductive age

Reproductive function in men with thyrotoxicosis appears to be unaffected after ¹³¹I therapy (20, 21).

Most studies have shown that ^{131}I treatment for differentiated thyroid cancer may cause transient impairment of testicular function (22-24). Gonadal damage may occur in those requiring multiple treatments, particularly with cumulative doses greater than 14 GBq of ^{131}I , and sperm banking should be considered in appropriate cases.

REPRODUCTIVE EFFECTS OF THYROID DYSFUNCTION IN FEMALES

Sex steroid metabolism

Hyperthyroidism

As in men, hyperthyroidism results in increased levels of SHBG (25, 26). Plasma oestrogen levels may be twofold or threefold higher in hyperthyroid women during all phases of the menstrual cycle (27). The metabolic clearance rate of 17β -oestradiol is decreased in hyperthyroidism due to increased binding of 17β -oestradiol to SHBG (28). Mean plasma levels of testosterone and androstenedione are elevated (29). The production rate of testosterone and androstenedione are significantly elevated, and the conversion ratio of androstenedione to oestrone, and testosterone to oestradiol, are increased in hyperthyroid women (30). Mean LH levels in both the follicular and luteal phases are significantly higher in hyperthyroid women than in normal females (31). Serum LH levels decrease to normal after a few weeks of treatment with antithyroid drugs (32). Baseline FSH levels may be increased (33, 34), although this is refuted by some studies (35, 36). In a study by one of the authors, the gonadotropin response to GnRH was increased before treatment of hyperthyroidism and remained slightly exaggerated 4 months after treatment in comparison with controls (33).

Hypothyroidism

Women with hypothyroidism have decreased metabolic clearance rates of androstenedione and oestrone and an increase in peripheral aromatization (37). The $5\alpha/5\beta$ ratio of the metabolites of androgens is decreased in hypothyroid women, and there is an increase in the excretion of 2-oxygenated oestrogens (38). The binding activity of SHBG in plasma is decreased, so that plasma concentrations of testosterone and oestradiol are decreased, although their unbound fractions are elevated. The alterations in steroid metabolism disappear when the euthyroid state is restored (39). Gonadotropin levels are usually normal (40). However, blunted or delayed LH response to LHRH has been reported in some hypothyroid females (35, 41).

Menstrual function and fertility

Hyperthyroidism

Amenorrhoea, oligomenorrhoea, hypomenorrhoea, and anovulation can occur in hyperthyroidism. The frequency of menstrual abnormalities in recent studies differs from earlier series. In one recent study, however, we found irregular cycles in only 46 (21.5%) out of 214 thyrotoxic patients. 24 had hypomenorrhoea, 15 poly-, 5 oligo-, and 2 hypermenorrhoea. None had amenorrhoea. From a similar number of normal controls, 18 (8.4%) had irregular periods, and of these 12 had oligomenorrhoea (42). These results are inconsistent with what is generally believed and written in the classic thyroid textbooks concerning the frequency and pattern of menstrual disturbances in thyrotoxicosis (43, 44) and indicate that such opinions should be revised. Hyperthyroidism in women has been linked to reduced fertility, although most thyrotoxic women remain ovulatory according to the results of endometrial biopsies (45). We measured progesterone levels, a fertility parameter, in the middle of the luteal phase of the cycle in 74 women of reproductive age, 37 of whom had Graves' disease and 37 of whom were euthyroid controls matched for age and weight. We found that progesterone levels were decreased before treatment in comparison with controls and were unrestored 4 months after carbimazole therapy (46).

Hypothyroidism

In women of fertile age, hypothyroidism results in changes in cycle length and amount of bleeding, that is, oligo- and amenorrhoea, polymenorrhoea, and menorrhagia. A recent study (47), found that 40 (23.4%) out of 171 hypothyroid female patients had irregular cycles. From those, 17 had oligo-, 6 hypo-, 5 amenorrhoea, and 12 hypermenorrhoea/menorrhagia. None had poly- or hypermenorrhoea. Although this finding indicates that the frequency of menstrual disturbances in hypothyroidism is approximately three times greater than in the normal population, this is still much lower than the findings of previous similar studies. Furthermore, we found that the main menstrual irregularity was oligomenorrhoea (42.5%), which is also inconsistent with what is generally believed or written in classic thyroid texts (40, 48). Severe hypothyroidism is commonly associated with diminished libido and failure of ovulation (45).

The use of radioiodine in the management of hyperthyroidism and thyroid cancer in female patients of reproductive age

Studies on pregnancy outcomes and offspring of patients previously treated with ^{131}I for thyroid carcinoma failed to reveal any significant ^{131}I -related effects (49-53). In one recent study, Schlumberger et al. (54) presented data on 2,113 pregnancies conceived after exposure to 30-100 mCi of ^{131}I given for thyroid cancer or thyroid remnant ablation. The incidences of stillbirth, preterm birth, low birth weight, congenital malformation, and death during the first year of life were not significantly different between pregnancies conceived before and after radioiodine therapy. These data do not establish that no risk exists, but they indicate that the risk is less than other more common hazards of pregnancy. Also, they indicate that the risk of a second tumor or of damage to the gonads of women treated with ^{131}I is low and of no clinical significance. Fertility in the long term is not disturbed and ^{131}I treatment is not contraindicated for this reason. Nevertheless, it should be avoided for at least one year after exposure to ^{131}I , because of the increased risk of miscarriages (55). Following therapeutic administration of ^{131}I to the mother, breastfeeding should be discontinued immediately (56-58).

TABLE 1 Summary

Hyperthyroidism appears to cause sperm abnormalities (mainly reduction in motility), which reverse after restoration of euthyroidism.

Radioiodine therapy for thyroid cancer may cause transient reductions in sperm count and motility, but there appears to be little risk of permanent effects provided that the cumulative dose is less than 14 GBq.

The effects of hypothyroidism on male reproduction appear to be more subtle than those of hyperthyroidism and reversible. Severe, prolonged hypothyroidism in childhood may be associated with permanent abnormalities in gonadal function.

Hyperthyroidism is associated with menstrual disturbances in female patients, mainly hypomenorrhoea and polymenorrhoea. The frequency and pattern in contemporary studies is at variance with views expressed in classic thyroid textbooks.

Hypothyroidism may cause oligomenorrhoea, amenorrhoea, hypo- and hypermenorrhoea/ menorrhagia. Severe hypothyroidism is commonly associated with diminished libido and failure of ovulation. The frequency of these disturbances is much lower than findings of older similar studies. The incidences of stillbirth, preterm birth, low birth weight, congenital malformation, and death during the first year of life are similar between pregnancies conceived before and after ^{131}I therapy for thyroid cancer. Pregnancy should be avoided for at least one year after the ^{131}I . Therapeutic administration of ^{131}I should be followed by immediate cessation of breastfeeding.

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THYROID HORMONE AND LUNG DEVELOPMENT

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Introduction

Lung formation is a continuous process but has been sub-divided, for descriptive purposes, into developmental phases. In humans, the phases are embryonic (4 to 7 weeks), pseudoglandular (7 to 16 weeks), canalicular (16 to 24 weeks), and a terminal sac phase (24 weeks-term). During the embryonic phase, the endodermal foregut outgrowth subdivides dichotomously establishing the lobar bronchial tree and, in the pseudoglandular phase, grows rapidly to its completed pattern. During the late canalicular phase (22 to 24 weeks) specialization of the respiratory portion of the lung begins, with dilation of the airways and differentiation of the epithelium to type I (gas exchanging) and type II (surfactant producing) pneumocytes. The development of the respiratory airway continues through the terminal sac phase, initially with the formation of saccules, but with further sub-division to the formation of alveoli, which can be seen as early as 29 weeks gestation (1).

Pseudoglandular phase of lung development

Until very recently the effect of thyroid hormones on airway branching morphogenesis and early lung cellular differentiation, and the specific developmentally regulated factors that control these processes, had not been studied. During the early pseudoglandular period of murine lung development T3 accelerates embryonic lung cellular differentiation resulting in a prematurely advanced epithelium, and reduction in new branch formation (2). These are novel observations. But the question remains: are the thyroid hormone levels at this stage of development in humans sufficient to influence lung development? Serum T4 and T3 levels in the early human fetus are a 100 fold lower than maternal values and the efficacy of such low concentrations to contribute to the regulation of development has been questioned. However, FT4 values in fetal first trimester coelomic and amniotic fluids, and fetal serum up to 17 weeks gestation are at least one third of those biologically active in their euthyroid mothers (3). The human fetus at these gestations is almost solely dependent on a transplacental thyroxine supply; hence both T4-binding protein levels and the maternal serum T4 determine fetal FT4 levels. Maternal hypothyroxinemia in early pregnancy can be associated with irreversible effects on infant neurodevelopment, and perhaps now disordered lung branching morphogenesis should also be considered?

Late canalicular and terminal sac phases of lung development

Survival of human preterm infants is related to gestational age (0% at 20 weeks, 90% plus at 30 weeks), which in turn is determined by adequacy of the maturation of the lung for gaseous exchange during the late canalicular and terminal sac phases. Inadequate lung maturation results in respiratory distress syndrome (RDS). In more mature preterm infants this is predominantly caused by a deficiency of surfactant, but in infants <30 weeks gestation it is further compounded by inadequate differentiation of the epithelium, and a limited gas-exchanging surface area. The role of thyroid hormones in lung development has concentrated predominantly on these late fetal events, particularly the regulation of surfactant production. In a series of in vivo studies in animal models it has been established that T4 and T3 accelerate surfactant production and fetal lung maturation. Acceleration of fetal lung maturation and surfactant phospholipid production also occurs in vitro in different species, and in different model systems, including human lung explants and fetal type II pneumocytes, but without changes in surfactant protein levels.

The development process of pulmonary alveoli formation, through septation of saccules, is similar in all mammals, although the timing of this varies considerably among species. Little is known about the regulation of these events. However, there have been important recent advances in this research area with evidence for differential regulation of pulmonary alveoli formation by oxygen, corticosteroids, and retinoids, including the specific expression of retinoic acid receptors (4). Thyroid hormones also appear to have a role in this process of alveolar formation. In rats, alveolar formation is predominately

postnatal, and administration of T3 to newborns accelerates the pace of septation resulting in a greater alveolar surface area, and smaller alveoli, without affecting total lung volume. Propylthiouracil inhibits septation and thyroxine administration overcomes this inhibition (5). The evidence for the role of thyroid hormones in the regulation of alveolar formation originates from before the mid 1980's and clearly should now be updated and integrated with more recent advances in molecular regulation of this process.

Respiratory distress syndrome

The association between serum thyroid hormone concentrations and respiratory distress syndrome (RDS) has been extensively studied (6). Early reports suggested that thyroid hormone concentrations in cord blood of premature infants with RDS were low relative to gestational age and birth weight matched controls suggesting a cause and effect relationship. These findings were not supported in later studies (7) and it is probable that the control populations in the earlier reports were inadequately matched. Congenitally hypothyroid mice (hyt/hyt) have delayed lung maturation (8), and the most convincing human evidence comes from a case report of a pregnancy with maternal and fetal heterozygote Pit-1 deficiency; the index infant had delayed bone age, respiratory distress syndrome with chronic lung disease, and neurodevelopmental delay (9).

Several studies have however demonstrated depressed postnatal TT3 and TT4 concentrations in infants with RDS. These changes are believed to reflect reduced protein binding associated with reduced TBG concentrations relative to healthy controls. However, FT4 concentrations in infants with RDS are variable; they have been shown to be lower than, similar to, or higher than those in healthy infants. Variations in FT4 assay methods in part explain these differences (10).

Thyroid hormone therapy in RDS

An early study of thyroid hormone therapy (T3 and T4) in infants with RDS demonstrated a reduction in mortality from 29% to 6.6% in the treatment group. This finding has not been reproduced in subsequent studies of either T3, or T4 therapy. In addition, no beneficial effect on duration of ventilation or incidence of chronic lung disease has been identified (11). The results of a recent multi-centre randomised trial of T3 and hydrocortisone in preterm infants (Thorn Trial) showed no beneficial effect over placebo on mortality, ventilator or oxygen dependence (12).

Estimates of the prevalence of non-thyroidal illness in premature infants range from 30% in low birth-weight to 60% in very low birth-weight infants. The evidence to date suggests that neonatal morbidity including RDS has a major impact on postnatal thyroid function, with more severe illness associated with more marked hypothyroxinaemia and a worse outcome. For example, infants who subsequently die have TT3 and TT4 concentrations two to three folds lower than survivors of similar gestational ages (13). There is currently no evidence that thyroid hormone therapy has a beneficial role in neonatal non-thyroidal illness.

Transient hypothyroxinaemia in preterm infants is characterised by a temporary post-natal reduction in serum T4 with normal levels of TSH. The aetiology remains unclear but is unlikely to be solely secondary to non-thyroidal illness, and may have contributions from the withdrawal of maternal-placental thyroxine transfer, developmental constraints on the synthesis and peripheral metabolism of iodothyronines, iodine deficiency and hypothalamic-pituitary-thyroid immaturity. The challenge is to understand these individual component parts, how they are inter-related, and then to develop appropriate therapies. Transient hypothyroxinaemia is present in the majority of infants less than 30 weeks gestation, and is associated with neurodevelopmental deficits; if the means of assessing the respiratory system in sick preterm infants had the same degree of sophistication as later tests of psychomotor function then perhaps associations could also be found with other lung dysfunctions in these infants.

Antenatal glucocorticoids and TRH

Maternal antenatal glucocorticoids before preterm delivery reduces the incidence of respiratory distress syndrome. There is evidence that thyroid hormones potentiate surfactant production and lung maturation induced by glucocorticoids. The translation of these in vitro observations to in vivo studies of the effect of thyroid hormones on fetal lung development is that the ability of T4, T3 or TSH to cross the placenta in different species remains controversial. Exogenous TRH crosses the placenta and increases fetal TSH and T3 production. The initial clinical studies of combined prenatal TRH and corticosteroids appeared to reduce chronic lung disease in preterm infants, but subsequent studies in North America and Australia (ACTOBAT) and more recently in Europe (14) have shown no additional

benefit of TRH, and in the latter studies the infants had a poorer neurodevelopmental outcome. It is known that postnatal administration of TRH between 16 hours and 28 days postpartum results in marked increases in TSH, T3 and / or T4 in both preterm and term infants from 24 weeks gestation onwards. In addition maternal administration of TRH prior to preterm delivery has demonstrated that the pituitary is responsive to TRH as early as 24-28 weeks gestation. This has been interpreted as consistent with a tertiary (central of hypothalamic origin) rather than a primary cause of postnatal hypothyroxinaemia in preterm infants (15).

Cooling and other birth stresses are natural stimulants of the hypothalamic-pituitary-thyroid axis. In term infants following delivery there is a marked post-natal surge of serum TSH levels at around 30 mins of age, with further later increments in T3 and T4 levels.

Concluding remarks

Few studies have investigated preterm infants in sufficient detail, gestational ages and numbers, to allow the complete temporal description of the relationships within the hypothalamic-pituitary-thyroid axis, but the evidence to date suggests preterm infants have an attenuated pituitary-thyroid response. Further carefully controlled investigations of this axis in preterm infants are clearly required to allow the development of preventative therapies, which maximise the role of thyroid hormones in adaptation to extra-uterine life.

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TREATMENT OF THYROID HORMONE RESISTANCE SYNDROMES

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TREATMENT OF THYROID HORMONE RESISTANCE SYNDROMES

Resistance to thyroid hormone (RTH) is an inherited disease characterized by a reduced responsiveness of target tissues to thyroid hormone action. RTH is a rare syndrome, being up to now described in about 700 patients belonging to 250 unrelated families, with an estimated incidence of 1:50,000 live births (1). Molecular studies have clarified that RTH is linked to alterations of thyroid hormone receptor beta (TRb) (2, 3). In fact, sequence analysis of TRb gene showed that 80-90% of RTH patients harbors mutations of this gene located on chromosome 3 and coding for two TR isoforms, b1 and b2. Most of the described mutations are located within 3 "hot spot" regions in the ligand binding domain of the receptor. Thus, mutated proteins result in either a reduced affinity for T3 or an impaired interaction with the cofactors involved in the transcriptional machinery (4, 5). In accordance with the dominant autosomal inheritance of RTH, almost all patients express one normal b-receptor. Resistance in RTH patients occurs because the mutant receptor inhibits the activity of the normal a and b-receptors, the clinical phenotype being most severe in the case of homozygosity for the mutant receptor. The deletion of the entire TRb gene, due to a nonsense mutation in codon 1 is accompanied by a normal phenotype in heterozygous subjects (i.e., lack of dominant negative effect), whereas full clinical and biochemical features of RTH are manifest in homozygous patients. The resistance at pituitary and hypothalamic level results in a biochemical picture characterized by elevated thyroid hormone (TH) levels in the presence of detectable thyroid stimulating hormone (TSH) concentration. Despite of normal TSH concentration, goiter is usually present, mainly due to increased bioactivity of circulating glycoprotein (6). Clinical manifestations are extremely variable, the majority of patients being euthyroid according with a generalized form of RTH (GRTH), while a minority of them presents with thyrotoxic features standing for a less pronounced resistance at the peripheral tissue level (PRTH). Moreover, there is only one case reported in the literature of isolated resistance to peripheral tissue level (PTRTH), presenting with a severe hypothyroid state (7). In some patients with either GRTH or PRTH, the clinical presentation may vary over the time and may present with different features on various tissues and organs. Such a variable phenotype does not correspond to a given genotype as patients harboring the same TRb mutation may have either form of RTH (8).

Hence, there are no pathognomonic signs and symptoms of RTH and the clinical picture depends on the degree of compensation achieved by high concentrations of TH in different tissues.

Early recognition of RTH is important, since its management differs from that of the other disorders that may enter into the differential diagnosis with RTH, i.e. TSH-secreting pituitary tumors and the various forms of hyperthyroidism accompanied by diffuse goiter. Besides, even if the classification of RTH patients in GRTH or PRTH may arise from uncertain signs and symptoms, it remains useful in order to define the necessity and the choice of treatment.

In this article, we review the most common therapeutic approaches to patients with GRTH or PRTH from childhood to the adulthood. Emphasis will be given to the most recent approaches based on the molecular engineering of natural agonist of nuclear receptor that turned out to be potent and selective ligands of mutant receptors involved in the disease.

GRTH: primum non nocere

The great majority of subjects with RTH adequately compensate the abnormal function of the mutant receptors by increasing thyroid hormone levels, thus achieving an euthyroid state, goiter remaining the only manifest abnormality. Consequently, any therapeutic approach prompted to reduce the elevated thyroid hormone concentrations should be avoided since it may aggravate the clinical picture leading to hypometabolic signs and symptoms.

Unfortunately, despite of the increasing awareness of this rare disorder, the presence of goiter frequently accompanied by tachycardia induced many clinicians to misleadingly diagnose the presence of Graves' disease in about 50% of RTH patients. Inappropriate treatments with antithyroid drugs, or even more invasive treatments, such as thyroid ablation by radioiodine or surgery were thus undertaken (3).

Administration of thyroid hormones, mainly L-T₄, is appropriate in patients in whom the increased endogenous TH production does not result in a sufficient supply to all peripheral tissues, as well as in patients erroneously treated with thyroid ablation. When L-T₄ therapy is undertaken in a patient with RTH, the optimal dosage is extremely variable among individuals. Sometimes a dosage as high as 1000 mg per day may be necessary to achieve the desired effects, i.e. to overcome tissue resistance (1). The achievement of the optimal L-T₄ dosage is documented by monitoring the clinical features of the patient, as well as by measuring several parameters of peripheral TH action (i.e., sex hormone-binding globulin: SHBG, angiotensin converting enzyme: ACE, carboxyterminal crosslinked telopeptide of type I collagen: ICTP, soluble interleukine-2 receptor: sIL-2r, cholesterol, osteocalcin, etc.). Useful may be also the measurement of TSH concentration that should remain into the normal range and not be suppressed. Finally, a possible increase of the pituitary size or even the appearance of a pituitary tumor should be carefully monitored. In fact, the resistance at the pituitary level creates a continuous stimulus on thyrotrophs to secrete TSH. As documented in a single patient, L-T₄ therapy successfully reduced pituitary hyperplasia in an RTH patient (9). Therefore, the pituitary monitoring by MRI may be an additional tool to follow L-T₄ therapy in RTH patients.

When tachycardia is the unique sign of RTH, treatment with cardioselective b-blockers is indicated. Among these drugs, agents inhibiting peripheral T₄ to T₃ conversion, thus worsening of the hypothyroidism present in certain tissues, should be avoided. For this reason, cardioselective compound, such as atenolol devoid of effect on peripheral T₄ conversion, appears to be more useful (3).

PRTH: controlling thyrotoxic features

As already stated, distinguishing between the GRTH and PRTH is of crucial importance since the management of these two forms is different. Admittedly, the presence of complex clinical features of tissue-specific hyperthyroidism, as well as the insufficient sensitivity of the indices evaluating peripheral thyroid hormone action in patients with PRTH, renders the treatment of these individuals very difficult. PRTH patients may take advantage from reduction of thyroid hormone levels. Many efforts were therefore spent in finding a therapy for PRTH able to block pituitary secretion of TSH, thus resulting in a slight reduction of TH. In 1983, we suggested the use of triiodothyroacetic acid (TRIAC), a TH byproduct with predominant thyromimetic effect on pituitary secretion as compared to that on the peripheral tissues (10). In particular, it has been demonstrated that TRIAC has a higher affinity for TR_b1 than does T₃, while the two compounds have a similar affinity for TR_a1. Moreover, TRIAC seems to selectively augment the function of TR_b1 without affecting TR_a1, thus influencing the activity of the only TR receptor involved in RTH. Cotransfection studies also showed that TRIAC is more effective in increasing the function of mutant TR_b1 or in overcoming its dominant negative effect than T₃, thus providing further experimental support to the use of TRIAC not only in PRTH, but also in selected cases of GRTH (11).

TRIAC, as well as another thyroid hormone analog, the dextro-thyroxine (D-T₄) (12), have been extensively employed in PRTH patients (10). In fact, both these agents have been shown to suppress TSH levels leading to reduced secretion of thyroid hormone and restoration of the euthyroid state in the majority of patients, although failures have also been reported. In our series of RTH patients treated with TRIAC, only one did not show clinical improvement, despite of normalization of TSH and FT₄ concentration, thus suggesting that clinical symptoms are not always related to the presence of PRTH. In some other cases, even in the presence of unchanged immunoreactive levels of TSH, both serum FT₄ and hyperthyroid features normalized. In fact, TRIAC treatment leads to the normalization of TSH bioactivity, which is also recorded under T₃ administration. Finally, the results obtained with the two analogs may be different in the same individual. In fact, a beneficial effect of D-T₄ in a boy with PRTH who failed to respond to TRIAC, has been reported (13).

When patients present tachycardia, despite of the reduced thyroid hormone level, cardioselective beta-blockers may be associated following the criteria discussed before.

Treatment of children with RTH

Increased endogenous TH may not be sufficient to supply the requests of peripheral tissues, especially in infants with GRTH. Although general criteria for treatment of infants with GRTH is still lacking, in

young children presenting with growth and/or mental retardation, the administration of supraphysiological doses of L-T4 may be beneficial to overcome the high degree of resistance in some tissues (14). It is evident that such therapy needs careful monitoring in order to avoid overtreatment. In this respect, particular attention has to be paid to TSH levels, as well as to a number of indices of peripheral TH action and pituitary MRI, as previously discussed. No alternative treatment to L-T4 administration has been so far discovered.

American scientist reported that 70% of RTH children and 50% of adults met criteria for attention-deficit hyperactivity disorder (ADHD) (15). Other European and American authors have not confirmed these data. Even in the absence of the clear linkage between the two disease, treatment with TRIAC seem to be useful to control symptoms related to ADHD, as documented by us at least in one RTH patient (personal observation).

In utero diagnosis and treatment

Although the diagnosis of RTH is now possible both in utero (16) and at birth (17), the indications for treatment of fetuses and newborns are still under investigation. In fetuses with RTH who are small for gestational age, an indication to the treatment can be envisaged. Since TRIAC has been documented to cross the placental barrier (18), administration of this analog to the mother may be undertaken only after molecular confirmation of RTH in the fetus. In newborns, retarded bone development and failure to thrive indicate the need of L-T4 administration at supraphysiological doses.

Previously suggested treatments

Several agents acting at various levels have been suggested in the past for the treatment of RTH, in particular PRTH. Lowering serum thyroid hormone levels by antithyroid drugs invariably causes dramatic increase of TSH levels followed by a consistent increase in goiter size and possible pituitary hyperplasia. This approach should be therefore avoided or considered as the last resort. Administration of T3 was also suggested, but this produces daily peaks of high levels of T3, which may maintain signs, and symptoms of thyrotoxicosis (19). Corticosteroids constantly cause severe inhibition of hypothalamic-pituitary-adrenal axis function and cushingoid features (20). Furthermore, trials with either the dopaminergic drugs, such as bromocriptine, or somatostatin analogs have been performed (21), but it appears that during a prolonged administration, all these drugs rapidly lose their efficacy in reducing TSH levels, and therefore the TH hypersecretion (22). In particular, somatostatin analogs appear extremely useful in controlling signs and symptoms of hyperthyroidism in patients with TSH-secreting pituitary adenoma. On the contrary, except for the first few days after injection, RTH patients rapidly escape from the inhibitory effect on TSH secretion. Moreover, we tried to improve the refractoriness to thyroid hormones by chronically administering pharmacological doses of vitamin A to RTH patients, but no modification of clinical and biochemical indices was obtained (23). The rationale for vitamin A treatment is that of enhancing the availability of retinoids, in particular 9-cis-retinoic acid which is the ligand of RAR that dimerizes with TR β in binding to TH receptor response elements (TREs) on gene controlled by TH, thus augmenting the action of thyroid hormones on gene expression.

Future of RTH therapy

Mutations associated to RTH have been shown to reside within or around the hormone-binding pocket of the receptor, thus disrupting its normal transactivation function. Unfortunately, clinical treatment of RTH with supraphysiological concentrations of T3 to recover mutant TR β proteins activity may lead to overstimulation of TR α receptors responsible for undesired effects such as tachycardia. Hence, compounds having high affinity and selectivity for mutant TR β isoforms, and not for TR α , are sought for RTH therapy. Using known receptor agonists as a structural scaffold, potent hormone analogues can be rationally designed to complement a mutant form of thyroid hormone receptor. GC1 is a potent nonhalogenated thyromimetic compound of particular interest because of its preferentially binding to TR β over TR α (24). Unfortunately, it shows a reduced activity toward the mutant TR β receptors than to the normal ones. On the basis of site-models generated from the T3/TR β crystal structure, a neutral alcohol HY1 has been designed, which resulted to be a potential subtype selective ligand for the mutant receptor TR β (R320C) (25). In particular, HY1 is more potent than GC1, it is capable of eliciting substantial transactivation response from the mutant TR β at concentration showing only partial activation of TR α .

In this respect, it is possible to think about therapies based on modifications of known agonists to obtain highly potent and selective ligands capable to compensate the action of the mutant receptors implicated in RTH.

Finally, molecular engineering provided novel therapeutic approaches based on the ability to clone individual types of gene, transfer them into recipient cells and express them, or to design new proteins, or even to inhibit specifically the expression of a predetermined and characterized gene *in vivo*. Considering that pathophysiology of RTH is based on the dominant negative effect of the mutant receptor on the normal one, and taking into account that the deletion of the entire TR β gene due to a mutation in codon 1 is accompanied by a normal phenotype, it is possible to speculate that genetic strategies prompted to inhibit mutated TR β gene expression may be considered as a specific and individual therapy for RTH patients.

Table. Suggested therapeutic approaches for resistance to thyroid hormone (RTH) patients.

Drugs	Untoward effects and limitations
TRIAC	Effective in almost all patients
D-T4	Effective in almost all patients
T3	Production of daily peaks of very high T3 concentrations, which contribute to maintain clinical hyperthyroidism
Bromocriptine	Transient effect owing to TSH escape from inhibition
Sms analog	Transient effect owing to TSH escape from inhibition
Corticosteroid	Cause of severe inhibition of hypothalamic-pituitary-adrenal axis function and cushingoid features
Antithyroid drugs	Cause of further increase in TSH circulating level with consequent increase of goiter size and to hyperplasia at pituitary thyrotroph level
b-blockers	Effects limited to b-adrenergic blockade. Propranolol inhibits peripheral conversion of T4 to T3, causing a worsening of tissue hypometabolic state. Cardiac selective compounds, such as atenolol devoid of effect on peripheral T4 conversion, appear to be more useful.

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WHAT MAKES A THYROID CELL A THYROID CELL ?

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When G. Hennemann asks you to cover such a subject for next year the answer is obviously yes and then you wonder why you accepted it. Probably G. Hennemann had in mind our repetitive remarks at the ETA congress that thyroid cell lines should not be considered as "the thyroid cell"... The question what makes a thyroid cell a thyroid cell can be subdivided in :

1) What is the thyroid cell ?

As any cell, the thyrocyte is defined by its program i.e. by the proteins that it contains and by their spatial organization. This begs the question : what does it do ? As any differentiated cell, the thyrocyte contains the specific proteins needed for its specialized function, the synthesis and secretion of thyroid hormone. Thus the iodide symporter NIS, iodide channels such as pendrin, and probably others, the matrix of thyroid hormone synthesis, thyroglobulin, the H₂O₂ generating system THOX1 and THOX2, thyroperoxidase, the ill defined endocytosis apparatus and iodotyrosine dehalogenase are all present in the thyrocyte. Some of these proteins can be found in other cells but the combination is unique.

Similarly the specific control by and role of TSH implies the expression of TSH receptor in thyrocytes (with some exceptions in some species, adipocytes, retroorbital fibroblasts ?). Thus the thyrocyte is defined by the unique expression of a combination of proteins with specific functions.

As the thyrocyte is the only cell that perceives the physiological signals of TSH and iodide it must, by necessity, be the transmitter of such information to other cells in the thyroid such as endothelial cells, fibroblasts, macrophages, etc (unless TSH receptors exist in thyroid endothelial cells ?) [1].

2) What does it look like ?

The protein content of the thyrocyte cannot explain its function without the complement of spatial organization. Unlike the other endocrine cells the thyrocyte localizes all the elements of hormone synthesis at its apical border: iodide, thyroglobulin, H₂O₂. Their toxicity could otherwise greatly harm the cell. On the other hand, the follicular structure allows the elements of this synthesis to remain concentrated. Otherwise, as in isolated cells or in monolayer cultures, the efficiency of the synthesis is annihilated by diffusion. These constraints imply a rigorous cell localization of the specific proteins NIS and TSHR at the basal side, facing the extracellular fluid, iodide channels, THOX and TPO at the apex and thyroglobulin in the lumen. The lumen also allows both the storage of the prehormone thyroglobulin and an incremental iodine coupling to thyroglobulin and hormone synthesis according to the iodide supply. This mode of storage in a matrix protein requires an endocytotic rather than exocytotic mechanism of secretion.

3) Where does it come from ?

The steps of thyroid development and their molecular correlations have been beautifully illustrated by Di Lauro and his team. Migration of the primary committed cell from the floor of the pharynx to its cervical site requires the TTF2 transcription factor. Differentiation development and the appearance of the specialized proteins NIS and TSH receptor, thyroglobulin and TPO expressions require the TTF1, Pax 8 and probably yet unknown transcription factors. None of these factors is entirely specific for the thyrocyte, but their combined presence is... It is the combination that defines the cell, just as the combination of a few letters defines a word [2].

4) What makes it tick ?

Just as the constraints of its biochemistry fit in with the special structure of the secretory thyrocyte, the

constraints of physiology fit in with its regulation.

First, the two main factors involved in the regulation are TSH, which relays the information on thyroid hormone requirements as appreciated by the hypothalamus and hypophysis, and the iodide plasma levels which inform about the supply that is necessary for thyroid hormone synthesis. Both factors are acting on virtually all the steps of thyrocyte function and on its growth. TSH accomplishes this through its receptor and iodide mostly through iodinated derivatives such as iodoheptadecanal and perhaps iodolactones by still poorly defined mechanisms. Apart from these agents some other locally acting factors such as IGF also contribute to the regulation. Neurotransmitters different from one species to another have receptors on thyrocytes but their physiological role, if any, is unknown [1].

Second, the thyroid secretes a slow acting long lived prohormone thyroxine with a delayed transcriptional action that sets a durable level of activity rather than, as other hormones, eliciting a response to external or internal factors. The pituitary TSH-thyroid-thyroxine axis behaves as a thermostat maintaining basal metabolism and various biosyntheses. Therefore, the regulation of the thyroid does not need to be very fast. Nevertheless, perhaps as a remnant from our evolutionary past when acute thyroid hormone secretion was needed to respond to cold, the effects of TSH are both rapid and delayed. However, unlike neurotransmitter receptor with their rapid on/off responses and, contrary to its sister hormones LH and FSH., the effect of TSH is prolonged and the TSH receptor is little desensitized or downregulated. Hence the stimulatory effects of TSAb... In most other systems, which desensitize rapidly and strongly (eg the insulin receptor, the nicotinic receptor), even stimulatory antibodies have an overall inhibitory effect. Also, unlike these receptors, the TSH receptor has a basal activity. After all, in finalist terms, our body always needs some thyroid hormone.

Third, as most physiological systems, the thyroid responds to a stimulus by rapid activation, and, if the stimulus is important and long lasting, by a delayed multiplication of the elements i.e. the cell mass and the number of cells. The two types of responses are elicited by the same stimuli, themselves resulting from physiological demands.

5) Is there a good model ?

Our main interest is evidently the human thyroid cell in vivo and its dysfunction in disease. Various models are proposed to study in depth this cell. The best, by far, but difficult to obtain is the human thyroid cell in thyroid slices with its normal organization. However the cells do not survive more than a few hours in this model. Also, as most follicles remain closed their lumen remains inaccessible [3]. Thyroid cells in primary culture are more remote from physiology but can be studied over days. Unless reconstituted in follicles they do not allow studies on function (see 2). Animal thyroid slices and cultured cells are a good substitute for human preparations but their regulatory circuits may be different (eg pig thyrocytes multiply in the presence of iodide but not TSH !). The usual cell lines (FRTL5, WRT and PCCl3 cells) are even more remote from physiology. Their convenience for study is inversely proportional to their relevance. Their publication/output to experiment ratio is by far the best. For instance, their growth regulatory circuits are completely scrambled with TSH cyclic AMP and IGF1 using almost the same pathways. However, they allow genetic experiments and, in so far as the investigated pathway is similar to the human cell, their study can provide much useful, and otherwise impossible to get, information. However in the interpretation of results one should be aware of the limitations, and always restrain from using the word cell line in the introduction and thyroid cell in the title, abstract and discussion. Models are models and none of them justifies the word "the thyroid cell"...; with species differences there is no such thing [4]. When possible, the best strategy to study thyroid cells is to investigate mechanisms in vitro, to validate results in vivo in transgenic and knockout mice and, if possible, use human disease to validate the concepts. This is the strategy that proved the Ret-PTC role in papillary carcinoma [5], the TSH receptor/cAMP role in autonomous adenoma [6], the TTF1-Pax8 differentiation and TTF2 thyroid migration concepts [2].

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THE THYROID HORMONE RECEPTOR FAMILY: INSIGHTS FROM KNOCKOUTS

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THE THYROID HORMONE RECEPTOR FAMILY: INSIGHTS FROM KNOCKOUTS

Abstract

Thyroid hormone receptors (TRs) are nuclear receptors that act as thyroid hormone (T3)-regulated transcription factors. Several TRa and TRb receptor isoforms are encoded by the Thra and Thrb genes, respectively, and evidence from targeted mutagenesis in mice indicates that each gene plays specific roles in development and homeostasis. In addition, combined mutations produce exacerbated or otherwise modified phenotypes indicating that together these genes co-regulate an extended array of actions. This interplay between receptor genes provides a versatile mechanism underlying the diverse biological responses to T3.

The TR family

It has been a long-standing puzzle how the thyroid hormonal signal elicits its remarkably wide but nonetheless specific range of actions. The appealing possibility that tissue-specificity is determined at least in part by a family of receptors with differing spectra of actions has been implicit since the cloning of the first TR cDNAs in 1986 (1,2) indicated the existence of two related receptor genes. These genes, denoted Thra and Thrb in mice (or THRA and THRB in humans) display differential but overlapping patterns of expression. Furthermore, they each express alternative receptor isoforms suggesting the operation of a complex code of receptor pathways in vivo (3-5). But what exactly are these receptor roles and how is labour divided in this receptor family? Targeted mutagenesis in "knock-out" mice has recently suggested answers to some of these questions.

The TR family includes three Thrb products: TRb1 which is widely expressed, TRb2 which is restricted to the retina, pituitary and cochlea (3-5) and TRb3, found in an osteosarcoma cell line, lung and kidney (6). Thra encodes a widely expressed TRa1 receptor. In mammals, Thra also encodes an enigmatic C-terminal splice variant, TRa2, that does not bind T3 nor transactivate (3), as well as some minor, truncated products (7). Although Thra and Thrb are differentially expressed during development, both genes become widely co-expressed in many adult tissues, suggesting that they serve both specific and common roles.

Unique and common functions of TR isoforms

Targeted mutagenesis has indeed demonstrated independent roles for Thrb and Thra (Figure)

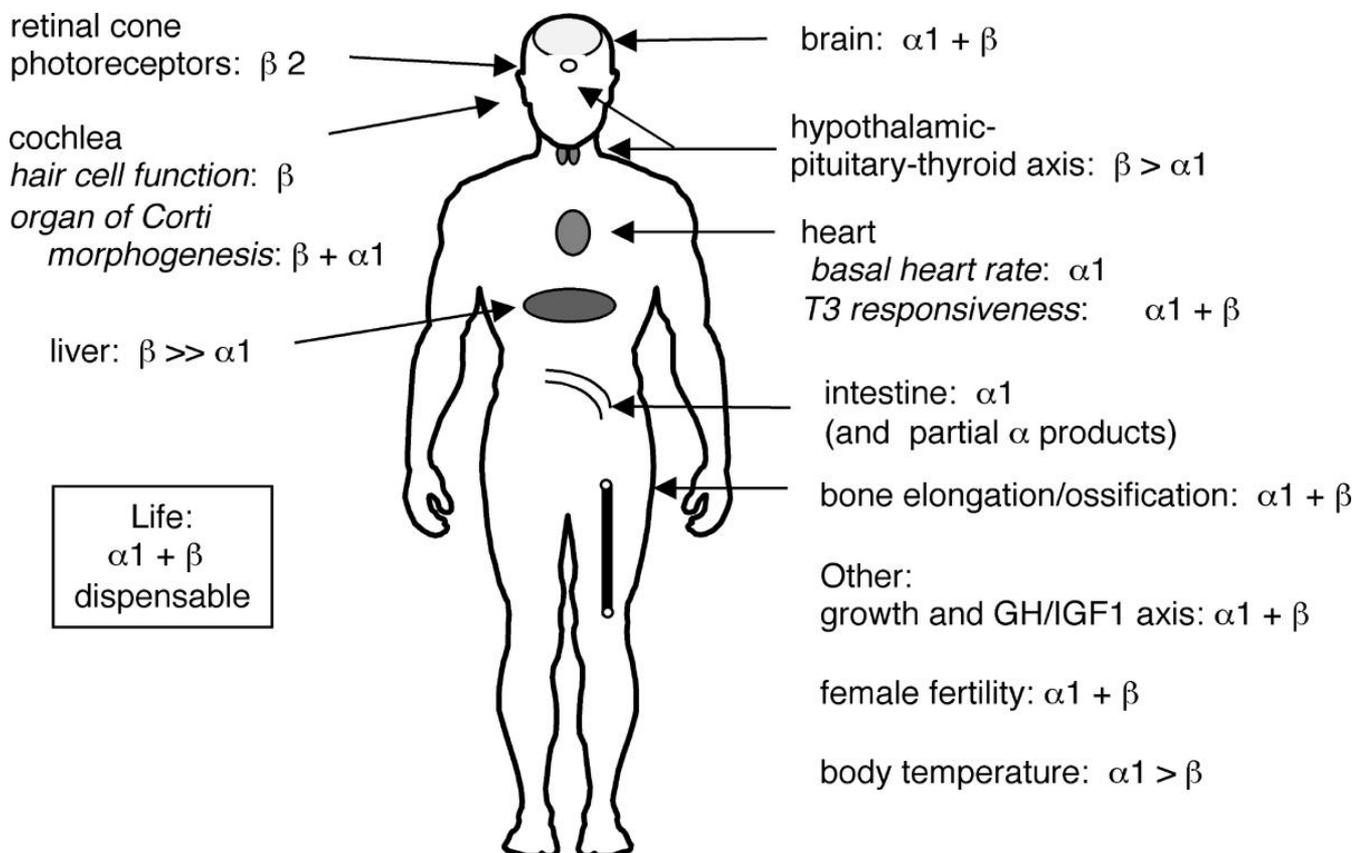


Figure. Some in vivo functions of TR α 1 and TR β receptors.

An overview of the roles of TR α 1 and TR β , as suggested by phenotypes of TR knockout mice (extrapolated onto a human diagram, for simplicity, although not all functions need be identical between species). Receptor-specific functions include the role of TR β 1 in liver, and TR α 1 in heart. Most systems show varying contributions from both TR α 1 and TR β . Some functions show a full cooperation, or compensation, between TR α 1 and TR β and no phenotype is detected unless all TRs are deleted (e.g. fertility, growth). Brain requires more detailed study, but current evidence indicates at least some individual roles for both TR α 1 and TR β . Most mice lacking all known TRs show normal longevity, indicating, perhaps surprisingly, that TRs are dispensable for life.

TR β receptors primarily mediate the feedback control of the pituitary-thyroid axis (8-11), liver metabolism (12-14) and the development of the cochlea (15,16) and of retinal photoreceptors (17). In contrast, TR α 1 has a major role in thermoregulation (18,7), basal heart rate (19,18,20,21), gut maturation (22,23) and lymphocyte development (24). TR β and TR α 1 also have important individual roles in the brain, an important but complex target of T3 (25-29).

It is notable, however, that many of the obvious phenotypes that might have been predicted from what is known of the defects arising in hypothyroidism only arise in the combined absence of TR α 1 and TR β receptors. The deletion of all known TRs results in dwarfism, pituitary hormone abnormalities, bone immaturity, female infertility and exacerbated defects in the pituitary-thyroid axis (11,30), liver (31), cochlea (16), skeletal muscle myosins (32), optic nerve (33) and brain (34). These worsened phenotypes indicate that TR β and TR α 1 co-regulate many of the same physiological functions in vivo. Indeed, upon closer inspection, both receptor genes play at least some role, although in widely varying proportions, in most systems studied. The failure to detect many of these functions in single gene knockouts may be explained by the extent to which phenotypes are masked by receptor compensation. Functions that appear as more strictly receptor-specific prove to be rarer (but critical), and include the almost complete reliance of liver T3 responses on TR β 1 (13,31,14), the role of TR β 2 in the differentiation of retinal cone photoreceptors (17), the role of TR α 1 in setting basal heart rate (18) and of various TR α products in gut maturation (23).

How unique are TR isoforms?

Given that TR β and TR α 1 receptors have closely related DNA binding and T3 binding domains, how might they mediate their individual functions in vivo? There is some in vitro evidence that the slight structural distinctions between TR α 1 and TR β lead to preferences in the regulation of a few target

genes, through subtle differences in their DNA binding specificity or their interaction with transcription cofactors (35). However, this is not generally applicable because TRa1 and TRb transactivate similarly through most known target DNA elements *in vitro*. Regardless of the potential of TRa1 and TRb receptors to regulate the same or somewhat different subsets of genes, distinct receptor isoforms probably mediate many tissue-specific functions simply because of their varying expression levels and tissue-specific distributions. An example is the requirement for TRb2 in the development of cone photoreceptors (17), which probably reflects the specific expression of TRb2 in the immature photoreceptor cell layer at a time when there is relatively little expression of any other TR in these cells. A corollary of this view is that the overexpression of one TR isoform should be able to compensate for the loss of another TR, and this has recently gained support from a mutation in *Thra* that deletes TRa2 but causes overexpression of TRa1 (36). When bred into mice deleted for *Thrb*, this *Thra* mutation has the remarkable result of largely correcting the deafness and thyroid dysfunction of *Thrb*-null mice (37). This is probably because the overexpression of TRa1 substitutes for loss of TRb (the loss of TRa2 itself is not the critical corrective factor because another *Thra* mutation deleting both TRa1 and TRa2 does not rescue the *Thrb*-null phenotype; (7)). The liver defects of *Thrb*-null mice are only partly corrected by this *Thra* mutation, perhaps consistent with distinct zonal distributions of TRa1 and TRb1 within the liver which preclude compensatory expression of TRa1 in the pertinent cell types (38,39). This need not exclude some additional role for the structural distinctions between TRa1 and TRb receptors in tuning the response in some tissues.

If TRs are to an extent interchangeable, then at least in some tissues, the total mass of receptors present in a cell may be the primary determinant of the response to T3 rather than the precise composition of the receptor mass. The possession of two receptor genes, endowed with alternative splicing and promoter usage, thus allows control of an extended array of functions, and probably a subtlety of regulation, that could not be achieved with only one receptor gene. This ability to express independently a family of receptors provides versatility in regulating the timing, distribution and levels of expression of TRs. This view is consistent with the presence of the same two receptor genes across all vertebrate species (mammalian, avian, amphibian, fish) studied (40,41).

Other implications of TR knockouts

Studies on targeted mutant mice have yielded novel insights into the variety of TR pathways underlying T3 actions, but have other implications too. First, these mouse strains represent models for human disease, including the syndrome of resistance to thyroid hormone that is associated with THRB mutations (42,43). Newly described point mutations in mouse *Thrb* are likely to create models for the typical dominant form of this syndrome (44,26). Although no inherited human disease has yet been associated with THRA, mutations in mouse *Thra* point to some phenotypes that may be predicted in human patients, including cardiac (18,45) and neurological abnormalities (25,28,29). Finally, the delineation of the receptor-specificity underlying different physiological functions may suggest new means of targeting therapeutic intervention in thyroid disorders, if suitable agonists or antagonists specific for TRa1 or TRb can be developed (46,47).

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MOLECULAR PATHOGENESIS OF CONGENITAL HYPOTHYROIDISM

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Introduction

It has become evident that in the care of patients with congenital hypothyroidism detected by newborn screening a most objective and professional information on the disease and its consequences is a prerequisite for an optimal care of the patient and the family. The parents of a so far healthy appearing newborn may doubt that it is unexpectedly affected by a congenital disease requiring life-long treatment. Their questions focus not only on the consequences for the long-term development of their child, but also on the cause and the probability of inheritance. Most recently insight has been gained in the molecular pathogenesis of CH. These studies have led to the description of so far unexplained forms of CH, which are characterized by accompanying defects of other organs. In some of these patients early diagnosis and treatment was not able to result in a normal mental development, which then could be explained by the molecular defects in factors important not only for the embryonic development of the thyroid gland but also for other organs including the CNS. Therefore a complete up-to-date knowledge of the aetiology is mandatory for all endocrinologists and paediatricians involved in the primary counselling of families to ensure the optimal support for the individual child.

Epidemiology and classification of congenital hypothyroidism

Screening programmes report a rather constant incidence of permanent primary CH of 1 :3000 to 1:4000 newborns with the exception of a decreased prevalence in the African-American and an increased prevalence in the Hispanic population (1). Girls are more frequently affected than boys (female to male ratios ranging from 2 :1 to 4:1) (2,3)

The most common aetiology of primary CH is a spectrum of defective thyroid gland development (thyroid dysgenesis) including so called "athyrosis" without visible thyroid tissue in imaging studies, thyroid ectopy (frequently located in a sublingual position), hypoplasia with remnants of thyroid tissue in the normal position and also hemithyroidea. These forms represent 75-85% of all cases of permanent CH (3,4,5), while defects of thyroid hormone biosynthesis characterized by a normally sized or enlarged thyroid gland in the normal position are present in only 15-25% of the patients. A classification, which is based solely on the results of imaging studies, is not conclusive, because the available studies have limitations. For example thyroid scintigraphy, which is based on the presence of functional thyroid tissue, fails to detect remnants of hypo-or non-functioning thyroid tissue. Therefore the term "apparent athyrosis" (6) has been created to describe patients with no visible thyroid tissue in thyroid scans but demonstration of a thyroid gland in ultrasound studies as well as measurable serum L-thyroxine and thyroglobulin concentrations. Therefore, the results of the serum measurements of thyroid hormones and thyroglobulin should be taken into account when classification of congenital hypothyroidism is performed.

Table1: Classification of permanent congenital hypothyroidism

Reports on associated birth defects in patients with permanent CH vary in their frequencies ranging from a slight increase of 2.4% (7) in comparison to the normal population up to significantly increased frequencies of 20 to 25% (8,9). More recent studies have shown that the increased risk of associated malformations in patients with permanent CH is limited to patients with thyroid dysgenesis (3). These data underline the importance of a complete and thorough investigation of every newborn with the tentative diagnosis of CH for an associated congenital defect with special attention to the cardiovascular system in order not to miss accompanying problems, which may influence the long-term outcome.

Congenital hypothyroidism due to hypothalamic and pituitary defects

Congenital hypothyroidism due to hypothalamic and pituitary defects (central hypothyroidism) is estimated to occur in 1 in

50,000 newborns. The most prevalent cause of central hypothyroidism is a defective development of the hypothalamus or pituitary leading to multiple pituitary hormone deficiencies, while defects of hypothalamic and pituitary peptides and their receptors only rarely have been identified as the cause of central congenital hypothyroidism. However, congenital central hypothyroidism can be as severe as primary congenital hypothyroidism and a delayed treatment can result in irreversible mental retardation, even in the absence of hypoglycaemia caused by adrenal insufficiency or growth hormone deficiency.

Inheritance of congenital hypothyroidism

Congenital hypothyroidism in the era of newborn screening so far has remained a sporadic disease and is not regarded as a hereditary disorder because familial occurrence is rare. Even in patients with putative autosomal recessive defects of thyroid hormone biosynthesis familial cases have been described rarely and systematic molecular genetic studies of candidate genes of thyroid hormone biosynthesis in patients with normally developed or enlarged thyroid glands are scarce. Therefore, no data on the epidemiology of the different defects of thyroid hormone biosynthesis or on the possible modes of inheritance are available so far. Recent studies have described autosomal recessive inheritance (6) and familial dominant occurrence of thyroid dysgenesis (10). Moreover, an increased frequency of minor abnormalities of the development of the thyroid and pharyngeal derivatives has been described in first-degree relatives of patients with CH due to thyroid dysgenesis (11). But again epidemiological data on the prevalence of familial thyroid dysgenesis are scarce, but it has become apparent that thyroid dysgenesis, at least in a subset of the patients, is an inherited disorder.

Genetic defects in thyroid development

Studies of mouse models with targeted disruption of genes involved in the development of the thyroid gland have provided insight in the molecular mechanisms of organogenesis and, thereby, the basis for molecular genetic studies in human patients affected by thyroid dysgenesis.(12,13) In mice, normal organogenesis and migration have been shown to be dependent on the normal expression and interplay of at least three different transcription factors: NKX 2.1 (TTF-1), PAX-8 and TTF-2. Targeted mutagenesis of these transcription factors in mice furthermore has demonstrated associated developmental defects of other organs because none of these factors is exclusively expressed in the thyroid.

Thyroid dysgenesis due to PAX-8 mutations

The PAX-8 gene belongs to family of genes, which is characterized by a highly conserved paired-box DNA binding domain, which encode for proteins, which play an important role in the entire embryonic development. PAX-8 is expressed in the thyroid primordium, the mid- and hindbrain region and in the developing kidney. Mice homozygous for disruption of the PAX-8 gene are characterized by severe hypothyroidism and small hypoplastic thyroid remnant without follicular structures (14), while in heterozygous mice no abnormalities of thyroid development have been described. If these mice are not rescued with thyroid hormone replacement they die shortly after birth, but although PAX-8 is expressed also in the kidney and the CNS no obvious abnormalities of these organs have been described in homozygous or heterozygous knock-out animals. Screening for mutations in the PAX-8 gene of patients with congenital hypothyroidism has led to the identification of several patients with heterozygous mutations which have been inherited in a dominant fashion (15,16,17). Most patients do not present other developmental defects, but in two unrelated male patients one hypoplastic kidney and one renal agenesis was observed. The thyroid gland of the affected patients presents with different morphological phenotypes. Thyroid hypoplasia, cystic hypoplastic remnants and ectopic thyroid were described and the severity of hypothyroidism was mild to moderate. In the families of patients with congenital hypothyroidism due to PAX-8 mutations other carriers of heterozygous mutations had only mild adult-onset hypothyroidism or were euthyroid indicating incomplete penetrance, which has been reported also in other disorders related to defects of PAX proteins (16).

TTF-2 mutations and syndromic congenital hypothyroidism

TTF-2 belongs to a family of transcription factors characterized by a forkhead DNA-binding domain. TTF-2 is expressed in the thyroid and the anterior pituitary. In mice with homozygosity for targeted disruption of the TTF-2 gene both thyroid agenesis and thyroid ectopy was identified, indicating that also in humans athyrosis and ectopy may be regarded as different degrees of severity of the same molecular defect. Furthermore these mice have a cleft-palate which makes their feeding impossible and therefore early neonatal death is unavoidable (12). While screening of the TTF-2 gene of patients with CH without associated problems failed to demonstrate any mutation (18), the study of two siblings with the so-called "Bamforth syndrome" (19) including athyrosis and CH, developmental delay, cleft-palate, choanal atresia, bifid epiglottis and spiky hair

demonstrated homozygosity of a loss-of-function mutation of the TTF-2 gene in both siblings (20). The heterozygous parents were unaffected. Therefore TTF-2 mutations seem to be a very rare cause of CH in humans resulting in a specific syndrome with other organ manifestations.

Mutations of the NKX2.1 mutations in patients with congenital hypothyroidism and predominant neurological symptoms

The NKX2.1 (TTF-1, TITF-1 or T/ebp) -gene encodes a transcription factor of the homeobox domain containing genes of the NKX2 family. NKX 2.1 is expressed in the thyroid, forebrain, basal ganglia, pituitary and the lung. Targeted disruption of both NKX2.1 alleles led to a complex phenotype of newborn mice (13). These mice die shortly after birth because of respiratory distress resulting from a defective lung development with insufficient surfactant production. While homozygous newborn mice were lacking any thyroid tissue at birth (athyrosis), heterozygous mice exhibited no abnormalities of thyroid development. In addition in the homozygous mice the pituitary was completely absent and the development of the hypothalamic region was abnormal. Due to the early death of homozygous mice a study of hypothalamic-pituitary function or neurological testing could not be performed. The search for mutations in the NKX2.1 gene in patients with CH did not reveal abnormalities (21). However, two studies were published on patients with deletions of chromosome 14q13 and 14q12-13.3 encompassing the NKX2.1 locus, who presented with mild hyperthyrotropinemia, neonatal respiratory distress and pulmonary problems as well as unexplained ataxia and muscular hypotonia (22,23). However, in these reports it was suggested that the neurological problems observed in the patients might be due to the deletion of other genes located in the deleted chromosomal regions. These descriptions encouraged the investigation of the NKX2.1 gene of patients with congenital hypothyroidism, in whom the longterm outcome despite an early onset of treatment and adequate dosage was unfavourable due to pulmonary complications, severe muscular hypotonia and neurological symptoms, which were defined as choreoathetosis and in some patients to mental retardation. In so far in 6 of such patients heterozygous mutations of the NKX2.1 gene were identified (24). The phenotype of thyroid and pulmonary manifestations covers a wide spectrum ranging from hyperthyrotropinemia to severe CH due to thyroid agenesis and severe neonatal RDS requiring ventilation to a slight increase in pulmonary infections, while choreoathetosis presents with less phenotypical variation. Recently familial benign choreoathetosis without accompanying pulmonary or thyroid disorders has been attributed to NKX2.1 mutations as well (25). In respect of the severe phenotype of the NKX2.1 knockout mice, homozygosity for NKX2.1 mutations in humans is probably not viable. The mechanism by which heterozygous mutations cause the phenotype is most likely haploinsufficiency. Although the heterozygous NKX2.1 mice previously have been reported to be unaffected, a more recent study describes abnormalities of thyroid function and neurological development in heterozygous mice (26). The phenotypical variation observed in patients with heterozygous NKX2.1 mutations is most likely due to modifier genes with different expression in different genetic backgrounds. Thus, a new syndrome of congenital hypothyroidism, pulmonary problems and the predominant symptom of choreoathetosis could be attributed to mutations of the NKX2.1 gene.

These recent results have implications for the prognosis of patients with CH detected by newborn screening because the identification of defects of molecular mechanisms resulting in a defective development of the thyroid gland as well as of other organ systems will probably explain the less favourable outcome of some patients with CH.

Table 2 Molecular mechanisms causing thyroid dysgenesis

Defects of thyroid hormone biosynthesis

It is assumed that 15-25% of the patients primary CH is caused by loss-of-function mutations in genes encoding proteins which are involved in the synthesis of thyroid hormones in thyroid follicular cells.

Loss-of-function mutations of the TSH receptor

TSH is a prerequisite not only for normal thyroid hormone synthesis but also for proliferation. Accordingly, the inherited congenital hypothyroidism in the *hyt/hyt* mouse was shown to be caused by homozygosity for a loss of function mutation of the TSH receptor (P556L) leading to thyroid hypoplasia and severe congenital hypothyroidism (27). Partial resistance to TSH was identified in patients with euthyroid hyperthyrotropinemia (28). Subsequently, homozygosity or compound heterozygosity for several loss-of-function mutations of the TSH-receptor have been identified in patients with congenital hypothyroidism. The phenotypical includes a spectrum of severity ranging from mild forms to severe CH with "apparent athyrosis" on thyroid scan (29,30). With ultrasound studies, hypoplastic remnants of the thyroid are detectable and, therefore, the phenotype is similar to patients with PAX-8 mutations. The mode of inheritance is autosomal recessive, but elevated TSH levels have been described in some of the heterozygous carriers.

Defects of the sodium-iodide symporter

Iodide transport at the basolateral membrane of the follicular cell is the initial step of thyroid hormone biosynthesis. After cloning of the gene encoding the sodium-iodide symporter (NIS), homozygous and compound heterozygous mutations of the gene have been identified in patients with congenital hypothyroidism. (31,32). It seems that thyroid enlargement is not present at birth and the development of nodular goiter, which has been described in patients with NIS mutations, occurs in later life.

Thyroperoxidase (TPO)

Thyroperoxidase is a key enzyme in thyroid hormone biosynthesis catalysing the iodination of tyrosine residues of the thyroglobulin molecule and the coupling of iodotyrosines to T3 and T4. The autosomal recessive inheritance of loss of function mutations of the TPO gene has been described in the majority of patients with congenital hypothyroidism and a total or partial organification defect demonstrated by perchlorate discharge tests (33). The systematic study of patients with severe CH and a normally developed or enlarged thyroid also revealed a high proportion (60%) of autosomal recessive inheritance of TPO-mutations (34). In some newborns a goiter is present, in other patients with neonatal diagnosis the development of goiter is prevented by early thyroid hormone replacements. The factors leading to fetal goiter development or the factors preventing fetal goiter development in patients with defects of TPO are so far unknown. In some patients a mutation can be identified only on one allele. One of the possible explanations - partial uniparental disomy - has been reported so far only in one patient (35).

Thyroglobulin

Thyroglobulin (Tg) is involved in thyroid hormone synthesis and storage . Autosomal recessive inheritance of Tg defects has been identified so far only in a few cases of congenital hypothyroidism, most likely due to the difficulties of performing mutational screening of the large Tg gene (>300 kb, 48 exons, open reading frame of 2748 AA).The human phenotype is characterized by variable degrees of severity of CH and goiter development when thyroid hormone replacement is delayed.(36)

Pendrin

The functional role of Pendrin, an anion transporter, in the thyrocyte is the transport of iodide across the apical membrane into the follicular lumen. Pendrin is also expressed in the inner ear and the kidney, where its exact functions are unknown. The phenotype of the Pendred Syndrome is characterized by congenital sensorineural hearing loss, goiter and in only a minority of patients congenital hypothyroidism. Autosomal recessive inheritance of mutations of the Pendrin (PDS) gene have been reported in familial cases of Pendred Syndrome which presented with congenital deafness, hypothyroidism and goiter in some of the newborns (37). Targeted disruption of the PDS gene in mice resulted in deafness but not in impairment of thyroid function, indicating that in mice other mechanisms of iodide transport can compensate the lack of functional Pendrin at the apical membrane.

Molecular defects in CH due to hypothalamic and pituitary defects

Several defects of the hypothalamic-pituitary axis can be expected in patients with central hypothyroidism. It can be assumed that loss-of-function mutations of several genes would lead to selective central hypothyroidism : preproTRH, TRH receptor and TSH β gene.

Table 3 Molecular mechanisms of central hypothyroidism

TRH and TRH-receptor

The human TRH gene has been mapped to chromosome 3q13.3.-q21, but no human mutations have been described so far. It is possible that humans with a TRH defect probably will present with a rather complex phenotype due to the unpredictable symptoms of central TRH deficiency and deficiencies of other cleavage peptides of the pre-pro TRH molecule. Targeted disruption of the TRH gene in mice led to an unexpected mild phenotype with central hypothyroidism and hyperglycemia (38).

So far only one family with compound heterozygosity for loss-of-function mutations of the TRH receptor gene with central hypothyroidism has been described. The affected patient presented with severe congenital hypothyroidism, short stature and mental retardation, but initiation of treatment was delayed (39).

Thyrotropin

Congenital hypothyroidism caused by mutations in the TSH β chain is rare. Loss-of-function mutations of the TSH β gene present with a variable phenotype. Some patients only have mild congenital hypothyroidism (40,41), while others present with severe symptoms already in the neonatal period and are mentally retarded if thyroid hormone substitution is delayed. TSH is very low or immeasurable and cannot be stimulated by TRH. The most prevalent mutation identified in different populations, which results in severe congenital

hypothyroidism, is a 1 bp deletion (T) from codon 105 (TGT) of the TSHB gene, converting a cysteine to a valine residue (C105V) and yielding an additional 8-amino acid nonhomologous peptide extension on the mutant protein (42).

Developmental defects of the pituitary

Defects of pituitary development result in various forms of impaired secretion of pituitary hormones. Usually the clinical picture is characterized by multiple hormone deficiencies including thyrotropin, however the time course of manifestations of the different hormone deficiencies is highly variable and therefore congenital hypothyroidism maybe the first and leading symptom of a defective hypothalamic-pituitary development.

Pit-1

Pit-1 (human homologue: POU1F1) is a transcription factor important for pituitary development and pituitary hormone expression. Two animal models of pit-1 defects, the Snell and Jackson mice, are characterized by deficiencies of growth hormone, prolactin and TSH. Several reports on humans with loss-of-function mutations in the transcription factor Pit-1 have documented severe congenital hypothyroidism as the leading symptom in the newborn period with a delayed manifestation of growth hormone deficiency (43,44). The mode of inheritance is autosomal recessive if the mutation is located in the DNA-binding domain, while other mutations with a dominant negative effect are inherited in an autosomal dominant manner.

Prop-1

The expression of another pituitary specific transcription factor Prop-1 (Prophet of pit-1) identified in the Ames mice precedes the expression of pit-1. Prop-1 deficiency results in impaired GH, prolactin, TSH but also gonadotropin secretion. Autosomal recessive inheritance of loss- of- function mutations in patients with combined pituitary hormone deficiencies have been reported (45). However, the severity of the phenotype varies widely and the manifestation of the different hormone deficiencies follows an unpredictable time course. In some patients the late occurrence of ACTH deficiency has been reported. Severe congenital hypothyroidism as the leading symptom so far has not been described.

LHX3

More recently we were able to describe the first human mutations of the LHX3 gene encoding for a LIM homeodomain containing transcription factor involved in the development of the anterior pituitary (46). Previously, targeted disruption of the LHX3 gene in mice had resulted in a disturbed development of the anterior pituitary with conserved function of the corticotrophs. In these consanguineous pedigrees, the mutations were inherited homozygously. In contrast, screening for mutations in sporadically occurring combined pituitary hormone deficiencies no abnormalities of the LHX3 gene were identified. Most importantly, as a peculiar finding a short neck with limited ability to rotate the head was observed in all affected children but no abnormality of the spine could be visualized on X-ray or MRI. In these patients congenital hypothyroidism was the first and leading symptom, which preceded the diagnosis of growth hormone or gonadotropin deficiency.

Thus congenital hypothyroidism due to hypothalamic-pituitary defects can be caused by different molecular mechanisms. However, the symptoms can be as severe as in primary congenital hypothyroidism and a delayed treatment can result in irreversible mental retardation.

Type of CH	Ultrasound Appearance	Thyroid scan	Serum TSH	Serum T4	Serum TG
Thyroid dysgenesis					
Athyrosis	No thyroid< tissue	No uptake	Increased	Undetectable to very low	Undetectable to Very low
Ectopy	No thyroid tissue	Ectopic uptake	Increased	Low to Low normal	Low to normal

Hypoplasia	Hypoplastic remnant	No uptake	Increased	Undetectable to low normal	Undetectable to Normal
Defects of hormone biosynthesis	Normal or enlarged thyroid	No uptake or normal uptake, Normal or enlarged volume	Increased	Undetectable to low normal	Undetectable to increased
Central hypothyroidism		Decreased uptake Decreased volume	Undetectable Or low	Undetectable to low normal	Undetectable to normal

Table 1 Classification of permanent hypothyroidism

	TSH-receptor	PAX-8	TTF-2	NKX2.1
Protein family	G-protein coupled receptor	Paired domain Transcription factor	Forkhead domain Transcription factor	Homeodomain Transcription factor
Expression pattern	Thyroid Pituitary? Hypothalamus? Adipose tissue?	Thyroid Mid - and Hindbrain Kidney	Thyroid Anterior pituitary	Thyroid Forebrain Pituitary lung
Phenotype in knockout mice	Thyroid hypoplasia CH	Thyroid hypoplasia Early death	Thyroid agenesis or Thyroid hypoplasia Cleft palate Early death	Thyroid agenesis Pituitary aplasia Forebrain defects Disturbed lung Development Neonatal death
Human thyroid phenotype	Thyroid hypoplasia Severe to moderate CH Hyperthyrotropinemia	Thyroid hypoplasia Cystic rudiments Ectopy Severe to mild CH	Thyroid agenesis Severe CH	Thyroid agenesis Thyroid hypoplasia Normal thyroid Severe to moderate CH Hyperthyrotropinemia
Manifestation in other human organs	-----	Developmental defect of the kidneys	Cleft palate Bifid epiglottis Choanal atresia Spiky hair	Choreoathetosis Respiratory distress Pulmonary infections Mental retardation
Inheritance	Autosomal recessive	Autosomal dominant	Autosomal recessive	Autosomal dominant
Chromosome	14q31	2q12-14	9q22	14q13

Table 2 Molecular defects of thyroid development

	TRH-receptor	β-TSH	POUF1	PROP1	LHX3
Chromosome	8q22	1p13	3p11	5q	9q34
Inheritance	Autosomal Recessive	Autosomal Recessive	Autosomal Recessive or Autosomal Dominant	Autosomal Recessive	Autosomal Recessive
Pituitary hormone Deficiency	TSH	TSH	TSH GH Prolactin	TSH GH Prolactin Gonadotropin (Corticotropin?)	TSH GH Prolactin Gonadotropin
Other Manifestations	Mental Retardation ?	Mental retardation ? (Sensorineural Deafness ?)	Mental retardation ?	Pituitary mass	Rigid cervical spine Limited head Rotation Mental retardation? Pituitary mass

Table 3 Molecular mechanisms of central hypothyroidism

Summary

These recent results have implications for the counselling of parents and patients with CH detected by newborn screening. With the rapid advancement of diagnostic tools for molecular genetic studies it should be possible in the near future to include the investigation of candidate genes in the diagnostic work-up to ensure a correct counselling of the parents and early focussed support for affected patients. From the current knowledge the hypothesis can be challenged that long-term unfavourable consequences for patients with CH despite early and adequate treatment might result rather from a common cause of defective thyroid and brain development than from fetal or perinatal hypothyroidism.

Congenital hypothyroidism in the era of newborn screening so far has remained a sporadic disease and is in general not regarded as a hereditary disorder because familial occurrence has remained rare.

Since the majority of patients with CH due to thyroid dysgenesis is not affected by the syndromic types of CH caused by mutations in known transcription factors, unidentified genes may be involved or a polygenic inheritance of CH has to be postulated. Therefore congenital hypothyroidism due to thyroid dysgenesis has to be considered as a hereditary disorder, but the mode of inheritance and the modifying genetic and environmental factors are far from being clarified. Further systematic studies are necessary to assess the prevalence of mutations in known candidate genes for the defects of thyroid hormone biosynthesis, thyroid organogenesis and hypothalamic-pituitary development in patients presenting with CH.

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ETIOLOGY OF GRAVES OPHTHALMOPATHY

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Why are the clinical manifestations of Graves ophthalmopathy (GO) present in only a subset of patients with Graves' disease? If the orbital fibroblast (OF) is the target cell of the autoimmune attack, what is the autoantigen? Is it really the TSH receptor (TSH-R)? Are TSH-R autoantibodies causally involved? Or is GO a predominantly T-cell mediated disease, but why and how are T-lymphocytes then homing specifically to the orbit? These questions are still largely unanswered, and the precise etiology and immunopathogenesis of GO remains an enigma.

GENETIC AND ENVIRONMENTAL FACTORS

It has been demonstrated that most if not all Graves' hyperthyroid patients without apparent eye changes have subclinical GO (as evident from enlarged eye muscles on orbital imaging), and that most if not all euthyroid GO patients have laboratory evidence of thyroid autoimmunity and Graves' hyperthyroidism will develop in a sizable proportion. The difference between Graves' hyperthyroid patients with or without clinical manifestations of GO thus seems to be quantitative rather than qualitative. The next obvious question is whether genetic and environmental factors determine the degree of expression of GO in Graves' disease, see fig.

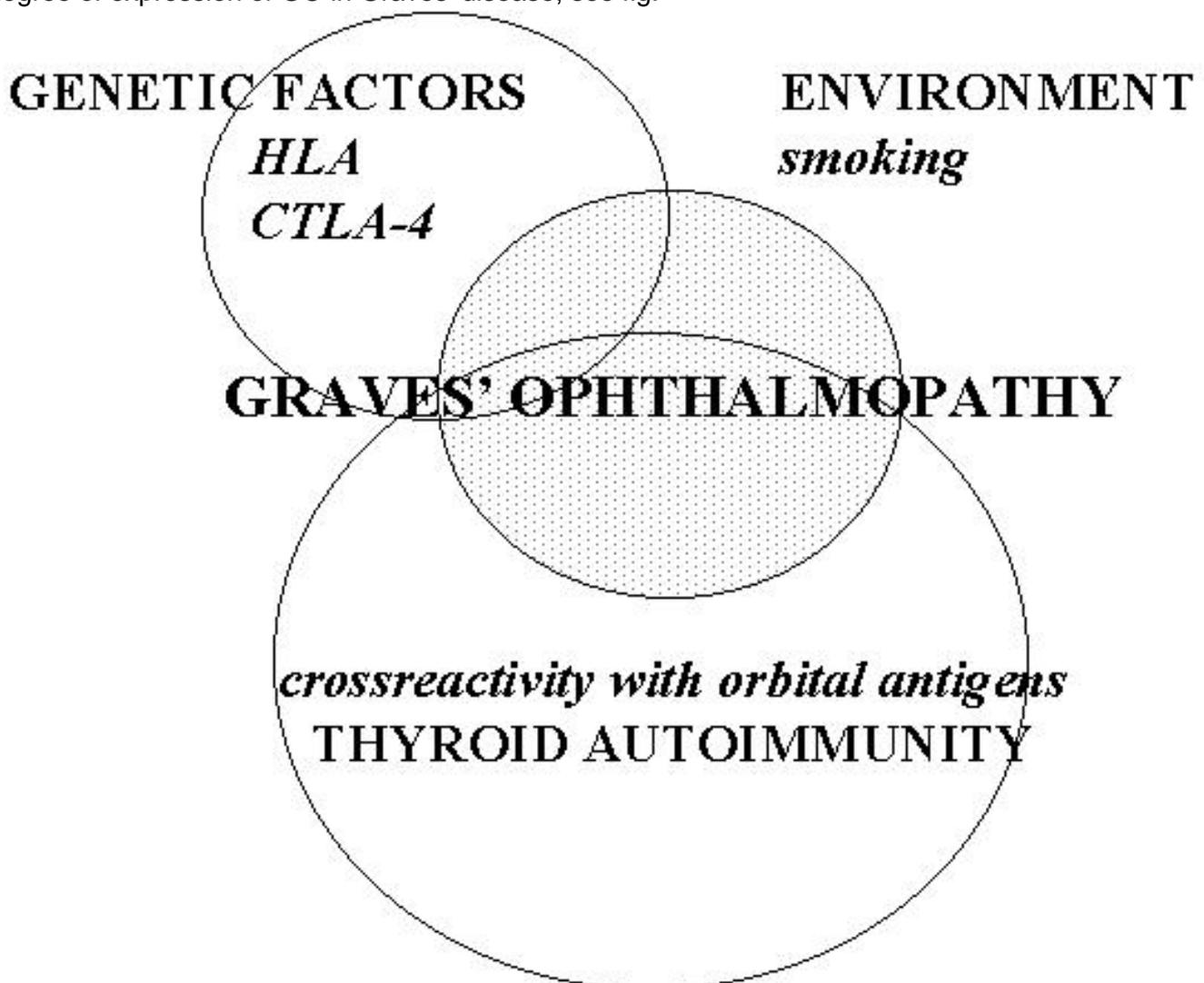


Figure. Interrelationship between genetic, environmental and immunological factors involved in the pathogenesis of Graves' ophthalmopathy. Little is known on gene-environment interaction and their precise interplay with the immune system

The search for genetic susceptibility loci in GO has so far been disappointing as the proportion of polymorphisms in a number of candidate genes is similar in Graves' patients with or without clinically apparent eye disease, although there are a few exceptions. HLA-DPB/*201 is less prevalent in GO patients (3%) than in non-GO patients (21%) or controls (30%), but the protective effect of this HLA-DPB allele is weak (1). In contrast, the A polymorphism in the CTLA-4 gene (A/G at codon 17) confers a risk to GO as the strength of the association of the G allele with GO increases with the severity of GO: odds ratios are 1.49 for eye changes of class 1 (no signs, only symptoms) and 2 (soft tissue involvement), 1.67 for class 3 (proptosis) and 4 (extraocular muscle involvement), and 3.06 for class 5 (corneal involvement) and 6 (optic nerve involvement) compared with Graves' disease patients without orbital signs (2). Other studies however cannot confirm this finding (ref?). An association with the CTLA-4 polymorphism has also been observed in other autoimmune diseases like type 1 diabetes mellitus and Addison's disease, and is thus not specific for GO but rather confers a risk for autoimmune disease in general. It thus remains difficult to explain the phenotypic appearance of GO in Graves' disease patients from a specific genotype. It could be that polymorphisms in a number of genes which discriminate little when considered one by one, provide in combination a more meaningful pattern. In contrast, environmental factors are apparently a major determinant of the occurrence of manifest GO. For, the prevalence of smokers is 65% in Graves' hyperthyroid patients with GO, 43% in Graves' hyperthyroid patients without GO, and 34% in controls. The risk to develop Graves' hyperthyroidism in relation to smoking is relatively small (odds ratio 1.9, 95% CI 1.1-3.2), but the odds to develop GO in smokers is much higher (7.7, 95% CI 4.3-13.7) (3). The highest odds ratios are observed in the patients with the most severe eye disease. The risk is also higher in current smokers than in former smokers, suggesting a direct and immediate effect of smoking. Indeed, one study reports a very high relative risk of 7.0 (95% CI 3.0-16.5) for development of diplopia in heavy smokers (>20 cigarettes per day), whereas the risk is not significant in ex-smokers who had also smoked >20 cigarettes per day (RR 1.9, 95% CI 0.5-7.7)(4). The data suggests that discontinuation of smoking may prevent GO to a certain extent.

The mechanisms by which cigarette smoking enhances the development of GO remain largely unknown. Systemic effects of smoking include polyclonal activation of B and T cells resulting in production of co-stimulatory cytokines. Serum concentrations of some cytokines (like IL-1RA) and adhesion molecules (like sICAM-1) are indeed higher in smoking than in nonsmoking patients with GO, but this may just indicate more severe ophthalmopathy in smokers (5,6). Local effects of smoking may affect orbital fibroblasts (OF). OF in vitro produce more glycosaminoglycans (GAGs) when cultured under hypoxic conditions (7), produce more IL-1 when exposed to tobacco glycoprotein, and express more HLA-DR at their surface when exposed to tar (8). Another factor carrying a small risk for developing or worsening of eye changes is ¹³¹I therapy (9). It is plausible that the release of thyroid antigens following radiation injury triggers autoimmune reactions in the orbit via T-cell activation and prolonged increase of e.g. TSH receptor antibodies.

ORBITAL FIBROBLASTS: THE TARGET CELLS OF THE AUTOIMMUNE ATTACK

Swelling of extraocular muscles and retrobulbar fat are the hallmark of GO. Biopsies of affected muscles show a marked expansion of the endomysial space caused by an increased number of collagen fibers interspersed with granular amorphous material containing hyaluronic acid. Orbital connective tissue contains 254 ± 16 μ g GAG/g wet tissue (as opposed to 150 ± 13 μ g GAG/g in controls), largely due to an excess of chondroitin sulfate and hyaluronic acid (10). GAGs by virtue of their polyanionic charge attract much water and contribute markedly to the swelling of retrobulbar tissues. GAG production by OF is greatly enhanced by a number of cytokines, as evident from in vitro studies. The lymphocytic infiltrate in the orbit of GO patients is composed predominantly of CD4+ and CD8+ T-cells with a few B-cells. Retrobulbar T-cells of GO patients recognize autologous OF but not eye muscle extracts in a MHC class I-restricted manner (11). Homing of T-cells to the orbit is facilitated by the expression of HLA-DR and adhesion molecules in endothelial cells and OF, which is enhanced by IL-1a, TNF α , IFN γ and Graves IgG). Extraocular muscle fibers do not demonstrate immunoreactivity to adhesion molecules. These findings have led to the widely accepted view that OF are the primary target cells of the autoimmune attack in the orbit.

THE ORBITAL AUTOANTIGEN: IS IT THE TSH RECEPTOR?

One of the conceptually intriguing findings of the last decade has been the detection of the TSH-R in orbital adipose/connective tissue specimens of GO patients and - to a much lesser extent - of controls. The TSH-R is fully expressed at both the mRNA and the protein level, and is also functional as evident

from an increase of cAMP in response to TSH. The story evolves with the demonstration that in vitro differentiation of preadipocytes (of GO and normal tissues) is associated with enhanced expression of functional TSH-R (12). Further evidence in favour of the TSH-R as the long sought-after shared antigen between the orbit and the thyroid comes from animal experiments. Orbital changes (consisting of infiltration by lymphocytes and mast cells, edema, dissociation of muscle fibers, accumulation of adipose tissue and TSH-R immunoreactivity) occurred in none of NOD mice but in the majority of BALB/c mice upon treatment with TSH-R primed T-cells (13). Genetic immunization with TSH-R cDNA of NMR outbred mice (more comparable with the outbred nature of humans) produced orbital pathology in a small minority of the female mice (14). Elevated T4 levels and thyroid stimulating antibodies (TSAb) were induced in the latter but not in the former experiment. Taken together, the animal experiments suggest that orbital pathology depends on sex, genetic background and a Th2-response to the TSH-R, but does not require TSAb. Although the TSH-R is presently favoured as the causative autoantigen in GO, definitive proof is lacking. It could well be that the intrathyroidal immune response generates autoimmune reactions to still other thyroid antigens which by cross-reactivity with orbital antigens cause the orbital changes.

Screening of human eye muscle expression libraires have identified a number of antigens which are recognized by antibodies circulating in the serum of GO patients. However, the wide distribution of these antigens in various tissues and the rather high prevalence of antibodies against these antigens in serum of subjects without Graves' disease distract from their role as a major autoantigen in GO. The same holds true for several orbital fibroblast antigens other than the TSH-R: autoimmune reactions against these antigens are likely secondary and of uncertain pathogenetic significance (15,16). An old hypothesis explains the autoimmune damage in GO from thyroglobulin transported through a retrograde lymphatic route from the thyroid to the orbit. Indeed, thyroglobulin of thyroid origin has recently been found in about half of retrobulbar fibroadipose tissue samples (but not in extraocular muscles) of GO patients, and not in patients without thyroid or eye disease. Interestingly, the presence of thyroglobulin was related to previous treatment with radioiodine (17). TgAb in Graves' disease patients, however, are not related to the presence or absence of GO, and Tg-TgAb immune complexes have not been demonstrated in orbital tissue of GO patients. It is thus unlikely that Tg plays a primary role in the pathogenesis of GO.

Still other autoantigens might be involved. Sera of Graves' patients with or without GO contain autoantibodies against IGF-1 binding sites on OF (18); their biologic significance is unknown. A very recent study reports that Graves IgG is capable to induce IL-16 and RANTES (a C-C type chemokine) expression in fibroblasts derived from the orbit, thyroid or skin of Graves' patients; this T-cell chemoattractant activity was not observed in fibroblasts from subjects without thyroid disease (19). The findings suggest a particular phenotype of fibroblasts and a more generalized connective tissue involvement in Graves' disease. The induction of IL-16 by Graves IgG is inhibited by rapamycin; rapamycin also inhibits IGF-1 receptor mediated signaling. So, the IGF-1 receptor is another attractive candidate autoantigen.

HUMORAL OR CELL-MEDIATED AUTOIMMUNITY IN GO?

Th1 and Th2 derived cytokines may reflect cellular and humoral immunity, respectively, albeit the distinction is incomplete with many overlaps. Both Th1 and Th2-derived cytokines are elevated in sera of patients with untreated GO (5), but serum concentrations may not accurately reflect what is going on in the orbit. Recent studies clearly indicate predominant Th1 immune reactions in early GO whereas humoral immunity (Th2 type) might play a greater role in later stages (20). The human data are thus at variance with the experimental animal models which by virtue of strong IL-4 and IL-10 thyroidal immunoreactivity suggest a Th2-like response to the TSH-R in the development of ophthalmopathy in these animals. It has been argued, however, that DNA vaccination with plasmid generally leads to a Th1-biased immune response (19). In GO patients who had been euthyroid for at least two months, serum TSAb is directly related to proptosis measurements and the clinical activity score (21), but these findings provide at best circumstantial evidence for humoral immunity in GO. Although the data remain conflicting, it might well be that GO is primarily a T-cell dependent disease, but that humoral immunity is playing an important contributory role at certain stages of the disease.

CONCLUSION Despite much progress, the precise immunopathogenesis of GO remains unknown. The orbital fibroblasts are most likely the primary targets of the autoimmune attack; upon stimulation by cytokines from infiltrating T-cells they produce excessive amounts of very hydrophilic glycosaminoglycans which cause swelling of retrobulbar tissues. It remains to be elucidated why T-

cells are homing to the orbit. Recent studies implicate Graves' IgG, which can induce chemoattractants for T-cells in fibroblasts, albeit requiring a specific phenotype of fibroblasts to do so. The nature of the autoantigen in the fibroblast is still an open question, but the TSH receptor is a promising candidate. In early GO there exists a predominant Th1-like cytokine profile in orbital tissues, and a marked expression of a functional TSH receptor which is enhanced during cytokine-induced differentiation of preadipocytes into adipocytes. Thus, in humans, GO looks like a T-cell mediated disease (at variance with data from recently developed animal models), although TSH receptor antibodies may propagate autoimmunity at later stages. The proposed sequence of events may ultimately prove to be wrong - this is the challenge for doing research! Genetic factors and especially smoking may determine the severity of the clinical expression of GO in patients with Graves' disease.

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THYROXINE REGULATES ITS OWN ACTIVATION BY FEED-BACK CONTROL OF

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Thyroxine (T4) is the main iodinated product of thyroid secretion, a long-lived pro-hormone molecule that must be activated by deiodination to the short-lived biologically active T3 in order to initiate thyroid hormone action. This deiodination reaction occurs in the phenolic (outer or 5')-ring of the T4 molecule and is catalyzed by two selenocysteine (Sec)-containing iodothyronine deiodinases, i.e. D1 and D2. As a counter point to the activation pathway, both T4 and T3 can be irreversibly inactivated by deiodination of the tyrosyl-ring, a reaction catalyzed by D3, the third member of the selenodeiodinase group. The coordinated changes in the expression and activity of these enzymes ensure thyroid hormone homeostasis and the constancy of T3 production, constituting a major mechanism for adaptation to limitations in the supply of iodine, starvation and changes in environmental temperature (reviewed in (1)).

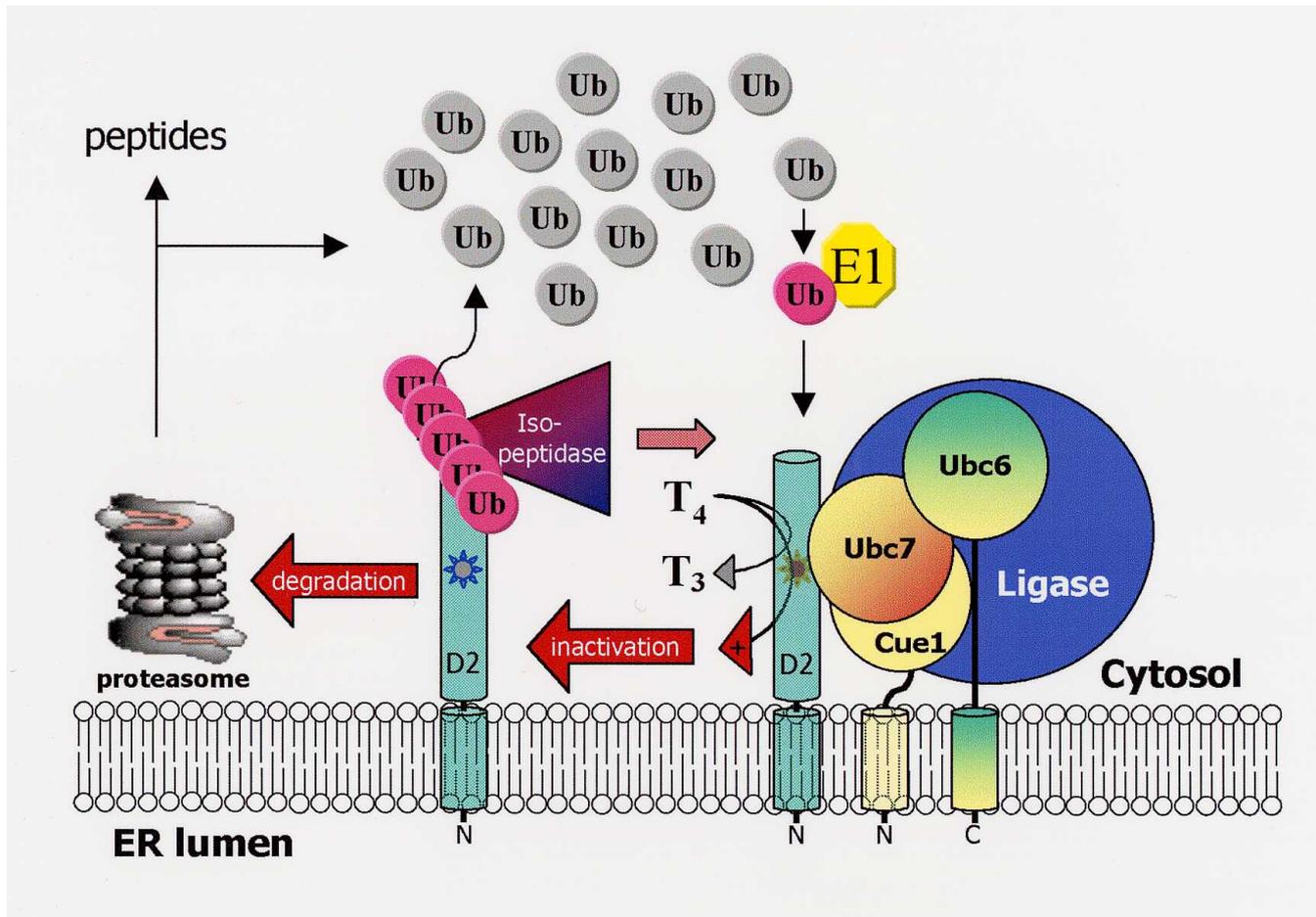
D2 is considered the critical homeostatic T3-generating deiodinase due to its substantial physiological plasticity. For example, D2 responsiveness to cAMP constitutes the basis for its rapid neural stimulation in several tissues, linking D2 expression with the hypothalamus and widening the spectrum of environmental and endogenous stimuli that can potentially influence adaptive T3 production (1). D2 is a 32 kDa type 1, endoplasmic reticulum (ER)-resident membrane protein that has a short luminal NH₂-terminus and a single transmembrane domain within its first 40 amino acids. The bulk of the enzyme is in the cytosol, including its Sec-containing catalytic active center (2). In immunocytochemical studies using confocal microscopy its distribution is typically perinuclear and it colocalizes with ER-resident binding protein when transiently expressed in HEK-293 and NB-2A cells, and in a human mesothelioma cell line (MSTO-211H) where it is endogenously expressed (2; 3).

A number of transcriptional and post-translational mechanisms have evolved to ensure limited expression and tight control of D2 levels, which is inherent to its homeostatic function. The D2 mRNA in higher vertebrates is more than 6 kb in length, containing long 5' and 3' untranslated regions (UTRs). The D2 5'UTRs are greater than 600 nucleotides and contain 3-5 short open reading frames (sORFs), which reduce D2 expression by as much as 5-fold (4). Alternative splicing is another mechanism that regulates D2 level as mRNA transcripts similar in size to the major 6- to 7-kb D2 mRNAs, but not encoding an active enzyme, are present in both human and chicken tissues. D2 levels can also be regulated by AUUUA instability motifs located in the 3'UTR of D2 mRNA as deletion of 3.7-kb from this region increases D2 activity approximately 3.8-fold due to an increase in D2 mRNA half-life (4).

D2 activity/mRNA ratios are variable, indicating that there is significant post-translational regulation of D2 expression. In fact, the decisive D2 property that characterizes its homeostatic behavior is a short half-life (~45min) (5) that can be further reduced by exposure to physiological concentrations of its substrate, T4, and in experimental situations, reverse T3 or T3 (5-11). This constitutes a rapid, potent generalized regulatory feedback loop that efficiently controls T3 production and intracellular T3 concentration based on how much T4 is available. The potency of the iodothyronines to induce loss of D2 activity mirrors the enzyme's affinity for the substrate, indicating that enzyme-substrate interaction must occur in order to induce loss of D2 activity.

How is loss of D2 activity mediated? Important metabolic pathways often contain key rate-limiting enzymes whose half-lives can be modified by selective proteolysis. This process is frequently mediated

by the ubiquitin (Ub)-proteasome system by which target proteins are marked for destruction by Ub conjugation.



Legend to the Figure:

Artistic representation of D2 ubiquitination and proteasomal degradation. Ub is ubiquitin; E1 is the enzyme that activates Ub; E2 is the Ub conjugase; Isopeptidase catalyzes the D2 de-ubiquitination; Ubc6 and Ubc7 are E2s involved in D2 ubiquitination; Cue1 is a ER-docking protein for Ubc7; the star in D2 molecule represents the Sec-containing active center.

The ubiquitinated proteins are subsequently recognized and degraded by the proteasomes (12; 13). Indeed, ubiquitination and proteasomal degradation are deeply implicated in the post-translational regulation of D2 activity. The first evidence was obtained in GH4C1 cells in which the half-life of the endogenous D2 was noted to be stabilized by MG132, a proteasome inhibitor (14). Substrate-induced loss of D2 activity was also inhibited by MG132 in such cells, indicating that both conditions affecting loss of D2 activity were mediated by the proteasomes. This implies that the loss of D2 activity is, at least partially, due to proteolysis, a premise that was confirmed after the levels of immunoprecipitable labeled D2 were shown to parallel D2 activity, both under basal conditions and after exposure to substrate (15).

Selection of specific proteins for proteolysis is usually achieved at the level of Ub conjugation, a process that involves recognition of as yet undefined amino acid-sequences in the target protein by the ubiquitinating enzymatic machinery. The first step is activation of Ub by ATP, a process catalyzed by the E1 enzyme. The next step, target recognition, is coordinated by the combined actions of a series of Ub-conjugating enzymes (E2s) and Ub-ligases (E3s). There are approximately a dozen E2s or E2-related proteins, which share a conserved catalytic domain of approximately 150 amino acids (16). Individual E2s are involved in different cellular processes and, therefore, in the ubiquitination of different classes of substrate proteins. E3s, on the other hand, are more abundant and with no overt sequence homology, are thought to be largely responsible for the high degree of specificity of protein ubiquitination (13).

Evidence of D2 ubiquitination and E1 involvement was obtained in cells expressing a temperature

sensitive E1. At the restrictive low temperature, which inactivates the E1, D2 activity and protein levels are stabilized, even when protein synthesis is inhibited or D2 substrates are present (17). Ub-D2 conjugates are high molecular weight proteins (100-300 kDa), easily identified in lysates of cells transiently expressing an epitope tagged D2. In such a system, Ub-D2 conjugates behave as expected, i.e. they are increased in cells exposed to D2 substrates such as T4 and decreased if E1 activity is blocked. Exposure to the proteasome uptake inhibitor MG132 also increases Ub-D2 conjugates because proteasome blockade does not interfere with ubiquitination. An important observation is that, under a number of conditions, D2 activity in a cell lysate correlates with the levels of D2 protein and not Ub-D2, indicating that D2 is inactivated by ubiquitination. Interestingly, under the same conditions, D1 and D3 are not ubiquitinated that is in agreement with their relatively long (>12 h) half-lives (17).

To identify additional proteins involved in D2 ubiquitination, a selenocysteine to cysteine mutant of D2 was expressed in the yeast *S. cerevisiae*, a cell model in which the Ub-proteasome system is well characterized and D2 displays its typical cellular and molecular properties. Especially important, D2 was highly ubiquitinated and maintained its short half-life and sensitivity to substrate exposure, and its degradation was blocked by inhibitors of the proteasome uptake. Interestingly, D2 was stabilized in yeast strains that lack specific E2s, Ubc6p or Ubc7p. Both of these E2s are involved in the ER-associated degradation (ERAD) process, in agreement with the fact that D2 is an ER-resident protein. Importantly, in the yeast that lacked the E2 proteins the substrate-induced loss of D2 activity and proteolysis were also impaired. On the other hand, no difference in D2 levels was detected in a yeast strain deficient in Ubc1p, an E2 involved in the degradation of unfolded protein, thus confirming that D2 ubiquitination in the yeast system is specific (18). The involvement of the mammalian homologues of Ubc6p and Ubc7p in D2 ubiquitination was confirmed in HEK-293 cells (19).

At this writing there is little information regarding the identity of specific structures in the D2 molecule that confer this remarkable metabolic instability and substrate sensitivity. Fusion of the FLAG sequence to the COOH terminus of D2 prolongs its half-life and increases the size of the Ub-D2 pool 20- to 30 fold but only increases D2 activity modestly. However, no change in half-life is observed if the FLAG tag is fused to the NH2 terminus, located within the ER. This result argues that D2 ubiquitination is not an irreversible signal to its degradation as not all Ub-D2 undergoes proteolysis. Enzymatic de-ubiquitination of Ub-D2 occurs *in vitro* (20) and could explain recycling *in vivo*. Through this pathway Ub-D2 can be reactivated through Ub isopeptidases to D2, explaining the prolongation of the activity half-life of the COOH-FLAG-tagged D2. This implies that the reservoir of inactive Ub-D2 and the pool of active D2 are normally in a dynamic equilibrium that could shift toward active D2 if proteasomal uptake is blocked or toward the formation of inactive Ub-D2 conjugates when cells are exposed to substrate (Fig. 1). This also potentially constitutes a novel mechanism that could account for the speed with which changes in D2 activity take place after stimulation or substrate exposure since synthesis or proteolysis are not required for these adjustments.

These results indicate that in order to understand the activation pathway of thyroid hormone we must have a thorough knowledge of the signals and components of the ubiquitin-proteasome system. Surprisingly, this system is not involved in D1- and D3-catalyzed thyroid hormone activation and inactivation, respectively, demonstrating how much we have to learn about these important aspects of thyroid physiology.

Acknowledgement

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CAUSES OF HOT THYROID NODULES

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Hot thyroid nodules

Hot or autonomously functioning thyroid nodules (AFTN) are benign thyroid tumors that can be found in up to 50% of adult goitres in iodine deficient areas . They are named according to their ability to function without thyrotropin (TSH).

Radioiodine or ^{99m}Tc-pertechnetate imaging (scintiscan) of the thyroid gland is performed to determine increased radionuclide uptake of thyroid epithelia compared to surrounding parenchyma. AFTN most frequently manifest as toxic multinodular goitres (TMG) - a heterogeneous clinical disorder that includes besides hot nodules the presence of nodules with normal or decreased (cold nodules) iodine uptake on scintiscan.

TSH and cAMP signaling

In normal human thyroid tissue TSH stimulates all aspects of thyroid physiology, i.e. iodine uptake and metabolism, thyroid hormone synthesis and release (1). TSH signaling is mediated through cyclic adenosine-monophosphate (cAMP) and phospholipase C (2). Increased cAMP or TSH produces goitre (3, 4) and stimulates proliferation of thyroid epithelial cells in vitro (5-7). Moreover, in vivo data for chronic stimulation of the cAMP cascade available since the introduction of transgenic models also show increased thyroid proliferation (8-10). Importantly, cooperative interaction of TSH and insulin/insulin-like growth factor (IGF)-I signaling is a significant aspect of thyroid physiology (11) that very likely combines systemic and local stimulation.

Constitutive activation of the cAMP cascade

General activation of cAMP signaling in thyroid epithelial cells is known to cause the development of goitre. In contrast, a focal increase of cAMP very likely leads to the development of benign thyroid tumors. Moreover, molecular defects (e.g. mutations) resulting in activated signaling through the cAMP cascade might be responsible for autonomous function of thyroid epithelial cells (12). Hence, identification of somatic mutations in the TSH receptor (TSHR) and Gsa protein which conferred constitutive activation to the cAMP cascade provided a molecular explanation for AFTN (13, 14). Clinical relevance of TSHR mutations has been also demonstrated in the context of germline mutations causing autosomal dominant nonautoimmune hyperthyroidism (15). The finding of constitutively activating mutations in a number of G protein-coupled receptors formed a general principle in the genetics of endocrine disease (16, 17). We maintain a database (http://www.uni-leipzig.de/~innere/TSH/frames_en.htm) that lists all known TSH receptor mutations found in autonomously functioning thyroid nodules or autosomal dominant nonautoimmune hyperthyroidism (18)

Likelihood of other somatic mutations

We have recently screened 75 hot thyroid nodules for somatic TSHR mutations with the more sensitive denaturing gradient gel electrophoresis method and found somatic TSH receptor mutations in 57% and Gsa mutations in 3% of the thyroid lesions (19). To speculate on the etiology of mutation negative nodules we used heterozygous polymorphisms in X-chromosome-linked markers to decide the clonal origin of thyroid tissue from female patients. Mutation negative autonomously functioning thyroid nodules could comprise polyclonal lesions which do not evolve from a single mutated cell. However, 50% of mutation negative cases from female patients show a monoclonal origin when tested for X-chromosome inactivation (19, 20) which indicates a mutation in a gene other than the TSH receptor or

the Gsa protein. Candidate genes for the development of toxic thyroid nodules are located in the cAMP cascade (e.g. other G protein subunits, adenylylcyclase, phosphodiesterase, protein kinase A). However the number of isoforms for each of these components makes a systematic screening rather painstaking. Alternatively, overexpression of signaling proteins like the TSH receptor, Gsa subunits, adenylylcyclases or downstream effector molecules could also be a cause of autonomously functioning thyroid nodules. Moreover, an altered turnover of the TSH receptor could play a role in aberrant TSH signaling leading to increased cAMP accumulation. In addition, possible molecular defects in the synergistic insulin/IGF-I cascade are also awaiting investigation. New strategies are therefore mandatory to evaluate the role of signaling proteins downstream of the TSH receptor or in other interacting cascades as candidate genes for the development of autonomously functioning thyroid nodules. One approach is a better understanding of the signaling in thyrocytes that most likely generates a number of promising candidates that could reward a mutational screen. Efforts to define the set of players using cDNA expression arrays point to a general distortion of signaling in thyrocytes of hot and cold thyroid nodules (21).

Iodine deficiency and nodular growth

Epidemiological data reveal a clear difference in the incidence of TMG between a region with iodine deficiency to one with normal iodine supply in the diet (22). With a delay of several years the incidence of TMG is reduced after iodine supplementation in an iodine deficient area of Switzerland (23). Moreover, there is a strong correlation with age since older patients show a much higher percentage of nodular transformation in iodine deficient and iodine sufficient areas (24). Finally, nodular transformation usually occurs in an already enlarged thyroid independent of the cause of hyperplasia (25). These common characteristics indicate that an early stimulus causes enlargement of the thyroid in which the environment for nodular structures develops that appear after a long period of time (up to 30 and more years).

Hyperplastic growth and mutation

Animal models of hyperplasia caused by iodine depletion show increase in functional activity and a tremendous increase in thyroid cell number which very likely orchestrates a burst of mutation events. A higher replication rate will more often prevent mutation repair and implement these mutations into the genome resulting in small cell clones bearing a mutation. Over time this very likely increases mutagenic load of the thyroid which eventually targets genes crucial for thyrocyte physiology. If the TSH receptor or the Gsa protein is affected, autonomously functioning thyroid nodules are likely to develop from small cell clones. This is supported by the finding of TSH receptor mutations in 'hot' microscopic regions of euthyroid goiters (26).

Propagation of mutated cell clones

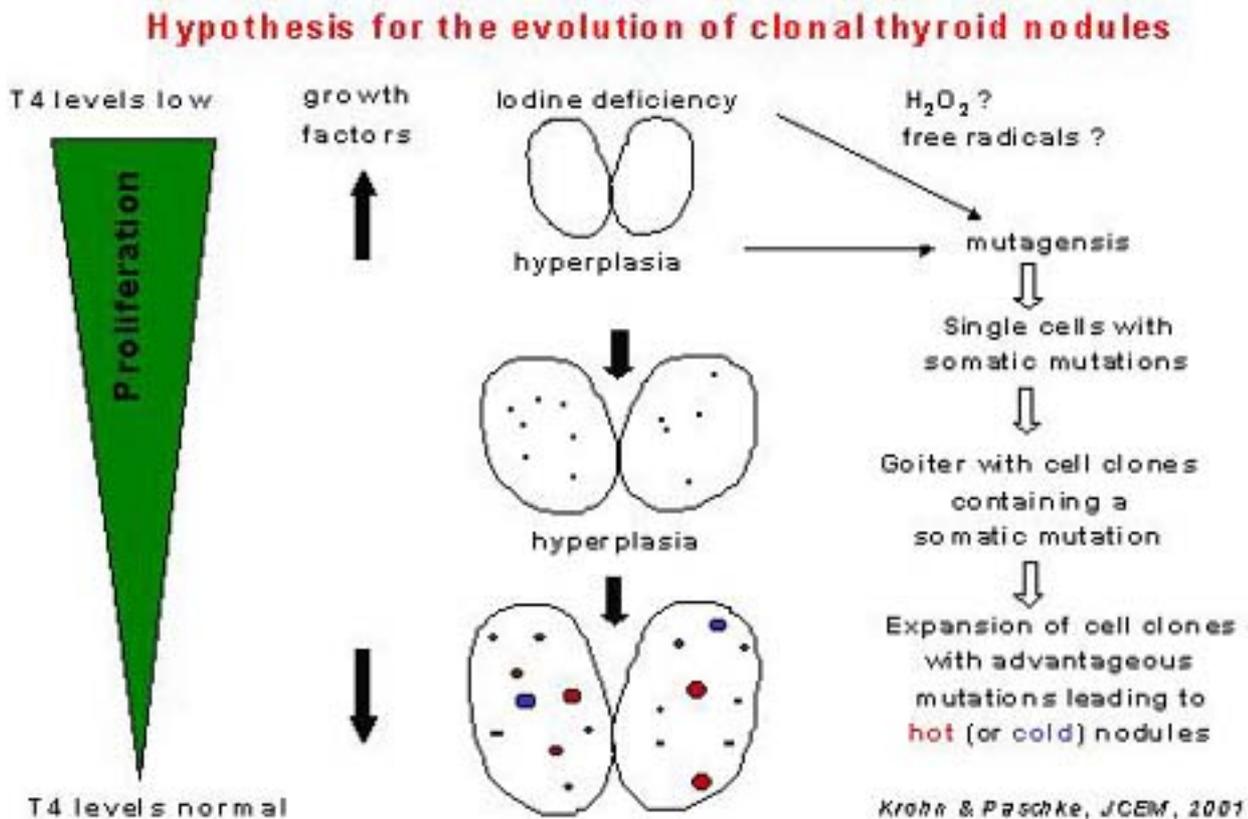
Hyperplasia goes along with increased growth factor expression like IGF-I, TGF- β 1, basic FGF and EGF (27-30). As a consequence cell proliferation associated with hyperplasia generates small thyroid epithelial cell clones. However, changes in growth factor expression are only transient and decline with prolonged goitrogen stimulation in line with mitotic activity (31). After this initial burst in growth factor expression small cell clones might only proliferate if they produce autocrine factors (e.g. IGF-I) and thereby compensate reduced paracrine stimulation. A constitutive activation in these cells (e.g. with a mutation in the TSH receptor, Gsa protein or Ras oncogene) could be the cause of growth factor expression as suggested for IGF-I after TSH/cAMP stimulation (32-34). Moreover, in contrast to a single constitutively activated cell where production of an autocrine factor might not be sufficient to cause self stimulation, a small cell clone might produce enough autocrine factors for self sustained proliferation. The constitutively active clone might therefore overcome inhibitory paracrine influences (e.g. lack of co-producing an activating factor) (?) from surrounding normal cells as suggested by Dawson and Wynford-Thomas (35). Interestingly, also growth factor expression stimulated through activating mutations in the TSH receptor most likely fades with time and is no longer detectable in late stages of thyroid nodules (36) although proliferation is still increased (37, 38).

In general, thyroid hyperplasias caused by goitrogens (e.g. propylthiouracil in animal experiments (39) or goitrogens in food (40) and in drinking water (41)) instead of iodine deficiency could also initiate the suggested line of events and lead to thyroid nodules which are also diagnosed in iodine sufficient

areas.

Conclusion

The sequence of events that very likely causes thyroid nodules (not only AFTNs) is as follows. (I)



Diffuse thyroid hyperplasia is a result not only of iodine deficiency but also of other goitrogens. (II) Due to increased proliferation during this stage of thyroid hyperplasia, mutagenesis is increased thus resulting in a higher number of cells bearing a mutation. Some of these mutations confer constitutive activation of the cAMP cascade (e.g. TSH receptor and Gsa mutations) that stimulate growth and function or other cascades that stimulate only proliferation (e.g. Ras oncogene). (III) In a proliferating thyroid, growth factor expression (e.g. IGF-I, TGF- β 1 or EGF) is increased. As a result cells divide and form small clones. After increased growth factor expression ceases small clones with activating mutations will further proliferate if they can achieve self-stimulation by expression of growth factors. They could thus form small foci which will develop into thyroid nodules (for a more extended review see Krohn and Paschke (42).

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CYTOKINES AND AUTOIMMUNE THYROID DISEASE

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CYTOKINES AND AUTOIMMUNE THYROID DISEASE

Autoimmune thyroid disease (ATD) affects up to 1% of the population and comprises several disorders, including autoimmune hypothyroidism (AH), Graves' disease and the extrathyroidal complications manifested as thyroid-associated ophthalmopathy (TAO) and pretibial myxoedema (PTM).

Cytokines and the thyroid

Cytokines play a central role in co-ordinating immune reactions and have multiple cellular sources, both immune and non-immune in origin. CD4⁺ T helper lymphocytes are classically divided into Th1 cells, mainly producing interferon- γ (IFN γ) and interleukin-2 (IL-2), which direct immune responses toward cell-mediated immunity. In contrast, Th2 cells secrete predominantly IL-4, IL-5 and IL-13 and promote humoral type immune responses (1). Th3 cells, producing mainly TGF β ₁, have been recently described and found to have an important role in the protection and recovery from autoimmune diseases (2).

Intrathyroidal cytokine production in vivo

Cytokine mRNA expression in thyroid tissue has been investigated using different techniques including Northern blotting, slot-blot analysis and reverse transcription-polymerase chain reaction (RT-PCR). Gene expression of IL-1, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, IL-13, IL-14, IL-15, IL-16, IFN γ , TNF α and a number of chemokines have been shown in both GD and HT tissue samples (3,4,5). Generally, a mixed Th1 and Th2 pattern has been found in the samples analysed, although some studies, using quantitative techniques, have shown a Th1 response in HT and a Th2 response in GD (6). However, the analysis of mRNA can be misleading, as gene expression does not necessarily correlate with protein production. To address this issue, immunohistochemical methods have been applied which demonstrated cytokine protein production by a variety of cells in ATD tissue in vivo. IFN γ was found in infiltrating lymphocytes, IL-1 in endothelial cells, whereas IL-1, IL-6 and TNF α were produced by thyroid follicular cells (TFC) (3).

A problem with the above studies is that surgery in ATD is infrequent and used late in the disease, in cases that may be atypical, making the general applicability of the results uncertain. Also, the potential for modulation of cytokine profile by antithyroid drugs or thyroxine, used to treat GD and HT preoperatively, should be taken into account.

Cytokine production by intrathyroidal inflammatory cells and TFC in vitro

Cell fractionation studies and in vitro cultures have been used to address the relative contribution of different cells to the intrathyroidal cytokine pool. However, it should be borne in mind that complete resolution of the cells can be difficult and contamination of a purified population with other cells can lead to artefacts. Also, a purified cell population may behave differently in vitro as it lacks the support of potentially crucial cells present in vivo. Gene expression of IL-2, IL-4, IL-6, IL-8, IL-10, IL-13, IL-14, IL-16, IL-17, IFN γ and TNF α has been demonstrated in ATD-derived lymphocytes (ITL), whereas IL-1 seems to be produced by other inflammatory cells (3,4). Protein production of IL-2, IL-4, IFN γ and TNF α was demonstrated in GD- and HT-derived ITL, further indicating a mixed Th1/Th2 response in ATD (3). However, subsequent work suggested a Th1 response in HT-derived ITL and Th2 response in GD-derived ITL when larger T cells (presumably activated) were analysed (7). This was refuted by Okumara et al (8), who demonstrated that GD-derived ITL have a mixed Th1 and Th2 characteristics. On balance, the concept of a predominance of a Th1 and Th2 response, in HT and GD respectively, is almost certainly an over-simplification as features of cell-mediated and humoral immunity can be found in both disease entities.

TFC themselves seem to have the ability to produce more cytokines than any other endocrine cell, and may, therefore, directly modulate the inflammatory reaction in ATD. TFC express mRNA of IL-1, IL-6, IL-8, IL-12, IL-13, IL-14, IL-15, IL-16, IL-17 and TNF α , in addition to a number of chemokines (3,4,9) and produce IL-1, IL-6, IL-8, TGF β and TNF α protein in vitro. The level of mRNA expression and protein production of cytokines and chemokines by TFC in culture can be modulated by cytokines, TSH, iodine and complement attack.

Immunological effects of cytokines

Pro-inflammatory effects

Cytokine production by TFC in vitro can be stimulated by IL-1, IFN γ and TNF α , and this may be important in increasing the size and the activity of the infiltrate in vivo. Cytokines enhance the expression of adhesion molecules on TFC and can stimulate nitric oxide (NO) and prostaglandin (PG) production by these cells, which may further have a role in localising and augmenting the inflammatory

reaction (3).

MHC class I expression is upregulated on TFC by IFN γ and TNF α treatment in vitro, which may play a role in tissue destruction through T-cell-mediated cytotoxicity (3). IFN γ also enhances MHC class II expression on TFC in vitro, which may have a role in enhancing the proliferation of non-B-7-dependent T cells, but may also have a protective role as detailed below.

Apoptosis seems to play a role in ATD, in particular HT (10). Cytokines can upregulate proapoptotic genes and downregulate antiapoptotic genes on TFC predisposing these cells to apoptosis.

Protective effects

TFC are resistant to complement-mediated cell lysis, an effect mediated in part by the expression of several protective proteins, which can be upregulated by cytokines (3). IFN γ and TNF α treatment of TFC in vitro renders these cells resistant to cell-mediated cytotoxicity, whereas TGF β_1 inhibits T cell proliferation and thyroid autoantigen recognition (3). Finally, the induction of class II expression on TFC by cytokines may have a protective effect through the induction of peripheral tolerance, as in most circumstances TFC fail to express the costimulatory signals (B7-1 and B7-2) that are necessary for the activation of naive T cells, thereby rendering these cells anergic.

Functional effects of cytokines

IL-1 and IL-6 enhance TFC proliferation in culture but can also have inhibitory effects if cells are stimulated with TSH, emphasising the complex interaction of these molecules in vivo. IFN γ and TNF α inhibit TFC growth and proliferation, without affecting cell viability (3). IL-1, IFN γ and TNF α all inhibit sodium-iodide symporter (NIS) gene expression and iodide uptake in TFC culture (11). TPO gene expression and TG production are decreased by cytokine treatment of TFC in vitro, which may affect iodine organification in vivo (12). IFN γ can also downregulate TSH-R gene expression (13).

These in vitro studies are probably too simple to reflect the complex in vivo situation as they only investigate the effect of a limited number of agents on TFC. Furthermore, the lack of information on the intrathyroidal concentration of cytokines in ATD poses a question mark over the interpretation of the in vitro data.

The immunological and functional effects of cytokines on TFC are summarised in the Table.

Proinflammatory effects	Protective effects	Functional effect

upregulation of MHC class I and II expression	upregulation of MHC class II expression	enhancement and inhibition of proliferation
induction of adhesion molecules expression	protection from cell-mediated cytotoxicity	downregulation of NIS gene expression and inhibition of iodide uptake
stimulation of PG, NO and cytokines production	protection from complement-mediated cell lysis	downregulation of TPO and TSH-R gene expression
predisposition to apoptosis	inhibition of T cell proliferation and autoantigen recognition	inhibition of TG production and iodine organification

Table: Immunological and functional effects of cytokines on thyroid follicular cells.

In vivo administration of cytokines and their effects on thyroid function

IFN α treatment for malignancy or chronic hepatitis is associated with impairment of thyroid function, ranging from the appearance of thyroid autoantibodies alone to the development of frank thyroid dysfunction (14). IFN α -induced changes in thyroid function are usually reversible, although permanent thyroid disorder may develop. A recent study has shown that antibody negativity after IFN α treatment has a protective role against the development of thyroid dysfunction over the years. In contrast, high level of thyroid autoantibodies after IFN α treatment, in particular the combination of TPO and TG antibodies, is a predictive factor for the development of thyroid dysfunction (15).

IFN β treatment for multiple sclerosis has also been associated with thyroid dysfunction(16), but this remains controversial (17). Finally, IFN γ administration in man has not been associated with any thyroid dysfunction or the development of thyroid autoantibodies (18).

IL-2 treatment can result in both hypo- and hyperthyroidism with an increase in thyroid autoantibodies (19). The administration of IL-6, a known acute phase protein, to healthy volunteers results in changes in thyroid function similar to those seen in non-thyroidal illness (20), which may explain the frequent occurrence of thyroid abnormalities in critically ill patients.

Serum cytokine levels in patients with ATD

Levels of IL-5 are increased in GD and HT sera (21), whereas IL-6, IL-8 and IL-12 concentration is increased in GD sera compared with controls (22,23). Increased serum levels of IFN γ , IL-4, IL-6, IL-10 and TNF α were found in GD compared with controls indicating a mixed Th1/Th2 response in this

disease (24).

An advantage of investigating serum cytokine levels in ATD is the ability to analyse cytokine profile early in the disease process, when the immune response is presumably more specific. However, serum cytokine levels may not reflect the intrathyroidal cytokine profile as levels of some cytokines may be very low in the periphery (falling below the detection sensitivity of the assay), despite high intrathyroidal concentrations.

Cytokines in TAO

Cytokine production in orbital tissue and orbital-derived lymphocytes

Early work using immunohistochemistry suggested a Th1-like cytokine profile in TAO tissue.

Subsequent studies using RT-PCR have shown mainly a Th2 response in retrobulbar tissue (25). Natt and Bahn (26) proposed that Th1 cytokines predominate in the early stages of the disease to be replaced by Th2 cytokines later in the disease process. The importance of Th2 response in TAO has been demonstrated in animal models of the disease (reviewed by Ludgate in *Hot Thyroidology*). More refined work has demonstrated a Th1 response in retrobulbar muscle (RBM) and a predominance of a Th2 response in retrobulbar fat (RBF) tissue (27).

In vitro studies have shown IL-2, IL-4, IL-10 and IFN γ production by orbital-derived lymphocytes, further suggesting a mixed Th1/Th2 response in this disease (25). In addition to lymphocytes, retrobulbar fibroblasts have been shown to produce proinflammatory cytokines and IL-1 receptor antagonist (IL-1RA) in vitro (28,29). Production of the latter can be stimulated by radiotherapy and this could be one mechanism by which radiotherapy exerts its beneficial clinical effect by blocking the action of IL-1.

Immunological and functional effects of cytokines in orbital tissue

Retrobulbar fibroblast proliferation is stimulated by cytokines, including IL-1a, IL-4, IFN γ and TGF β (25,30). Fibroblast function is also modulated by cytokines, as IFN γ , TNF, TGF β , IL-1 and IL-4 all induce glycosaminoglycan (GAG) production (25,31), whereas IL-1RA inhibits GAG synthesis (31).

In addition to these direct effects, cytokines may indirectly affect the inflammatory process through augmenting adhesion molecule, MHC class II, heat shock protein (HSP) and TSH-R expression in retrobulbar tissue. Cytokines increase adhesion molecule expression in retrobulbar fibroblasts in vitro (32), which could be a mechanism responsible for orbit-specific lymphocyte recruitment in TAO. MHC class II expression in cultured fibroblasts from TAO tissue increases with IFN γ treatment. Moreover, enhanced class II expression is more prominent in retrobulbar fibroblasts compared to abdominal fibroblasts taken from the same patient, possibly explaining the selective involvement of the retrobulbar

connective tissue in TAO. IFN γ and TNF α also enhance HSPs expression in TAO-derived fibroblasts but not fibroblasts from normal controls (32). The expression of TSH-R, a putative autoantigen in TAO, increases in orbital fibroblasts from TAO patients by IL-6 treatment, which may have a role in the initiation of the disease (33). Finally, cytokines can stimulate fibroblasts to produce metalloproteinase inhibitors (34) indicating that excessive accumulation of extracellular matrix in orbital tissue in TAO is due not only to increased production but also to impaired degradation.

The immunological and functional effects of cytokines in TAO are summarised in Table 2.

Proinflammatory effects	Functional effect
upregulation of MHC class II and adhesion molecule expression	stimulation of fibroblasts proliferation
upregulation of TSH-R expression	induction of glycosaminoglycan production
stimulation of heat shock protein expression	stimulation of metalloproteinase inhibitor production

Table 2. Immunological and functional effects of cytokines in retrobulbar tissue.

Summary

Cytokines, produced by both the inflammatory infiltrate and TFC, seem to play a crucial role in ATD. They can augment the inflammatory reaction through stimulation of intrathyroidal T and B cells and induction of immunological changes on TFC including the upregulation of MHC class I and II and adhesion molecules expression. Also, cytokines stimulate TFC to produce cytokines, NO and PG, which can further augment the inflammatory reaction and tissue destruction. Moreover, these molecules modulate the growth and function of TFC, and therefore, can be directly implicated in clinical thyroid dysfunction seen in ATD. Cytokines have also a role in the extrathyroidal complications of ATD, most importantly TAO. T cells are recruited to retrobulbar tissue in TAO patients, probably recognising an antigen (or antigens) shared with thyroid tissue, which may well be TSH-R. These T cells are activated and produce cytokines, which result in perpetuation of the inflammatory process through a number of mechanisms, including an increase in MHC class II, HSP, adhesion molecules and TSH-R expression in retrobulbar tissue. Moreover, cytokines increase fibroblasts proliferation locally and help

the recruitment of new inflammatory cells, thereby further augmenting the inflammatory reaction. In addition to cellular growth, cytokines increase the accumulation of extracellular matrix in orbital tissue through their stimulatory effects on GAG and metalloproteinase inhibitors production by retrobulbar fibroblasts. Therefore, modulation of cytokine production or blocking cytokine action in retrobulbar tissue may offer a new therapeutic approach in this difficult disease.

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MATERNAL HYPOTHYROXINEMIA AND NEURODEVELOPMENT: TO SCREEN OR NOT TO SCREEN; TO TREAT OR NOT TO TREAT.

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MATERNAL HYPOTHYROXINEMIA AND NEURODEVELOPMENT: TO SCREEN OR NOT TO SCREEN; TO TREAT OR NOT TO TREAT.

Human Rights:

1) "Every child has the right to an adequate supply of iodine to ensure his (or her) normal development."

Of particular importance in this context is the right of the unborn child.

2) "Every mother has the right to an adequate iodine nutrition to ensure her unborn child experiences normal mental development."

Declarations emanating from the Convention on the Rights of the Child, United Nations Assembly, New York, 1989; World Summit for Children, United Nations, New York, 1990: Declaration for the Survival, Protection and Development of Children; World Conference on Micronutrients: Eliminating Hidden Hunger, Montreal, 1991, UNICEF, WHO, FAO, ICCIDD; World Conference on Nutrition, Rome, 1992, WHO, FAO

All European States ratified these documents and have the same obligation of ensuring the above rights as they have, for instance, of providing clean and safe drinking water (37).

PART 1

Our position versus maternal hypothyroxinemia:

The second of the above statements, the one referring to the unborn child, was based on the overwhelming evidence then available, that many of the world's children had neurodevelopmental deficits of varying degrees, caused by iodine deficiency of the mother early in pregnancy. We now know that the link between the neurodevelopmental problems and the iodine deficiency (ID) is the maternal inability to

provide enough T4 to the developing fetal brain, namely her hypothyroxinemia#. We also know now that problems arise not only with severe ID, but also with moderate and even with mild ID (6). Therefore, we believe that hypothyroxinemia early in pregnancy ought to be prevented whichever its cause and, if prevention is not possible, it ought to be treated. The corollary of this is that screening programs need to be instituted, because most of these women would not be identified on clinical grounds.

Some urgent actions:

I) Ensure the rights of the child and of the unborn child. Therefore

- 1) Enforce USI (Universal Salt Iodination, namely iodination of all salt used for human and animal nutrition, including the salt used in food industries (37)). This is usually, and unfortunately, outside the legislative competence of thyroidologists, but ETA should continue to insist on its urgency in all National and International forums, especially European.
- 2) Include iodine supplementation, as KI tablets (where available), or in vitamin-mineral mixtures, in the routine care of pregnant women from the onset of pregnancy through lactation, before pregnancy if possible.
- 3) These efforts should aim at urinary concentrations during pregnancy and lactation of 180 µg I/ L or 220 µg I/ g creatinine, or higher.

II) Promote effective screening of pregnant women to identify those with hypothyroxinemia or other thyroid disorders.

- 1) Routine determination of 1st trimester FT4 should be added to the screening algorithms already proposed (15) to detect thyroid antibody positivity, and increased TSH, that were already planned before later evidence has related maternal hypothyroxinemia to neurodevelopmental deficits (17, 34).
- 2) A consensus regarding the cut-off FT4 values using different methods is still needed.
- 3) The reference values using different methods should only use sera from pregnant women in different stages of pregnancy, whose iodine intake is adequate as confirmed by the urinary iodine data (see I, 3 above).

III) Avoid preventable pitfalls in the evaluation of results:

- 1) of screening programs already in progress

2) in planning further clinical trials testing the consequences of maternal hypothyroxinemia for neurodevelopment of the child, and the effectiveness of its prevention or treatment.

3). We believe the main problems are related to an a priori choice of the cut-off point for the serum FT4 value. If division into the test and control groups is based on frequently used statistical criteria, such as = or > the 2.5th percentile value, respectively, the children of the women with FT4 values > the 2.5th percentile, but below the 10th percentile, would have been included in the control group. This could well mitigate the difference in the developmental outcome between both groups of children, that was found in the study by Pop et al. (39).

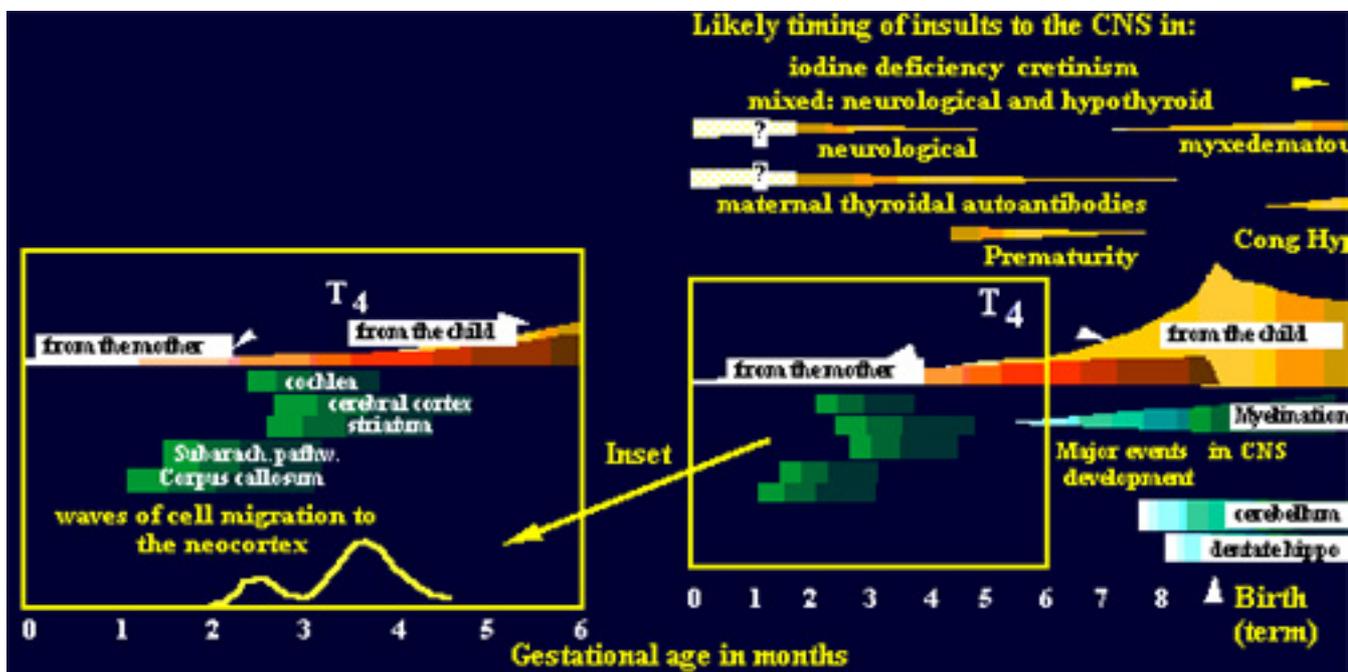
Part 2 of this paper contains more detailed proposals related to I)-III).

Frequent doubts and questions:

We have been personally involved for decades both in experimental work on the effects of thyroid hormones on the developing and adult brain, and the possible relevance of maternal-fetal thyroid hormone transfer, both in experimental animals and man. We have also been actively involved with neonatal thyroid screening programs and their follow-up, and in epidemiological studies in ID areas, including psychomotor development and hearing acuity of the inhabitants. For all these reasons it is sometimes difficult for us to understand why prevention of neurodevelopmental deficits related to the thyroid hormone deficiency, other than those caused by CH, have only become an increasing "hot thyroidology" problem during the last decade.

From written statements, questions asked, attitudes perceived at meetings and, last but not least, comments of reviewers of papers submitted for publication, we believe the following concepts merit being addressed. Most are further discussed in Part 2.

1) There is no proof that thyroid hormone is needed for early normal brain development in man. This is mostly based on the idea that, even if thyroid hormone receptors have been shown in 1st trimester cortex, thyroid hormone does not reach them in amounts likely to be biologically relevant. It is difficult to obtain this proof in man for obvious ethical constraints. This proof has been obtained in experimental animals (see Figure).



2) The widespread idea that low circulating FT4 is always accompanied by some concordant increase in TSH, even in the 1st trimester of pregnancy and that the term hypothyroxinemia is not clinically justified. This idea is contradicted by epidemiological studies in ID areas.

3) Difficulty in accepting that a hypothyroxinemic pregnant woman without further biochemical or clinical evidence of thyroid disease may be at risk of bearing a child with neurodevelopmental problems. Indeed, there is no proof that she herself needs higher circulating levels of FT4. The interventions that are being proposed might seem to be in conflict with medical practice, because there is no obvious "disease". But these interventions are not aimed mainly at the mother, but to ensure sufficient T4 for the fetal brain. Most pregnant women are given folate before and during pregnancy to avoid neural tube defects, without requiring any clinical or laboratory evidence that she needs the supplement for herself.

4) The idea that hypothyroxinemia early in pregnancy is only responsible for neurodevelopmental deficits in areas with severe ID, and therefore, not likely to occur in Europe. This idea is clearly untenable at present for most of Europe, as moderate and even apparently mild ID during pregnancy also affect neurodevelopment, and relatively few studies have been performed on the iodine intake of pregnant European women.

5) There is at present no consensus or precise definition of how severe the hypothyroxinemia has to be to significantly increase the risk of neurodevelopmental defects of the child. This is strictly true, but approximate values have already been reported. Defining a more precise cut-off point could be a priority of collaborative ETA studies outlined in Part 2.

PART 2.

The rest of this paper attempts to present evidence and discussions germane to the present issue. We will not again refer extensively to most of the epidemiological and experimental evidence (and bibliographic references) summarised in our previous reviews and comments (33, 34)

The following items will receive attention:

Introduction

Congenital hypothyroidism (CH) versus iodine deficiency (ID) cretinism.

Combined maternal and fetal hypothyroidism

Maternal hypothyroxinemia and neurodevelopment without severe ID

Hypothyroxinemia versus hyperthyrotropinemia of the pregnant woman

Likely causes of maternal hypothyroxinemia in Western populations

Mass screening of pregnant women during the 1st trimester

Reference values

The FT4 cut-off value

Introduction

Human studies, mostly epidemiological, have disclosed several different situations in which irreversible neurodevelopmental deficits have been related to thyroid hormone deficiencies early in human life (Table 1).

TABLE 1- THYROID HORMONE AND EARLY BRAIN DEVELOPMENT IN MAN.

CONDITION AND CONSEQUENCES	KNOWN PREVENTIVE MEASURES
Severe iodine deficiency (ID): neurological cretinism (deafness, mental retardation, cerebral palsy, etc)	Correction of the iodine deficiency before or very early in pregnancy (before midgestation)
Congenital hypothyroidism: mental retardation	Prompt postnatal treatment with T4
Maternal hypothyroxinemia: decreased I.Q. of progeny	Early correction of the maternal hypothyroxinemia

Combined maternal and fetal hypothyroidism: severe neurological damage, reminiscent of ID cretinism		Maintenance of normal maternal T4 throughout pregnancy, and prompt postnatal treatment of the newborn with T4
Thyroid hormone resistance syndrome: in many cases, mental retardation, deafness		Not yet feasible
Prematurity: neurodevelopmental impairment, cerebral palsy		Postnatal correction of transient neonatal hypothyroxinemia?

The present comments relate mainly to the first four conditions, although not necessarily in chronological order.

Congenital hypothyroidism (CH) versus iodine deficiency (ID) cretinism.

Prevention of major mental retardation by prompt postnatal treatment with T4, the aim of neonatal thyroid screening programs for CH established in most developed countries for over two decades, represents a prime example of the success of a strategy which benefits approximately 1: 3500-4000 babies. This contrasts with the permanent damage to the central nervous system (CNS) of the neurological endemic cretins (NC) born in areas of iodine deficiency (ID): the very severe CNS damage can only be prevented by adequate treatment during early pregnancy and not by postnatal treatment.

Fortunately, during the last decades epidemiological and experimental evidence have lead to a clearer understanding of the important differences between CNS deficits caused by ID, and those caused by untreated CH, and have provided plausible explanations for their different aetiologies (2, 6, 11, 17, 33, 34). It is now generally accepted that the mental retardation in untreated CH is caused by onset of cerebral thyroid hormone deficiency at birth, consequent to the interruption of the transfer of maternal thyroid hormones, mainly T4, the main precursor for the intracellular generation of T3 in the developing brain via type II 5'iodothyronine deiodinase (5'D-II). A normal maternal thyroxinemia and a normal response of cerebral 5'D-II activity to fetal hypothyroidism exert a protective effect on the fetal brain in utero, whereas maternal transfer of T3 does not have this protective effect. This hypothesis would explain the good results of treatment with T4, when it is started as soon as possible after birth, because it prevents postnatal thyroid hormone deficiency and its consequences. It also explains that even untreated CH infants do not have the severe neurological manifestations characteristic of NC (bilateral deafness, spastic diplegia, etc.), that are attributed to CNS damage occurring within the first half of pregnancy in

areas of ID. This early damage is the cause both of the severe neurological impairments of NC and the decreased mental development affecting the non-cretin population in areas with ID. Both are related to the low thyroxinemia of the mother within the first half of pregnancy, and not to her circulating T3 or TSH. We wish to point out that these women, at high risk of bearing a NC or a child with decreased mental and neuromotor development are not clinically hypothyroid, because their circulating T3 is normal or even slightly elevated, and may even prevent the appearance of "subclinical" hypothyroidism. TSH is not necessarily increased in ID, or increases to values that are usually lower than those of hypothyroid patients with a comparable degree of hypothyroxinemia and a concordant decrease in circulating T3. Unless other nutritional contributing factors are also present, that result in destruction of the gland (11), neither the cretin nor the non-cretin inhabitants with decreased mental development are clinically (or even "subclinically") hypothyroid, although their brain is likely to be selectively T3-deficient (20, 32). Thus, early irreversible neurodevelopmental problems of the child born from an ID mother are the consequence of the maternal hypothyroxinemia, whereas those of the untreated CH infant are caused by the hypothyroxinemia of the late fetus or the neonate, and may be prevented after birth.

Combined maternal and fetal hypothyroidism:

The contribution of maternal thyroid hormone to the normal neurodevelopment of the child is also evident from case reports that have appeared over the years from areas where ID, alone or combined with other factors, was not the underlying cause. In such cases, even prompt treatment with T4 after birth does not prevent neurodevelopmental disorders of the child. Some examples are: untreated women with TSH-receptor blocking antibodies, that are impairing both the maternal and the fetal thyroid (29, 44), a case of an inherited mutation of Pit-1 (5), and a case of unsuspected hypothyroidism in the mother of an infant with CH (7) For more on the possibility of combined maternal and fetal thyroid problems caused by autoimmune disease, see the recent editorial by Muller and Berghout (35) in a previous issue of *Hot Thyroidology*.

Maternal hypothyroxinemia without severe ID, and neurodevelopment

This term was introduced by Evelyn Man in her pioneering studies of the possible effects of low T4 during pregnancy on neurodevelopment of the progeny (27, 28). It was defined as a circulating level of T4 (then measured by the butanol extractable iodine, BEI) below the range usually found in women during the same trimester of pregnancy, whether or not clinical hypothyroidism was evident. We have used it to define women as hypothyroxinemic, using FT4 as criterion, whether or not clinical or subclinical hypothyroidism (increased TSH) is present. The results which strongly support the conclusion that

maternal hypothyroxinemia alone may have irreversible effects on neurodevelopment have been reviewed more extensively by us, and we will not repeat here the detailed description of the epidemiological studies, the principal ones being those of Man et al. (27, 28) and Haddow et al. (18) in the USA, and those of Pop et al. (39, 40) in The Netherlands. All these studies involve a large number of pregnancies (200, or more). Other studies are frequently quoted as contradicting this conclusion, mainly those of Montoro et al. (31), Leung et al. (25), and Liu et al. (26). We wish to point out that the first two did not actually quantify the neurodevelopmental outcome of the progeny, and the third study involved a very small number of children (8 in the study group, 9 sibling controls), unlikely to disclose the differences in developmental indexes of the magnitude reported in above studies (18, 27, 28, 39, 40). Table 2 simplifies the information that we obtained from published data or personal communication, as previously explained (34), and compares the results of the 4 different studies indicated above.

Table 2- Frequency of infants at risk for an I.Q. < 85 %, based on different criteria for the selection of their mothers. The Frequency* of CH is 1:3500-4000.

	I**	II**	III**	IV**
	BEI low vs normal	Anti-TPO pos vs neg	1st trim. FT4 £ vs >10th percentile	2nd trim. TSH ³ vs <98th percentile
% with IQ £85	33 vs 16	31.6 vs 15.9	50 vs 18	19 vs 5
OR with IQ£85 (95% CI)	2.6 (1.3-5.1)	4.8 (3.7-6.3)	4.6 (2.4-8.7)	4.5 (1.6-12.5)
% of pregnancies with risk factor	9.4	8.0	10	2.5
% of newborns at risk	3.1	2.5	5.0	0.5
Frequency ratio*	! : 32	1:40	1:20	1:150

CI, confidence intervals; BEI, butanol extractable iodine

* As referred to total live births; ** Study I (27, 28); II (40); III (39); IV (18)

Study I also comprised a group of children born to hypothyroxinemic women who were treated by thyroid replacement therapy. It is important to realise that none of the children had an IQ <85 %. In the study by Haddow et al. (18), there was also a group of children who were born to mothers who were treated during pregnancy: Their neurodevelopment was clearly better than that of the study group in all aspects, except for attention scores (assessed by the Continuous Performance Test), which was worse. These groups are not included in the simplified Table.

The main message of this Table is that impairment of neurological development related to maternal thyroid hormone status may be less severe than that reported for untreated CH, but it potentially affects 150-200 times as many children.

Hypothyroxinemia versus hyperthyrotropinemia of the pregnant woman

Another conclusion which may be drawn from Table 2 is that criteria related to circulating T4, FT4, and anti-TPO positivity (27, 28, 39, 40) permit detection of a larger number of infants at risk than an increased 2nd trimester TSH (18, 21). Obviously, screening and treating pregnant women on the basis of their anti-TPO positivity and hyperthyrotropinemia, as already proposed years ago (15), would represent an important step forward towards the prevention of neurodevelopmental deficits of many children. But we believe that the potential benefits of a screening routine that includes 1st trimester FT4 would benefit many more.

This opinion is not only based on the above epidemiological studies, but also on our present information regarding maternal-fetal transfer of thyroid hormone up to 17 weeks gestational age (3). Although the fetal samples included in this study were all obtained from women without any data suggestive of hyperthyrotropinemia or thyroid disorders other than a minor degree of ID, the results were in conceptual agreement with the following sequence of events. The concentrations of T4 escaping the placental "barrier" and reaching the embryonic fluids (coelomic and amniotic fluids and fetal serum) are correlated to maternal T4 or FT4. FT4 in embryonic fluids is determined by their concentrations of total T4 and of several T4-binding proteins, that are present at much lower concentrations than in the maternal circulation. As a result, the FT4 concentrations actually reach levels comparable to those that are biologically effective in adults. The type and concentration of the binding proteins are determined ontogenically, and unaffected by maternal thyroid status. Their binding capacity is far in excess of the total T4 reaching the embryonic fluids, so that FT4 levels depend ultimately on the maternal thyroxinemia.

Preliminary findings suggest that T3 and FT3 found in the embryonic fluids do not result from direct maternal-fetal transfer, but from embryonic metabolism of transferred T4. The FT4 available to the embryonic brain is likely to be the only substrate for the generation of the intracellular T3 that occupies cerebral nuclear receptors well before onset of fetal thyroid function (1, 13). The women with the lowest thyroxinemia levels, even when values are within a range that is normal in non-pregnant adults, would supply less T4 to the developing brain, and impair neurodevelopment.

We have studied (23, 24) the possible effects of maternal hypothyroxinemia, without clinical hypothyroidism, in two experimental rat models: a) the progeny of ID dams, that have low circulating T4, but normal T3, and no evidence of "clinical" hypothyroidism and b) the progeny of dams treated with a goitrogen for only 3 days, well before onset of fetal thyroid function. They had a transient minor decrease of circulating T4 and T3, but again showed no evidence of true hypothyroidism. In both models the migration of cells of the neocortex and hippocampus were clearly altered, with cells ending up in layers where they are never found in normal animals. This occurs at the expense of the cells reaching the different layers of the cortex and strata of the hippocampus. This is an irreversible change that could lead to functional deficits. Other investigators (9, 10) have also shown that early maternal hypothyroidism alters transiently the expression of several genes in the rat fetal brain.

In our opinion and despite previously expressed doubts (42), increasing evidence does support a biological role for maternal T4 during the first trimester. Although this cannot be verified in man, we have included an inset in Figure 1 which shows the timing of main waves of neuronal migration in the human neo-cortex (22).

Likely causes of maternal hypothyroxinemia in Western societies

Present information still identifies ID as the major cause of hypothyroxinemia, and possible neurodevelopmental defects. As indicated above, most Europeans, many doctors and health care personnel included, are not aware of this and believe there is no ID in their areas because they no longer see large goitres in young people or schoolchildren. But this does not necessarily mean that the iodine intake in their communities is high enough to meet the increased requirements of this essential micronutrient during pregnancy (12). The only way to evaluate this point is by measuring urinary iodine in a representative number of pregnant women, and this has not been done in most European countries, even when they might claim that they are free of IDD problems. Maternal hypothyroxinemia without TSH levels necessarily above the normal range have been described in countries such as Belgium (14, 16) and Spain (4). The latter studies have now been carried out both in an area where ID was recognised years

ago and the use of iodised salt promoted, and in the Community of Madrid, where studies in schoolchildren only disclosed a situation of mild ID in schoolgirls. In both Spanish studies, involving a total of 1200 pregnancies, it was found that about 40 % of the pregnant women had $< 90 \mu\text{g I / L}$, or $< 110 \mu\text{g I / g creatinine}$, values consistent with an intake less than half the recommended one for pregnant and lactating women. Their FT4 was lower, in all trimesters, than that of women receiving iodine supplements. Women who had 1st trimester FT4 below the 10th percentile of the values found in women with an adequate I intake (ca. $180 \mu\text{g I / L}$ or $220 \mu\text{g I / g creatinine}$) almost doubled the number found in the supplemented group. None of them had TSH values above the normal range. Their hypothyroxinemia would not have been detected by screening based on an elevated TSH. We believe it is quite significant from a European perspective, that an ongoing study in more than 400 pregnant women from Scotland (unpublished results from a EU Project on Neurodevelopmental Disorders in Premature Infants Caused by Thyroid Hormone Insufficiency - Molecular Basis for Diagnosis and Therapy, co-ordinated by Pof. R. Hume) discloses a similar situation in Dundee, Scotland. The United Kingdom is traditionally included among European countries free of ID.

It would appear that complying with the second of the above human rights is urgent. Iodine supplementation of pregnant women, even with the high amounts contained in iodised oils, is safe (43). Unless universal salt iodination (USI) is effectively implemented, permanent and adequately monitored, pregnant women should receive a daily iodine supplement of about 200-300 μg , an amount they are unlikely to obtain from iodised salt. It remains to be seen if this becomes unnecessary once USI has been introduced for several years, and women no longer on supplements actually reach successive pregnancies with thyroidal iodine stores adequate to supply the increased requirements throughout pregnancy and lactation.

There may be other causes of impaired thyroid function resulting in degrees of hypothyroxinemia, without a concordant increase in TSH, that would go unnoticed without mass screening based on FT4. Some, such as increased thiocyanate levels caused by smoking or intake of goitrogens, are likely to be mitigated or corrected if the iodine intake is adequate. Others might not. Shouldn't they be treated with T4 to attain a circulating FT4 that is normal for their stage of pregnancy, if iodine supplementation does not correct their hypothyroxinemia? This is being increasingly advised in other situations without clinical manifestations (30).

We have already referred to the editorial by Muller and Berghout (35) regarding autoimmune causes of maternal hypothyroxinemia. These authors also support the institution of appropriate screening programs, as many of these women would otherwise remain undetected.

Mass screening of pregnant women during the 1st trimester.

As is quite clear in all present conclusions, we believe that FT4 values are the criterion that should be used to detect as many women as possible, that are at risk of bearing a child with some degree of neurodevelopmental deficit with this underlying cause. A major problem that still has to be clearly solved is that we still do not really know which is the cut-off value to identify such women. On the one hand, this is due to the different values that are obtained when different commercial methods are used to measure FT4 in the same serum sample. On the other, it is also due to the scarcity of data for pregnant women in the different stages of pregnancy that we could use as an adequate reference, especially because the iodine intake has not been taken into consideration. Thus, for instance, Pop et al. use a 10th percentile as cut-off point, but they presented no evidence that all the women included in their study had urinary excretions concordant with an adequate I intake.

Reference values: Hot Thyroidology might be an adequate call for the ETA to institute an international effort to solve this problem. Both a 1st trimester sample of blood (spotted on neonatal screening filter paper) and serum, as well as a casual urine sample, should be obtained from 100-200 pregnant women who are receiving iodine supplements from before, or immediately after, onset of pregnancy. Adequacy of supplementation could then be confirmed by the determination of urinary I with the simplified method now available (38). Circulating serum FT4 should then be simultaneously measured with as many as possible of the commercial kits that are most frequently used. With mass screening as the aim, it would be especially important to include a kit measuring FT4 in spotted blood samples. Serum samples should also be tested to exclude those with increased TSH, or thyroid antibody positivity. Such a study would permit the definition of the normal percentiles for each of the different analytical procedures.

The FT4 cut-off value: This is a crucial problem, because the effectiveness of any screening program has to be confirmed. In many instances. Present evidence is not always considered sufficient to justify screening programs, and new trials are being carried out, or planned, to evaluate the effects of maternal hypothyroxinemia on neurodevelopment, and its correction through treatment. Despite our reservations regarding the I intake of all the women in the study (39), we could start by using point the 10th percentile FT4 value, as determined by the above collaborative study. If new trials are being carried out or planned we should only use as the control population children that were are born to mothers without ID during pregnancy. For evaluation of the results, a different approach could be used to determine the FT4 cut-off point. Instead of dividing the study group from the control group on the basis of a predetermined FT4 cut-

off value, the children could be assigned to the groups by their neurodevelopmental outcome: for instance, whether, or not, their IQ scores were < 85 %, or above. ROC curve analysis (receiver operating characteristics curve analysis) (19) could then be applied to define the specificity and sensitivity of different FT4 cut-off values, that separate the two groups. This would minimise the number of children that might have been inadequately included in the control group if we had used a pre-selected FT4 cut-off value, thus decreasing the odds ratio between the two groups.

FT4 that is low for the stage of pregnancy, whether or not clinical or subclinical hypothyroidism is present.

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INTERACTIONS OF THYROID HORMONE RECEPTORS WITH OTHER PROTEINS

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INTERACTIONS OF THYROID HORMONE RECEPTORS WITH OTHER PROTEINS

Abstract

Thyroid hormone receptors bind to the promoter regions of target genes and alter transcription by tethering a variety of coactivator and corepressor proteins. Although TRs generally are thought to bind DNA as heterodimers with RXR, it is unclear whether RXR always is essential, or whether there are functional differences between RXR-TR heterodimers or TR homodimers. T3-occupied TRs generally attract coactivators, but a very large number of such molecules has been identified, and an important challenge will be to define the specific roles of each of these. T3-dependent gene repression is less well understood, but may reflect the influence of specific cis-acting DNA elements to allow the liganded receptor to attract histone deacetylases.

Text

Thyroid hormone receptors (TRs) are members of the nuclear receptor superfamily closely related to retinoic acid and vitamin D receptors. The general structure of these receptors includes an amino terminal "A/B" domain of unclear function, a central DNA binding domain (DBD), a small flexible hinge region, and a carboxy terminal ligand binding domain (LBD). Two genes encode T3 receptors (TR α and TR β) that are nearly identical in their DBDs and LBDs, but unrelated in their A/B domains (the TRs also have splice variants). Although null mutations in mice [1,2] indicate that TR α and TR β subserve different functions in vivo, the mechanisms underlying this specificity are unclear. TRs themselves probably do not have inherent gene regulatory activity, but rather, function by attracting other molecules (generally proteins) to their target promoters. In fact, one plausible mechanism to explain in vivo functional differences between TR α and TR β would be differences in their protein interactions. This article will focus on selected aspects of three classes of TR-interacting proteins: dimerization partners that participate in DNA binding, corepressors, and coactivators.

Although TRs are capable of binding DNA in vitro as monomers, naturally occurring T3 response elements (TREs) have two receptor binding sites, consistent with the notion that TRs usually bind DNA as dimers. It is generally believed that retinoid X receptors (RXRs) are the dimerization partner for TRs [3,4]. RXRs also are nuclear receptors, and, distinct from their ability to heterodimerize with various other nuclear receptors, they can homodimerize and activate specific target genes in response to the RXR ligand 9-cis retinoic acid [5,6]. Evidence to support the central role of RXR in TR action is circumstantial: 1) in gel shift assays, RXR enhances the binding of TRs to DNA, and 2) in transfections, exogenous RXR enhances T3 induction of TR target promoters [3,4]. However, since RXR-null mammalian cells have yet to be described, it is not known what would really happen to TR function in the absence of RXR. At least some skepticism regarding the essential role for RXR is warranted for several reasons. First, TRs bind well to some TREs in the absence of RXR [7]. Second, gel shift assays do not really mimic the in vivo situation, where TRs are part of a larger complex of proteins which might stabilize the TR-DNA interaction. Third, under most circumstances, transfection of RXR only very modestly enhances T3 induction of reporter gene expression [8]. While this could indicate that endogenous RXRs are sufficient, there seems not to be enough endogenous RXR to support 9-cis retinoic acid induction of a reporter gene driven by an RXR response element. In yeast, TRs can activate some TREs in the absence of RXR [7], suggesting that T3 responsive genes manifest a spectrum of RXR dependence. Why might it matter whether TRs activate target genes as heterodimers with RXR or as homodimers? Genes that are dependent on RXR for T3 regulation could have the magnitude of T3 response modified by the availability of RXR. In addition, TR homodimers and RXR-TR heterodimers could attract different coactivator complexes, which again could permit differential regulation of subsets of T3 regulated genes within a cell. Further work is needed to better define the role of RXR in TR action.

Genes regulated by "positive" TREs are repressed by unliganded TRs and activated by T3-occupied TRs. The unliganded receptor dimer binds a corepressor complex that tethers histone deacetylases [9] to the TR, and hence, to the vicinity of the promoter. Deacetylation of histones tightens chromatin and impedes access to the promoter by the transcription machinery, thereby repressing transcription. T3 alters the conformation of the TR, causing this corepressor complex to be replaced by a coactivator complex. In a simple, commonly stated model, this coactivator complex includes a p160 protein (Steroid Receptor Coactivator-1, 2 or 3), CREB Binding Protein (CBP) and p300/CBP Associated Factor (PCAF), all of which have histone acetyltransferase (HAT) activity [10-13]. Histone acetylation loosens the histone-DNA interaction and thereby makes the promoter more accessible to the transcription machinery. A separate coactivator complex, variously called TRAP, DRIP, ARC or SMCC [14,15], binds in vitro to the ligand-occupied TR apparently in a mutually exclusive manner with the HAT complex. The TRAP complex does not have HAT activity and may interact with general transcription factors to induce gene expression.

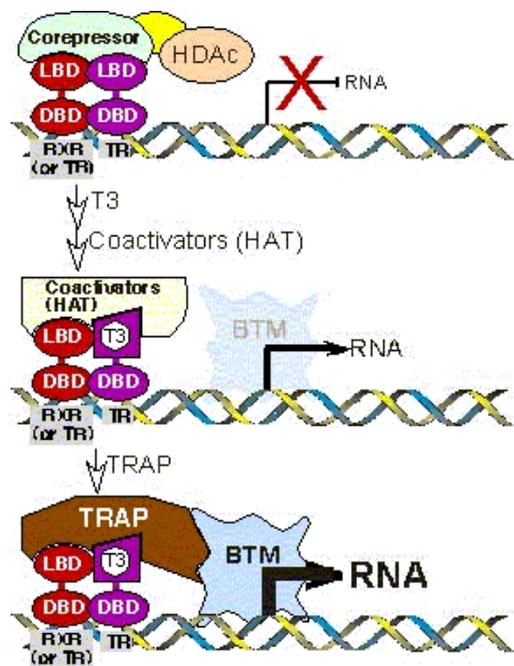


Figure. A model for gene regulation by T3 and TRs on "positive" TREs.

In the absence of T3, the TR binds as a heterodimer with RXR, or possibly as a homodimer, to a response element in the 5' flanking region of a target gene. The unliganded Ligand Binding Domain (LBD) binds to a corepressor protein, which tethers histone deacetylases (HDAC) to the vicinity of the promoter. Histone deacetylation, and perhaps other mechanisms, represses transcription.

The binding of T3 causes a conformational change in the TR LBD. The corepressor complex dissociates, and a coactivator complex with histone acetyltransferase (HAT) activity binds. Histone acetylation, and perhaps other mechanisms, alleviates the repression of transcription.

The HAT complex dissociates and is replaced by the multiprotein TRAP complex. This exchange might be facilitated by acetylation of HAT proteins. The TRAP complex interacts with the Basal Transcription Machinery (BTM) to induce transcription.

Many important questions remain unanswered regarding these coactivators. The commonly stated model suggests that the HAT complex binds the TR first, opening the chromatin structure and then being displaced by the TRAP complex (Figure 1). This needs experimental verification, as alternative models are possible. The dynamics of coactivator interactions with chromatin have been studied for estrogen dependent gene induction using chromatin immunoprecipitation [16]. These data indicate that p160 proteins and TRAP/DRIP are recruited simultaneously and rapidly to the promoter, but that CBP and PCAF are recruited subsequently. Further analysis suggested that the p160 and TRAP/DRIP proteins bind simultaneously to the same promoter molecules. These data are not consistent with the commonly stated model.

The importance of p160 and TRAP/DRIP proteins for thyroid hormone action has been confirmed by mouse knockout studies [17,18]. Many additional proteins have been discovered that bind to TRs and function as coactivators in transfections. How these fit into *in vivo* physiology is unclear. Although the sheer number of such coactivators precludes a complete discussion, several of particular interest will be mentioned. PGC-1 [19] is a coactivator that regulates mitochondrial function and biogenesis, and adaptive thermogenesis. It is expressed in tissues such as brown fat, muscle and liver, and is unusual in that it is massively induced by physiological stimuli such as cold exposure. PGC-1 and another coactivator denoted CoAA [20] contain an RNA binding domain, although whether RNA binding plays a role in TR coactivation is unknown. (The coactivator SRA functions as an RNA rather than a protein, but SRA appears to coactivate steroid receptors and not TRs [21]). CARM1 is a coactivator that binds to p160 proteins and that functions as a protein methyltransferase, methylating histone H3 and perhaps other proteins [22]. GT198 [23] is a coactivator that binds to the nuclear receptor DBD, rather than the LBD where most other coactivators bind. NRIF3 is unusual in that it coactivates TR and RXR, but not other nuclear receptors [24]. The *in vivo* functions of these and other putative coactivators remain to be defined.

Another complicating issue concerns the modulation of coactivator function by post-translational modifications. HATs acetylate many proteins in addition to histones. CBP had been found to acetylate SRC-3, which disrupts the interaction between the HAT complex and estrogen receptors, thus terminating estrogen-induced transcription [25]. A similar mechanism could extend to T3-induced genes. SRC-1 and presumably other coactivators are phosphoproteins [26]. Phosphorylation of SRC-1 via MAPK enhances its interaction with CBP and its function as a coactivator, at least for progesterone receptors [27]. Nuclear receptors themselves are subject to similar modifications. TRs are phosphoproteins, although a consensus has yet to be reached regarding the consequences of TR phosphorylation. TR phosphorylation has been reported to enhance the binding of TR homodimers but not RXR-TR heterodimers to DNA [28], to enhance the binding of RXR-TR heterodimers to DNA [29], or to diminish the binding of TR monomers

to DNA but not affect dimers [30]. For other nuclear receptors, however, the consequences of phosphorylation are more clear. For example, MAPK phosphorylation of the orphan receptor SF-1 enhances its binding to p160 coactivators and increases reporter gene induction [31].

Genes regulated by "negative" TREs are activated by unliganded TRs and repressed by T3-occupied TRs. TRH and the TSH subunit genes are the best studied, but the mechanisms involved remain poorly understood. Basic issues remain controversial, such as whether the TR must bind to the target gene to effect this regulation [32,33]. Adding to the confusion, transfection studies suggest that on these genes, classical corepressors induce gene expression by unliganded TRs, and classical coactivators repress gene expression by T3 [33,34]. This surprising result is consistent with the fact that mice with homozygous SRC-1 null mutations have thyroid hormone resistance, demonstrating a role for the "coactivator" SRC-1 in T3-mediated repression of TSH [18]. Recent studies of the TSH beta gene demonstrated T3-dependent binding of TR and histone deacetylase 2 to the promoter, and a physical interaction between these proteins mapped to the TR DBD [35]. Presumably, unusual aspects of the TSH beta promoter would enforce this unexpected interaction. This might suggest that the DNA sequence itself has an allosteric effect on the TR, dictating the proteins with which the TR interacts. In fact, allosteric effects of DNA on other transcription factors are well described [36], and suggest that DNA may best be viewed as another receptor ligand, inducing conformational changes that influence receptor-protein interactions.

Although great progress has been made in the field of thyroid hormone action, the added knowledge has led to new critical questions. The functions of TRs are driven by protein-protein interactions. Future studies of these interactions will be essential to advance our understanding of thyroid hormone action in health and disease.

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JUVENILE GRAVES' DISEASE: ¹³¹I AN OPTION OR NOT?

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Introduction

Graves' disease, the most common cause of hyperthyroidism in children and adolescents is an autoimmune disease in which stimulatory thyrotropin (TSH) receptor antibodies are secreted in excess (1,2) These antibodies mimic the action of TSH, leading to increased thyroid hormonogenesis and growth. In the absence of specific therapy for the immunological abnormality, treatment is aimed at inhibiting the ability of the thyroid gland to respond. The traditional approach to therapy is pharmacological blockade of thyroid hormone synthesis in anticipation that the immunological disease will go into a permanent remission and that permanent destruction of the thyroid can be avoided. Thyroid ablation, with surgery or radioiodine, is reserved for those patients in whom treatment with antithyroid medication has been unsuccessful.

In recent years an increasing number of pediatric endocrinologists, particularly in North America, have begun to favor the use of radioiodine over surgery to permanently ablate the thyroid gland and some have even advocated the use of radioiodine as initial therapy. Although radioiodine therapy is easy and effective, how safe is this approach and should it be used in children? This brief review will consider the advantages and disadvantages of the different therapeutic options with particular emphasis on a potential role for radioiodine.

Medical Therapy

The thionamide compounds (propyl thiouracil (PTU), methimazole(MMI) and carbimazole) exert their antithyroid effect by inhibiting the organification of iodine and the coupling of iodotyrosine residues on the thyroglobulin molecule to T₃ and T₄ (3). Carbimazole, used mainly in the United Kingdom, is rapidly converted to MMI, the active metabolite. MMI is preferred over PTU by many pediatric endocrinologists because for an equivalent dose it requires taking fewer tablets and because it has a longer half-life (2). As a result, MMI requires less frequent medication, an advantage particularly in non compliant adolescents. An additional advantage of MMI is that it results in a more rapid correction of the hyperthyroidism (3). On the other hand, PTU but not MMI inhibits the conversion of T₄ to the more

active isomer T_3 , a theoretic advantage if the thyrotoxicosis is severe (3). Because a therapeutic response is not observed for 4 to 6 weeks, a β -adrenergic antagonist drug such as atenolol or propranolol can be added in severe cases to control the signs and symptoms until the thyrotoxicosis is controlled. Alternately, an iodine preparation such as saturated potassium iodide solution (SSKI) or sodium ipodate can be used to inhibit the release of preformed hormone (and, in the latter case, block the conversion of T_4 to T_3). Patients are followed every 4 to 6 weeks until the serum concentration of T_4 (and total T_3) decreases or normalizes. When this occurs, one can either decrease the dosage of thionamide drug by 30% to 50% or, alternatively, wait until the TSH begins to rise and add a supplementary dose of l-thyroxine. Maintenance doses of PTU are given twice daily. MMI is administered once daily. Usually patients are followed every 4-6 months once thyroid function has normalized.

Mild toxic drug reactions (erythematous rashes, urticaria, arthralgias, fever and transient granulocytopenia), occur in 1% to 10% of patients (3,4). These side effects, considered to be allergic reactions, are more common in the first few months of therapy and in patients treated with higher doses of medication. Usually they subside spontaneously or with substitution of an alternative thionamide drug and can be treated with antihistamine therapy. Transient abnormalities in liver enzymes and mild leukopenia, reported to occur in as many as 25%-30% of children with Graves' disease (4), do not warrant discontinuation of the drug and probably are due, at least in part, to the underlying disease itself rather than to the medication. Although the frequency of such mild chemical abnormalities has been cited as an argument against medical therapy, usually these abnormalities resolve spontaneously and neither mild leukopenia nor a slight increase in liver enzymes presages the development of the rare, severe side effects of agranulocytosis or hepatitis, respectively. Thus routine monitoring of these parameters generally is not recommended (3), although some authors favor initial evaluation of the white blood cell count and liver enzymes prior to starting therapy. The overall incidence of agranulocytosis (usually defined as a granulocyte count <250 cells/ μ L) has been estimated to be 0.1% to 0.5% (3,4). There is some evidence that this life-threatening complication is more common in the first 3 months of therapy, in adults over the age 40 years, and in patients given larger doses of methimazole (>40 mg per day) (3,5). Hepatitis, on the other hand, has been reported to occur almost exclusively with PTU and may be more common in childhood (6). Exceedingly rare reported side effects of antithyroid medication include a lupus-like syndrome, polyarteritis, thrombocytopenia, aplastic anemia and

nephrotic syndrome. Like hepatitis, the latter very rare side effects, also, are more common in patients treated with PTU.

As long as patients are compliant, hyperthyroidism is readily controlled with antithyroid medication in >90% of individuals. Thus, the major difficulty with antithyroid drug therapy disease relates to the potential for relapse of the hyperthyroidism once therapy is withdrawn. The optimum duration of medical treatment is unknown, though usually patients are treated for at least 1 to 2 years. Relapses occur most commonly within 6 months of drug withdrawal. In adults, the rate of relapse plateaus at approximately 50% at 5 years (3). The rate of relapse in children may be somewhat higher. In one study, approximately 50% of children went into long term remission within 4 years, with a continuing remission rate of 25% every 2 years for up to 6 years of treatment (7). Favorable indicators that drug therapy can be tapered gradually and withdrawn include a small drug requirement, small goiter, lack of ophthalmopathy and lower initial degree of hyperthyroxinemia ($T_4 < 20 \mu\text{g/dL}$ (257.4 nmol/L); $T_3:T_4$ ratio <20). Recently, body mass index (BMI) <-0.5 SD (8) and older age (pubertal versus prepubertal age) (9) have also been associated with an increased likelihood of permanent remission. Children who develop Graves' disease prior to the age of 4 years tend to have particularly severe disease and are less likely to have a permanent remission (10). Persistence of TSH receptor antibodies, similarly, indicates a high likelihood of relapse (11, 12)). Initial studies suggesting that combined therapy (i.e., antithyroid drug plus I- T_4) might be associated with an improved rate of remission (13) have not been confirmed (14, 15)).

In addition to relapse of the hyperthyroidism, a small percentage of children treated medically will develop long term hypothyroidism.

Surgery

Surgery, the oldest form of therapy is performed less frequently now than in the past. An advantage of surgery is the rapid resolution of the hyperthyroidism. Pretreatment, usually with antithyroid medication for 4-6 weeks until the hyperthyroidism is controlled followed by iodide for 1-2 weeks, usually is recommended, although successful surgery has been reported after pretreatment with a β -adrenergic antagonist drug alone or in combination with iodide for only 10-14 days (3) Surgery has been associated with a higher morbidity rate than the other therapeutic modalities, greatly limiting its popularity. When the results of 6 separate studies involving more than 2,000 children treated with surgery were pooled (4), the most common complication (aside from temporary pain and discomfort, present in all patients) was transient hypocalcemia (10%). Keloid formation occurred in 2.8% of

patients. Other less common side effects of surgery were recurrent laryngeal nerve paralysis (2%), permanent hypoparathyroidism (2%), and, very rarely, death (0.08%). However, in a recent review of 82 children and adolescents from one institution, no instances of either recurrent laryngeal paralysis or permanent hypoparathyroidism were recorded and perioperative mortality was zero (16). Thus, when an experienced thyroid surgeon is available and modern methods of anesthesia and pain control are used, this therapeutic option is a safe and effective alternative. Unfortunately with the increased use of radioiodine therapy there has been a reduction in the number of experienced surgeons (17). Following total thyroidectomy the recurrence rate is <3% as compared with 10%-15% following subtotal thyroidectomy. Virtually all children treated with total thyroidectomy become hypothyroid, compared with approximately 80% of children treated with subtotal thyroidectomy. (4)

Radioactive Iodine

In recent years radioiodine therapy with ^{131}I is being favored increasingly by many pediatric endocrinologists for patients who fail medical therapy (4,18,19), and by some, even as the initial approach to therapy (20). The proponents of this form of therapy cite the relative ease of administration, the reduced need for medical follow up and the lack of demonstrable long term adverse effects. Although ^{131}I emits both γ and β -radiation it is the ionizing effects of the β particles with a path length of 1-2 mm that are responsible for destruction of thyroid follicular cells along with adjacent cells. Pretreatment with antithyroid medication is not necessary in most cases. An empirical dose of ^{131}I (3-15 mCi) can be used or the dose can be calculated, based on the formula:

Estimated thyroid weight in grams X 50-200 $\mu\text{Ci } ^{131}\text{I}$

fractional ^{131}I 24 hour uptake

A higher dose (150 μCi to 200 $\mu\text{Ci/gm}$) usually is recommended in children in order to completely ablate the gland and thereby eliminate any remaining thyroid cells that otherwise might undergo neoplastic transformation (4,19). Short-term complications of radioiodine therapy are mild and easily treatable. These include transient thyrotoxicosis 4 to 10 days after radioiodine administration and mild discomfort due to radiation thyroiditis. Vomiting has been reported (18). Preexisting enuresis may require special prophylaxis (18). One usually sees a therapeutic effect within 6 weeks to 3 months. Although worsening of ophthalmopathy, described in adults after radioiodine (21) does not appear to be

common in childhood, this may be related to the rarity of severe eye involvement in childhood and adolescence rather than to any true age-related difference. It is important to be sure that the adolescent is not pregnant, a contraindication of radioiodine. The major theoretic concern about using radioiodine therapy in children relates to the well-documented increased susceptibility of the young thyroid gland to the proliferative effects of ionizing radiation, a sensitivity demonstrated most strikingly in recent times by the consequences of the Chernobyl nuclear disaster. In areas exposed to increased radioiodine fallout, a 62 -fold increase in the incidence of papillary thyroid cancer was observed (22,23). This increased incidence of thyroid cancer was seen almost exclusively in children who were <10 years of age at the time of the reactor malfunction, being greatest in those <5 years of age or in utero. After age 6 years, thyroid adenomas were more frequent than carcinomas, and after the age of 12 years, the increased incidence of adenomas no longer was observed. Other well-known examples of the increased susceptibility to thyroid irradiation in the young include the increased incidence of papillary thyroid cancer in adults exposed to low dose external irradiation to the head and neck in childhood (24) and young adults treated during childhood and adolescence with mantle irradiation for Hodgkin's disease (25,26).

In thousands of adults treated with radioiodine, equivalent to several million patient years, no increased incidence of thyroid nodules, thyroid or other solid tissue malignancy or leukemia has been reported, nor is the frequency of birth defects increased (3,4). Longterm follow up of children, particularly young children <10 years of age, is much more limited. In a recent review of 11 studies involving 587 children and adolescents treated with radioiodine therapy between the ages of 1 and 18 years, benign solitary nodules were noted in 13 patients followed for 1 to 23 years (18). Similar results were obtained by the Cooperative Thyrotoxicosis Follow-up Study (27). In the latter study, an increased incidence of benign thyroid nodules was found only in children and adolescents treated for Graves' disease with radioiodine therapy. The increased frequency of benign thyroid nodules was greatest in the first decade of life and after the second decade was no longer seen. Four cases of thyroid malignancy following radioiodine therapy in childhood have been reported in the medical literature (4) Two of the 5 children were <10 years of age at the time of treatment and three of these individuals were treated with a low dose of radioiodine. No other serious long term complications of radioiodine therapy during childhood and adolescence have been reported.

In the aforementioned review of 587 children and adolescents treated with radioiodine therapy, hypothyroidism developed in 27%-92% and 0%-40% required retreatment. After 150 μ Ci to 200 μ Ci/gm the long term cure rate of hyperthyroidism has been reported to be 90% or greater and only 10% to

15% have been said to require retreatment. For a summary of indications and contraindications for radioiodine therapy, see Tables 1 and 2.

Table 1. Indications of Radioiodine Therapy in Childhood and Adolescence

Initial Approach

1. Underlying medical condition - e.g., heart disease (rare)
2. Serious behavioral problem - e.g., mental retardation, juvenile delinquency
3. Severe hyperthyroidism, unlikely to remit with medical therapy
4. Patient preference (older adolescent)

Secondary Approach

1. Failure of medical therapy
 - a. High dose requirement to achieve euthyroid state
 - b. Toxic drug reaction
 - c. Non compliance
 - d. Relapse of hyperthyroidism
-

Table 2. Contraindications of Radioiodine Therapy in Childhood and Adolescence

Relative

1. Large goiter
2. Significant ophthalmopathy
3. Young age (<10 years)

Absolute

Pregnancy

Summary & Conclusions

Controversy continues to surround the optimal treatment of Graves' disease in children and adolescents, and in particular, the role of radioiodine therapy. It is clear that there is no one approach that is perfect for every patient and that therapy needs to be individualized and discussed with the patient and/or his or her parents. In patients who are compliant and have mild or moderate disease, antithyroid medication remains an appropriate approach to treatment. Medical therapy is relatively safe and effective in controlling the hyperthyroidism in the majority of cases and there is a reasonable prospect that the disease will go into a permanent remission so that lifelong therapy will not be required. Most children can be maintained on a low, once daily dosage of MMI without any adverse effects and in many cases the drug can be withdrawn after 1-3 years without relapse of the hyperthyroidism for many years. Even in those children who do relapse following drug withdrawal a euthyroid state often can be maintained indefinitely with a low once daily dosage of MMI. In this latter group of patients, the goal may be simply to postpone definitive therapy to an older age when it will be safer and/or better tolerated. Since hypothyroidism and prevention of recurrence is a therapeutic goal of both radioiodine therapy and thyroidectomy in childhood, once daily medication with MMI is no more complicated than the daily thyroid replacement required after thyroid ablation.

When, then might therapy with ^{131}I be considered? The most important indication for radioiodine therapy in the pediatric age group is patients who fail treatment with antithyroid medication and in whom permanent thyroid ablation should be the therapeutic goal. Treatment failures include children who develop a toxic drug reaction, are non compliant, or in whom high doses of antithyroid medication are required to control the hyperthyroidism. Alternately, patients who relapse following discontinuation of medical therapy may choose permanent thyroid ablation, particularly if they are about to leave home to go to college. In these cases, radioiodine therapy is a reasonable choice in older adolescents, particularly if an experienced thyroid surgeon is not available, if the patient has an underlying medical condition that increases the risks of surgery or if the patient chooses this form of therapy. Rare situations in which radioiodine therapy might be chosen as initial therapy includes patients with severe hyperthyroidism who, it is felt, are unlikely to go into a permanent remission, or in whom it is felt the hyperthyroid state is to be avoided at all costs (e.g., delinquent or mentally retarded children, underlying medical condition, etc.). Radioiodine is contraindicated in pregnancy and should be used with caution in children with a large goiter in whom radioiodine therapy is less likely to be effective and

in the rare child with significant ophthalmopathy. In the latter case, a course of steroid therapy may be advised to minimize the risk of worsening the eye disease.(21)

The issue of radioiodine therapy in young children, particularly those <4 years deserves special mention. These patients, though rare, are particularly difficult to treat because they tend to have a particularly severe form of the disease with persistently high TSH receptor antibody levels for many years (10). Furthermore, in those in whom the disease develops in the first 3 years of life, permanent cognitive deficits and cranial synostosis have been reported (10). Not only are these children unlikely to undergo a permanent remission, but both the long term consequences of the hyperthyroidism as well the potential adverse effects of their persistently high TSH receptor antibody levels on the course of their eye disease are of concern. In this group of patients, therefore, early ablative therapy may be desired. Unfortunately, it is particularly in this patient group that radioiodine therapy should be used with the most caution, if at all. Not only is the potential risk of thyroid neoplasia the greatest in the younger age group, but radioiodine therapy is less likely to be effective and may even be harmful in view of the high frequency of a large goiter and significant eye disease, respectively in these patients. In summary, while there is clearly a role for radioiodine therapy in the pediatric age group, the best established indication is in older adolescents who have failed antithyroid medication or in whom permanent thyroid ablation is desired. Whenever radioiodine therapy is chosen in the pediatric age range, an ablative dose of ^{131}I should be used in order to destroy completely all thyroid tissue and thereby reduce the incidence of future neoplasia. Lifetime follow up is essential to ensure that the dosage of thyroid replacement used is sufficient to maintain the serum TSH concentration in the low normal range and to monitor the thyroid gland for the presence of nodules. Future collaborative studies aimed at gathering more long term safety and efficacy data in a larger group of patients will be of utmost importance to verify the safety of this approach, particularly in children <10 years of age.

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NEGATIVE REGULATION BY THYROID HORMONE

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Introduction

Thyroid hormone exerts many of its physiologic effects in cells at the level of transcription. Indeed, once it enters the nucleus triiodothyronine (T3) has the ability to either induce or inhibit gene expression. In the last number of years much work has begun to clarify the mechanism by which T3 is able to positively regulate gene expression. In contrast, T3 appears to inhibit gene expression through multiple mechanisms and it remains unclear which of these mechanisms predominates in vivo. This is further complicated by the fact that negative regulation by T3 occurs in many separate tissues including the CNS, heart and liver and on a wide variety of genes. Indeed a recent microarray study in murine liver suggests that more genes are negatively regulated than positively regulated [1]. Thus, an understanding of the molecular pathways by which T3 inhibits gene expression is critical to interpreting the physiologic effects of T3. This editorial will focus on the molecular mechanisms underlying negative regulation in the hypothalamus and the pituitary because T3's action there is essential for the maintenance of normal thyroid function. Before exploring negative regulation it will first be important to review the components of the T3-signaling system in positive regulation.

Positive Transcriptional Regulation by T3

T3 exerts its action on gene regulation through its cognate nuclear receptor family, the thyroid hormone receptor (TR) isoforms [2]. The TR isoforms are members of the nuclear receptor superfamily which includes the nuclear receptors for 1,25-vitamin D, retinoic acid, cortisol, the thiazolidinediones and the sex steroids. Humans possess two separate TR genes (α and β) which generate three T3 binding isoforms and 1 isoform which does not bind T3. Each of the TR-isoforms is similar in structure to other nuclear receptors which is heralded by an amino terminal domain which contains an activation function (AF-1), a central DNA-binding domain and a C-terminal ligand-binding domain. The TR α gene is present on chromosome 17 and gives rise to the TR α 1 and TR α 2 isoforms. TR α 1 binds T3 and, as demonstrated in mouse knockout experiments, plays a critical role in T3 action in the heart, bone, intestine and is also important in the regulation of body temperature [3, 4]. TR α 2 does not bind T3

because of an altered ligand-binding domain created by alternative splicing. It likely functions as an inhibitor of the T3-binding isoforms in certain situations [5]. The TR β gene is present on chromosome 3 and gives rise to the TR β 1 and TR β 2 isoforms both of which bind T3. TR β 1 and TR β 2 differ in the amino-terminal region (AF-1 domain) through alternative 5' exons. Genetic studies in mice demonstrate that TR β 1 is essential for hearing and as well for normal T3 action in liver and kidney while TR β 2 plays an essential role in T3 action in the pituitary, hypothalamus and retina where it is involved in the development of color vision [6-8].

The TR isoforms enhance gene expression by binding to positive thyroid hormone response elements (pTREs) in the promoters or regulatory elements of target genes usually as homo or heterodimers with the retinoid X receptor (RXR) another member of the nuclear receptor superfamily. pTREs contain two copies of the hexameric sequence AGGTCA arranged as either a direct repeat, inverted palindrome or as a palindrome. The TR isoforms are, for the most part, present in the nucleus bound to TREs regardless of the concentration of T3. In the absence of T3, akin to the hypothyroid state, the unliganded TR α 1 and TR β 1 isoforms are potent repressors of transcription when bound to pTREs. This repression is caused by the ability of these isoforms to recruit nuclear corepressors (NCoR and SMRT) which in turn recruit a multiprotein complex containing members of the histone deacetylase (HDACs) family [9, 10]. Through histone deacetylation these proteins modify chromatin and block transcription. TR β 2 does not function well as a repressor because its unique amino-terminus appears to inactivate corepressor function [11]. The binding of ligand to the TR causes a conformational change in its ligand-binding domain which results in the release of the corepressors and the recruitment of a wide array of proteins termed coactivators. These proteins include members of the p160 family, the DRIP complex, p300/CBP and CARM1 [12, 13]. Many of these proteins contain intrinsic histone acetyl transferase (HAT) activity which allows for re-modification of chromatin into a favorable position for transcription to occur. In addition, the coactivator CARM1 has methyltransferase activity which also modifies chromatin favorably [14]. Thus, T3 both reverses transcriptional repression and allows for transcriptional activation on pTREs such as those found in the α -myosin heavy chain gene in the heart and the type I deiodinase and spot14 genes in the liver.

Negative Transcriptional Regulation by T3

Normal thyroid function is dependent upon negative feedback by T3 at the level of the hypothalamus and the pituitary in order to regulate the thyrotropin-releasing hormone (TRH) and TSH α and β subunit

genes respectively. In the absence of T3 these genes are strongly stimulated and when hormone is added they are repressed. Because of their important role in normal physiology these genes have become model systems in which to explore negative regulation by the TR and more broadly provide further insight into the mechanism by which nuclear receptors in general mediate negative transcriptional regulation. Knockout studies in mice have confirmed the critical role of the TR β 2 isoform in negative regulation in the hypothalamus and pituitary where it is predominantly expressed and have also suggested that the coactivator SRC-1 (a p160 family member) is also necessary [8, 15]. Proposed mechanisms for negative regulation by the TR include: 1. direct binding of the TR to negative thyroid hormone response elements (nTREs); 2. interaction with and inhibition of other positively acting transcription factors; and 3. competition for a limiting quantity of a certain transcriptional cofactor such as CBP/p300.

The Thyrotropin-Releasing Hormone Gene

TRH is expressed in numerous locations in the CNS but is specifically regulated by T3 at the mRNA level in hypophysiotropic neurons present in the paraventricular nucleus (PVH) only [16]. The reason for this cell-specificity is not clear but extensive cell localization studies of the TR isoforms and their cofactors in TRH neurons have not been performed as cell-specific expression of TR β 2 and its cofactors may be necessary for negative regulation. The TRH neurons in the PVH are not only regulated by T3. Indeed, pathways which regulate food intake and metabolism, including the leptin, melanocortin and neuropeptide Y signaling pathways, engage the TRH neuron [17]. These pathways have the ability to stimulate or inhibit TRH transcription by modulating activity of transcription factors such as STAT and CREB which in turn may influence the activity of the T3 pathway. Thus, T3-mediated negative regulation cannot be viewed in isolation.

Transcriptional regulation of TRH is mediated by its regulatory sequences present in its promoter which is located 5' to the transcription start site. In transfection experiments performed in mammalian cells the unliganded TR, in contrast to its action on a positive TRE, activates the TRH promoter (and other genes which contain a nTRE) when linked to a reporter gene. The addition of T3 reverses this activation and causes repression. Mapping studies of the rodent and human TRH genes have identified a key element termed Site 4 which is present in the proximal promoter across species and is necessary for negative regulation by T3. Site 4 (TGACCTCA) and its surrounding nucleotides can bind both TR homodimers and RXR/TR heterodimers [18, 19]. Mutation or deletion of Site 4 blocks both ligand-independent activation and ligand-dependent repression of the TRH promoter. In addition to Site 4

another region of the human TRH promoter present in the first 55 bp of exon I also interacts with the TR and appears to also be involved in negative regulation. Taken together with the in vivo finding demonstrating the crucial role of TR β 2 in mediating regulation of TRH, these data suggest that the TR interacts directly with these sites to mediate negative regulation likely through the recruitment of corepressors and coactivators.

Surprisingly, when studied in mammalian cell culture systems NCoR and SMRT appear to be critical for ligand-independent activation of the TRH promoter while the coactivator complex appears to be necessary for ligand-dependent repression [20-22]. These activities contrast to their actions when recruited by the TR to positive TREs. However, these data are supported by mutagenesis studies performed on the TR. Mutations which block corepressor binding inhibit ligand-independent activation but not ligand-dependent repression. Conversely, selective mutations which inhibit coactivator binding prevent ligand-dependent repression only. Thus, two possibilities exist to explain these opposite effects of corepressors and coactivators on a negative TRE: 1. cofactors may have opposite effects on chromatin remodeling when bound to the TR on a negative TRE especially when one considers the role of TR β 2; and 2. the TR is acting principally off DNA (Figure 1) and functions to remove corepressors and associated HDACs in the absence of ligand, leading to activation.

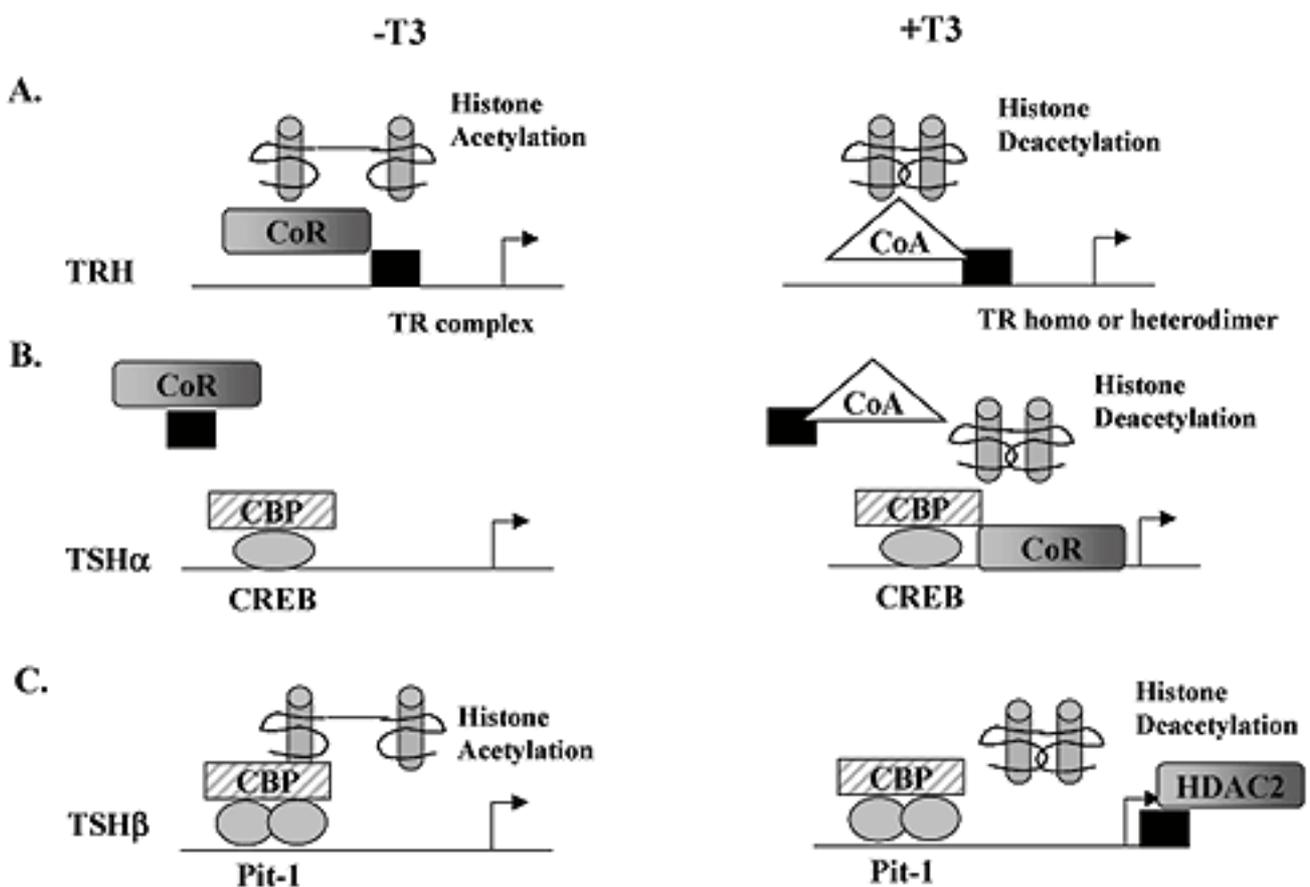


Figure 1: Models of Negative Regulation.

Shown are potential models of negative regulation on the TRH, TSH α and TSH β promoters.

- A.** The TRH promoter is regulated by TR binding sites in the proximal promoter (Site 4) and exon 1. Corepressors (CoRs) paradoxically activate transcription leading to histone acetylation and opening of chromatin in the absence of ligand, while the recruitment of coactivators (CoA) leads to transcriptional repression. Not shown is the role of Site 4 in mediating competition between the TR and CREB.
- B.** The TSH α promoter appears to be regulated independently of direct TR-binding leading to removal of corepressors from the promoter in the absence of ligand and their recruitment back in the presence of T3.
- C.** The TSH β promoter is down regulated by T3 through the recruitment of HDAC2 directly by the TR which binds as a monomer to a negative TRE in exon 1.

When T3 is added, the repression complex is then free to associate with other factors on the promoter while the coactivator complex is then drawn off by the liganded TR. Further work is needed to clarify this mechanism in vivo where other signaling pathways also engage the TRH neuron. Indeed, as discussed previously, melanocortin signaling via activation of the cAMP signaling pathway through phosphorylation of the transcription factor CREB (P-CREB) can activate TRH gene transcription. Remarkably, Site 4 is also capable of interacting with P-CREB and is necessary for melanocortin-mediated induction of the TRH promoter. T3 can inhibit this induction and it is likely that liganded TR β 2 competes with P-CREB for binding to Site 4 which would induce down regulation of TRH gene expression [17]. Thus, multiple mechanisms may be operative in the regulation of the TRH promoter by T3.

The TSH Subunit Genes

The TSH α and TSH β subunit genes are regulated at the level of transcription by T3 in the thyrotroph in the pituitary [23]. However it appears that different mechanisms may be operative. Both genes are also induced by the cAMP signaling pathway, though also through separate mechanisms. The TSH α promoter was originally shown to have a TR-binding site downstream of its TATA box [24]. However,

functional studies have demonstrated that this site is not important for transcriptional inhibition by T3. Tagami et al have recently demonstrated that two CREB-binding sites present in the proximal TSH α promoter are essential for negative regulation by T3 and that the TR works without binding to DNA. They have proposed that the unliganded TR is able to titrate away the repression complex containing corepressors and HDACs from the promoter (Figure 1). This allows for an increase in histone acetylation in the region of the promoter. T3 reverses this process and allows the repression complex to move back to the promoter leading to histone deacetylation which favors transcriptional inhibition [25]. Thus, the TSH α promoter remains the best example of a gene where the TR mediates negative regulation without binding to DNA.

In contrast to the TSH α gene, the rat and human TSH β genes contains a well described nTRE within exon I just downstream from the transcription start site that appears to bind TR monomers only. Initial descriptions of this region suggested that two TR binding sites are present but are spaced by 24 bp [26, 27]. Support for the role of the TR monomer in negative regulation of this gene also comes from experiments which show that RXR decreases both binding of the TR to the TSH β nTRE and negative regulation of the TSH β promoter by T3 [28]. Like the TSH α ; and TRH genes the TSH β gene is also induced by cAMP signaling (through its Pit-1 binding sites via interactions with CBP) which suggests that cross-talk between signaling pathways may also play a role in negative regulation of this promoter [29].

More recently Sasaki et al have demonstrated that cAMP-mediated activation of the TSH β promoter can be blocked by T3. Furthermore, they have established that a portion of the TSH β nTRE, which they term the Z region, is able to recruit the TR β isoforms and HDAC2 in the presence of T3 only to allow for inhibition[30]. This recruitment blocks cAMP-mediated activation of the TSH β promoter and enhances both the recruitment of histone deacetylase activity and apparent closing of the chromatin structure in mammalian cells. Remarkably, this recruitment of HDAC2 is independent of NCoR or SMRT and is mediated by the TR DNA-binding domain. Interestingly, the Z region sequence is conserved in a number of different genes including the TSH α gene but its role in mediating T3 regulation has not been established. Thus, like the TRH gene negative regulation of TSH β appears to require a specific nTRE.

Summary

Negative and positive transcriptional regulation by the TR isoforms is critical for the actions of T3.

Whereas the molecular mechanism involving positive regulation by the TR, including homo- or heterodimer binding to pTREs and cofactor exchange, have been well-worked out over the last number of years, the mechanisms underlying negative regulation still remain unclear. The TRH and TSH subunit genes provide important examples of genes negatively regulated by T3. Given that each appears to be regulated in a separate fashion by the TR it is likely that multiple mechanisms will be found to be responsible for the tissue specific negative regulation of genes by T3. Further insight into these mechanisms will come over the next few years as the availability of more knockout models increases and our ability to study protein-DNA interactions in vivo improves.

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THE ROLE OF THYROID OXIDASE (THOX) IN THYROID HORMONE SYNTHESIS

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Thyroid hormonogenesis and H₂O₂O generation

Thyroid hormone synthesis is dependent on the organification of iodide. Both thyroglobulin and iodide are substrates for this organification reaction that is catalysed by thyroid peroxidase (TPO).

It has been known for several decades that TPO has no activity without a source of H₂O₂ (1).

Several groups have studied the biochemical mechanism of H₂OO₂O generation. Although there was discussion on the underlying biochemical mechanism, all groups agreed on the fact that thyroidal H₂O₂ generation was Ca²⁺ dependent and involved a NADPH oxidase as electron transferring component to molecular oxygen (2,3 ,4).

Cloning of the human thyroid oxidase system

After elucidation of the full length amino acid sequence of human TPO from cloning experiments in the 1980's (5,6,7) it took over a decade before the cDNA and amino acid sequence of the human thyroid oxidase system was deduced.

After purification of a flavoprotein with NADPH- and Ca²⁺ dependent activity from pig thyroid plasma membranes, peptide sequencing and subsequent cDNA cloning, the cDNA sequence of a 138 kDa protein (p138^{T_{ox}}) was elucidated (8). Later p138^{T_{ox}} was demonstrated to be the incomplete version of THOX2, lacking over 1 kb at the 5' end of the mRNA.

Simultaneously two cDNAs encoding NADPH oxidases were cloned from thyroid based on the hypothesis that H₂O₂ generation in thyroid and leukocytes would be functionally similar. These cDNAs encode two highly similar proteins THOX1 and THOX2 of 1551 and 1548 amino acids respectively, see table.

Table: Differences and similarities between THOX1 and THOX2

	THOX1	THOX2
Gene size	36 kb	21.5 kb
Number exons	35 (2 non coding)	34 (1 non coding)
Number of amino acids	1551	1548
RNA expression	mainly thyroid	mainly thyroid
Antibodies available	yes, cross react with THOX2	yes, cross react with THOX1
Location protein	in thyrocytes intracellular and at the apical poles	
Regulation	upregulation by forskolin & downregulation by TSH	
<i>In vitro</i> functional activity	not successful yet	
Putative function	H ₂ O ₂ generation (based on presence of gp91 ^{phox} functional oxidase domains) crosslinking of tyrosine residues in thyroglobulin (based on homology with TPO and tyrosine crosslinking in <i>C elegans</i>)	
Role in thyroid pathophysiology	thyroid cancer: expression not related to progression congenital hypothyroidism: inactivating mutations reported	no data

The C-terminal part is clearly related to the leukocyte gp91^{phox}, and contains all the elements known to be essential for gp91^{phox} activity. The THOX1 and THOX2 N-terminus show 43% similarity with TPO (9). The same transcripts were cloned under the name DUOX1 and DUOX2 (10) and THOX2 was also identified by Serial Analysis of Gene Expression (SAGE) from normal thyroid tissue, followed by a computational subtraction approach (11).

Both THOX proteins contain consensus sequences for FAD- and NADHP-binding sites as well as heme binding sites and two EF-hand motives that may account for calcium binding activity. A striking unexpected feature of the THOX proteins is the 43 % similarity with TPO in the first 500 amino acids, although the distal and proximal histidine residues important for heme binding and peroxidase activity are replaced by serine in human THOX2 (12). The predicted structure shows 7 putative transmembrane regions pointing to intracellular localisation of the EF-hand motifs, the NADPH and FAD binding sites. In this model the glycosylated N-terminal domain with TPO similarity is located outside the cell, in the follicular space.

The THOX1 and 2 genes are located in close proximity at human chromosome 15q15 and are overall very similar. THOX1 has a telomeric position with respect to THOX2, and both genes have opposite transcriptional orientations. The THOX1 gene spans 36 kb and is composed of 35 exons from which the first 2 are non-coding. The THOX2 gene spans 21.5 kb and contains 34 exons from which 33 are translated to protein (13).

Tissue specific expression of the human thyroid oxidase system

Conventional Northern blotting shows that THOX gene expression is restricted to human thyroid, with some relatively low expression in trachea (14).

Blast analysis of expressed sequence tags databases and analysis of publicly available SAGE expression libraries also indicates THOX expression in some nonthyroid tissues. (8, 11, 14).

RT-PCR experiments show expression in many more tissues. Since it is difficult to judge the level of expression in relation to other tissue specific genes in most reports, the results seem to be subject to the rule: after enough PCR cycles almost everything is expressed everywhere.

Expression of THOX genes is variable among thyroid cancer tissues and does not seem to be related to the progression of the disease or the metastatic process (15).

Antibodies have been raised against THOX1 (9) and THOX2 (14) successfully, although both polyclonal antisera cross-react with their counterpart. They show that THOX proteins are located intracellularly and at the apical poles of thyrocytes and staining is heterogeneous within a follicle.

Regulation of THOX expression

The stimulatory effect on H₂O₂ generation has been linked to Ca²⁺ as a second messenger rather than to adenylate cyclase (). In several species regulation of THOX expression has been studied after forskolin and TSH stimulation. In pig, rat and dog thyroid cells cultures the addition of forskolin leads to upregulation of THOX mRNA. This effect is less pronounced for human thyroid cells. Methimazole treatment results in TSH increase and THOX2 downregulation in rats. (8, 9, 12)

The data show that although forskolin is regularly used to mimic TSH effects, such experiments are confusing the issue in case of THOX regulation.

There is no difference in THOX immunostaining in thyroid cancer tissues obtained in the same patient in both eu- and hypothyroid conditions, also suggesting that in vivo high TSH levels do not increase THOX expression in human thyroid (15).

Regulation of expression differs from the sodium iodide symporter in multinodular goiter, hypofunctioning adenomas and hyperfunctioning thyroid tissues (14).

There is a direct (inhibitory) effect of iodide and iodocompounds on the enzymatic activity of the thyroid NADPH oxidase. Whether this involves transcriptional down-regulation of the THOX1 and/or THOX2 genes was not investigated (17).

Conclusions and perspective

Full proof that THOX1 and THOX2, either alone or in combination, are involved in thyroid H₂O₂ generation is currently lacking. Based on the cDNA sequence, THOX1 and THOX2 are highly similar. Although both cDNAs are expressed in thyroid it is not clear whether both proteins are also present,

since available antibodies cross-react with both THOX proteins.

The C-terminal part of THOX2 has been identified based on the initial isolation of a thyroidal flavoprotein. The generation of enzymatic activity in THOX transfected cells has not been successful thus far. The inability to obtain full enzymatic activity in an in vitro reconstitution system is currently explained by the requirement of additional thyroid specific components necessary to get full protein processing and enzymatic activity in the thyroid ().

There is as yet little integration of the many biochemical data on the thyroid H₂O₂ generating system and the regulation of expression of the THOX genes. Both detailed investigations of the THOX1 and THOX2 promoter, as well as determination of the effect of the stimulation of thyroidal second messenger systems on THOX gene transcription will clarify these issues.

The *Caenorhabditis elegans* homolog of THOX1 is able to catalyse the cross-linking of tyrosine residues involved in the stabilisation of cuticular extracellular matrix and in *E. coli* ThOX1 is able to catalyse cross-linking of tyrosine ethyl esters (10). It is tempting to speculate that THOX proteins have a similar role in the cross-linking of (iodinated) tyrosine residues in thyroglobulin to form thyroid hormone or to generate the compact structure of the follicular thyroglobulin.

The definite role of thyroid oxidase in thyroid pathophysiology and thyroid hormone synthesis awaits the identification of THOX gene mutations in patients with hypothyroidism and iodide organification defects. There are scant reports on goiter and hypothyroidism in relation to decreased thyroidal H₂O₂ generation (19, 20) but in these cases the THOX genes have not been investigated. Since most patients with congenital hypothyroidism are treated early, the development of goitre in case of dysmorphogenesis does not occur. This means there is usually no thyroid tissue available and that genetic analysis of the THOX genes must be done from white blood cells. Since each THOX gene contains 33 coding exons and that the genes are highly similar also in a large part of the intron sequences, screening of the THOX gene sequences for mutations is a considerable task. There has been one preliminary report of inactivating THOX2 mutations in patients with congenital hypothyroidism due to a partial iodide organification defect (21).

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A ROLE FOR THYROID HORMONE IN THE PATHOGENESIS OF HEART FAILURE?

(amended version)

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Thyroid hormone and cardiac gene expression

In the heart, as in other tissues, the transition from the fetal to the adult phenotype is generally dependent on the perinatal rise of systemic thyroid hormone levels. The expression of key cardiac proteins remains dependent on TH in the adult as evidenced by the profound changes in cardiac performance in the transition from hypo- to hyperthyroidism. Most notably, T_3 stimulates the expression of the Ca^{2+} -pump of the sarcoplasmic reticulum (SR Ca^{2+} -ATPase, SERCA2), while reducing the expression of both the SERCA2-inhibitory protein phospholamban and the plasma membrane Na^+ - Ca^{2+} -exchanger. As a result, Ca^{2+} -transients shorten, relaxation rate increases and Ca^{2+} -filling of the SR goes up. The release of Ca^{2+} is also facilitated, through enhanced expression of the SR Ca^{2+} -release channel, the ryanodine receptor. Furthermore, the speed of contraction is increased through stimulation of expression of a faster myosin heavy chain gene ($MHC\alpha$), while the slower $MHC\beta$ isoform is repressed. These receptor-mediated, nuclear actions are responsible for the positive inotropic and lusitropic effects of T_3 . Together with the positive chronotropic effect of T_3 , the hemodynamic load placed on the heart increases and this is a major stimulus for growth of the cardiomyocyte (4). Since T_3 also reduces the systemic vascular resistance, the combined effects of T_3 on cardiac growth and contractility result in a rise in cardiac output adapted to the higher metabolic demand made by the organism. This form of hypertrophy is generally referred to as 'physiological'.

Cardiac gene expression in pathological hypertrophy

In contrast, when the increase in mechanical load of the heart is caused by chronic pressure and/or volume overload, due to hypertension, valvular dysfunction or loss of viable tissue following cardiac infarction, the ensuing hypertrophy often leads to decompensation and failure (pathological hypertrophy). Re-direction of cardiac ventricular gene expression in this condition is for many key enzymes the opposite of the changes induced by TH, i.e., a shift to slower contractile proteins ($MHC\alpha$ to $MHC\beta$), which is particularly evident in rodents, repression of SERCA2 and RYR and up-regulation of NCX and PL. The effects on the Ca^{2+} -regulatory proteins result in prolonged Ca^{2+} -transients,

increased diastolic Ca^{2+} -concentration and reduced peak Ca^{2+} -concentration. Together with the changes in expression of contractile proteins this is thought to be a primary cause of the systolic and diastolic dysfunction observed in pathological hypertrophy and failure in man and rat. Indeed, correcting the reduced expression of SERCA2 in transgenic mice and by gene transfer in rats is sufficient to prevent the progression to heart failure in models of pathological hypertrophy.

Multiple signal-transduction pathways are involved in regulating cardiac gene expression under normal conditions and in response to increased mechanical stress. These pathways converge on the promoters of target genes through signaling cascades or directly through hormone-binding transcription factors, as is the case for TH. At any time, the size and phenotype of the cardiomyocyte are therefore determined by the combined actions of multiple factors, and TH is one of them. In short, a significant part of the phenotype of pathological hypertrophy could be explained by reduced levels of TH, but is this the case in heart failure or, perhaps more importantly, during the development of pathological hypertrophy?

As in other non-thyroidal illnesses, TH-metabolism is altered in heart failure leading to a reduction of serum T_3 (reviewed in 1). The drop in serum levels is proportional to the severity of the disease and in one clinical study it was shown that daily administration of T_4 for 12 weeks improved several cardiovascular parameters in patients suffering from chronic heart failure. It is not known whether this involved correction of cardiac gene expression, but a recent study showed that T_3 could at least partially reverse such changes in rats which developed pathological hypertrophy following myocardial infarction. Normalizing serum T_3 restored the mRNA levels of $\text{MHC}\beta$, PL and Kv1.5 (a K^+ -channel), but higher doses of T_3 were needed to normalize $\text{MHC}\alpha$. Interestingly, SERCA2 mRNA did not respond at all. Reduction of serum T_3 could therefore account for some of the effects on gene expression in this model of acute MI. However, it is unlikely that changes in serum TH will play a role in the slow development of the pathological phenotype, for instance during chronic pressure overload, in the absence of overt illness. Indeed, the nearly complete shift in MHC-isoform expression and 50% reduction of SERCA2 mRNA, characteristic of pathological hypertrophy, was observed in a rat model of left ventricular pressure overload in the absence of changes in serum TH levels. Nevertheless, this does not rule out a role for TH-signaling, since the biological activity of TH is dependent on multiple processes and factors that may be regulated in a tissue-specific manner. Firstly, the TH levels of specific tissues may not reflect the systemic serum levels, and secondly, a complex interplay of DNA-binding factors ultimately determines the transcriptional efficacy of T_3 for individual genes or sets of genes.

What determines the biological activity of thyroid hormone in the heart?

The processes and factors that may determine the cellular level and transcriptional activity of T_3 in the cardiomyocyte are depicted schematically in Figure 1 and include active uptake of TH; metabolism of TH by deiodinases; the different TH-receptors (TR) and their dimerization partners (e.g. RXR), co-activators and co-repressors (e.g. N-COR); and cardiac transcription factors that interact with TH-receptors (e.g. GATA and MEF).

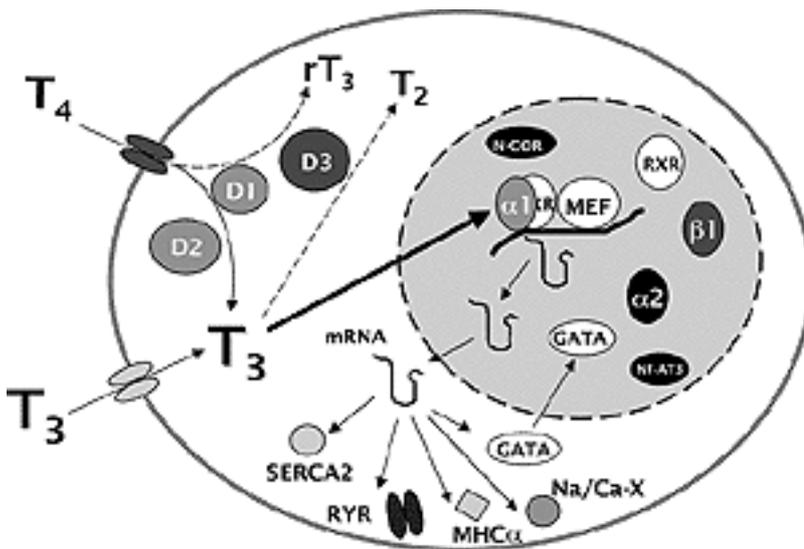


Figure. Determinants of the transcriptional activity of thyroid hormone in a cardiomyocyte.

Apart from some of the target genes of TH, the figure depicts the putative plasma membrane TH-transporters, the TH deiodinases type 1, 2 and 3 and their respective actions, the TR's: $-\alpha 1$, $-\beta 1$ and $-\alpha 2$, their heterodimerization partner RXR and co-repressor N-COR, the muscle-specific enhancer factor MEF, which can interact with TR's, the TH-dependent cardiac transcription factor GATA9 and NF-AT3, a partner of GATA implicated in redirecting gene expression in pathological hypertrophy.

Data are now accumulating that indicate that the expression of TR's is altered in pathological hypertrophy and failure. Although studies in humans are conflicting, a recent study in rat comparing pathological and physiological hypertrophy indicates a down-regulation of TR α 1, TR β 1, as well as the dominant-negative TR α 2 isoform, in chronic LV pressure overload. At least for TR β 1 the decrease in mRNA level was shown to result in less receptor protein. It was also found that MHC α was preferentially regulated by TR α 1, whereas MHC β and SERCA2 were more responsive to TR β 1 regulation. These observations could provide an explanation for differential responsiveness of cardiac genes to administration of T₃ (see MI study above), but whether they indicate a local hypothyroid condition, as suggested by the authors, remains to be shown. The effects of a true hypothyroid state result from the fact that unliganded TR's repress transcription from T₃-responsive promoters. The absence of TR's therefore creates less of a hypothyroid condition than the absence of T₃ as TR-knock out mice have shown. Consequently, data on the bio-availability of T₃ in pathological hypertrophy are needed.

As yet little is known about the active uptake of TH in the heart. Neonatal rat cardiomyocytes show active uptake of TH that is selective for T₃. The apparent absence of uptake of T₄ is in agreement with earlier isotope studies that showed that T₄ is not a source for local production in rat cardiomyocytes. In line with this, no appreciable increase in ventricular T₃ levels was found following overexpression in mouse hearts of type 2 deiodinase, which converts T₄ to T₃. Although D2 mRNA has been detected in human ventricle, D2 activity in human or rodent heart has not been reported. Very low levels of type 1 deiodinase have been found in rat heart. This deiodinase is primarily responsible for the production of serum T₃ from T₄ in liver and kidney, but it can also convert T₄ to the inactive metabolite reverse T₃ by inner ring deiodination. Yet, the negligible conversion of T₄ in rat heart suggests no functional role for this enzyme. Local TH metabolism therefore does not appear to be a likely site of regulation of T₃ bio-availability in the heart. However, data were recently presented at the 73rd annual meeting of the American Thyroid Association (Washington, November 2001) showing a marked induction of deiodinase type 3 (D3) activity in a model of right ventricular pathological hypertrophy and failure. This induction occurred exclusively in the RV and was paralleled by the characteristic changes in MHC-isoform expression and reduction of SERCA2 protein level. D3 degrades T₃ to inactive T₂ and the observed up-regulation would therefore provide a mechanism for reduction of intracellular T₃ levels. D3 is in many tissues expressed at higher levels in the fetal stage, including the human heart. Since cardiac growth is generally associated with at least a partial re-induction of the fetal gene program, it is perhaps not too surprising to find increased expression of this particular deiodinase. Whether this

results in an actual decrease in cardiac T₃ levels remains to be established. In any case, induction of D3 activity adds an interesting new twist to the tale of impaired TH signaling in pathological hypertrophy and it may be the cause of a true hypothyroid state of the cardiomyocyte.

What next?

Obviously, the complexities of TH-signaling in pathological cardiac hypertrophy are far from being unraveled. Based on the current data it may be speculated that the expression of genes that are involved in cardiac TH action, e.g., deiodinases and receptors, is altered as part of the response of the cardiomyocyte to chronic stress. This aspect of the response need not be beneficial under these circumstances, but may be an inescapable part of a growth program. As a result, the impaired signaling of TH may itself become a factor in driving some of the changes in gene expression in pathological hypertrophy, resulting in a hypothyroid-like phenotype. On top of that, as the severity of cardiac dysfunction progresses, reduction of serum TH levels may further aggravate the hypothyroid state of the cardiomyocyte. Since most studies in man and animal models have dealt with conditions of advanced hypertrophy and overt failure, detailed studies of the time course of changes in TH signaling and cardiac gene expression are needed first to establish if, and at what stage, TH might play a role in the pathogenesis of heart failure.

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THYROID AUTOIMMUNITY AND PREGNANCY OUTCOME

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Introduction

Several disorders that are historically associated with pregnancy loss - such as collagen vascular diseases (most notably systemic lupus erythematosus), chronic active hepatitis, inflammatory bowel disease, diabetes mellitus and thyroid disease - are autoimmune disorders (1). A number of studies have specifically addressed the relationship between thyroid autoimmunity and spontaneous pregnancy loss.

Autoimmune hypo- and hyperthyroidism also increase the risk for obstetrical complications. It appears that low birth weight; prematurity and eclampsia are associated with the severity of the thyroid dysfunction (2-5).

Thyroid failure (6) - and even low normal free thyroxine (fT4) levels! (7) - in early pregnancy are associated with impaired neuropsychological development. Considering the fact that thyroid autoimmunity often leads to the gradual development of permanent thyroid failure it becomes clear that the presence of thyroid autoimmunity has important repercussions on clinical practice in women of childbearing age (8).

In conclusion, there is accumulating evidence that thyroid autoimmunity is associated - causally or as an epiphenomenon - with adverse pregnancy outcomes. In this comment we will discuss some aspects of thyroid autoimmunity and pregnancy outcome. For an oversight of the factors that are involved in the pathogenesis of autoimmune thyroiditis, especially in relation to pregnancy the reader is referred a recent review (9).

Thyroid autoimmunity and pregnancy loss

Spontaneous pregnancy loss is common. It has been shown that the total rate of spontaneous pregnancy loss is 31% (10). An increasingly recognized factor in the aetiology of pregnancy loss is the presence of autoantibodies (11).

How might autoimmunity influence miscarriage? An extensive discussion of this issue is beyond the scope of this comment, for this the reader is referred to some excellent reviews (12;13). However, several points are worth mentioning at this point. Since the fetus expresses paternal MHC molecules adaptation of the maternal immune system is essential for a successful pregnancy. Such adaptation is accomplished through an increased production of cytokines that have immunosuppressive capacities. These cytokines are produced by so-called (CD4+) T-helper 2 (TH2) lymphocytes. Another subset of T cells exists that is also CD4+, these so-called T-helper 1 (TH1) lymphocytes are - unlike TH2 cells - well equipped to stimulate the cytotoxic and cytolytic arm of the cell mediated immune system (e.g. to activate macrophages and Natural Killer cells) via TH1 cytokines to kill target cells. During successful pregnancy there is a TH1 to TH2 shift characterized by down-regulation of the TH1 mediated effector arms of the immune system, concomitantly there is increased immunoglobulin production (9).

Interestingly, experimentally induced changes in the TH1 / TH2 balance during pregnancy can induce increased rates of miscarriage (14). From these data it is clear that an adequate adaptation of the immune system is of paramount importance for a successful pregnancy to occur.

Stagnaro-Green et al. studied 552 consecutive women in the first trimester of pregnancy and found that the presence of thyroid peroxidase (TPO) and/or thyroglobulin (Tg) antibodies in the first trimester of pregnancy is a risk factor for spontaneous fetal loss (17% vs. 8.4% in controls) (15). These results were confirmed by Glinioer et al. who found a higher rate of spontaneous abortion in 45 women with thyroid autoantibodies compared to 603 controls: 13.3% vs. 3.3% (16).

In a prospective study of 54 women who conceived after in vitro fertilization (IVF) we were unable to

find a significant association between the spontaneous abortion rate and the presence of TPO antibodies before pregnancy. Although miscarriages occurred in 33% of TPO antibody positive women and in only 19% of the TPO antibody negative women, the difference was not statistically significant (17). Our results thus contradict those of the two studies mentioned above and several biases can be proposed to explain this discrepancy (18). Firstly, the prevalence of thyroid autoimmunity was low in our pregnant women and if present the severity of the thyroid autoimmune process was mild. Secondly, we determined TPO antibodies before pregnancy, while in the other studies antibodies were determined during pregnancy. In view of the immunologic changes that occur during pregnancy (see ref. 7 for review) these differences in study design have probably led to inclusion of women with less severe forms of thyroid autoimmunity which might - at least in part - explain the discrepancy. In women with a history of habitual abortion the presence of non-organ specific autoantibodies, notably of antiphospholipid and anticardiolipin antibodies, has been associated with fetal loss (19). Data on the relationship between thyroid autoantibodies and habitual abortion are conflicting. Several studies found an association between TPO antibodies and recurrent first-trimester fetal loss (20-24). However, others could not confirm this observation (25;26). Interestingly, Vaquero et al. have recently investigated the role of mild thyroid abnormalities in women with thyroid antibodies and recurrent first trimester abortions (27). In this small study the authors showed that treatment with thyroid hormone was more effective than treatment with intravenous immunoglobulins. These data might be taken to suggest that mild degrees of thyroid insufficiency and not thyroid autoimmunity per se - is causal in the association between the presence of thyroid antibodies and recurrent abortion (27;28). In conclusion there are presently sufficient data showing an association of thyroid autoimmunity in early pregnancy and subsequent 'incidental' miscarriage. However, the data on the presence of thyroid antibodies and recurrent abortion are conflicting. Most likely, 'incidental' and recurrent abortion represent distinctive entities.

Autoimmune thyroid dysfunction and obstetrical complications

Despite the association between decreased fertility and hypothyroidism 2-2.5% of pregnant women have elevated TSH levels (29-31). Hyperthyroidism in pregnancy is less often encountered: approximately only in 1 of 1000-2000 pregnancies (2;3;32)

When women with hypothyroidism do become pregnant it appears that the most prevalent disorder is pregnancy induced hypertension (33-36). Other complications that have been described in some but not all studies are placenta abruptio, postpartum haemorrhage, stillbirths, low birth weight and significant anaemia (33;35;36). Considering treatment it is important to note that thyroid hormone replacement with T4 significantly improves - but not diminishes - the excess risk of obstetrical complications (33).

Hyperthyroidism during gestation is also associated with obstetrical complications such as low birth weight, prematurity and eclampsia (2). It is outside the scope of this editorial to discuss the treatment of hyperthyroidism during pregnancy, for this purpose the reader is referred to some excellent recent overviews (2;33).

Thyroid autoimmunity and offspring

Several studies have shown that the presence of thyroid antibodies is a powerful risk marker for the transition from subclinical to overt hypothyroidism (8;37). Indeed, women with thyroid antibodies are at risk of becoming hypothyroid during pregnancy with its increased demand for thyroid hormones (34). In a recent study by Pop et al. it has been shown that children born to women with maternal serum fT4 levels below the 10th percentile at 12 weeks gestation (irrespective of elevation of TSH and/or presence of TPO antibodies), had significantly lower neurodevelopmental scores compared to children of mothers with higher fT4 values. It is important to note that women with low fT4 levels at 12 weeks gestation were largely affected by autoimmune thyroiditis. However, there was no correlation between neurodevelopmental scores of the infants and maternal fT4 at 32 weeks gestation which is a puzzling finding in view of the expected deterioration in thyroid function during pregnancy in women with autoimmune thyroiditis (7;34). Whatever the explanation for this unexpected finding the fact remains that after appropriate statistical analysis fT4 levels below the 10th percentile at 12 weeks gestation represented a significant risk factor for impaired psychomotor development.

Haddow et al. have extended the findings of Pop et al. by (6). These investigators provided evidence that children born to mothers with hypothyroidism during the second trimester of pregnancy, as determined by an elevated TSH, have lower IQ-scores and more educational difficulties at age 7-9 than

children born to mothers with normal TSH levels during pregnancy. In their study 25,216 serum samples were prospectively collected and 47 women with TSH-levels at or above the 99th percentile of the values for all pregnant women were identified. Additionally 15 women with TSH values between the 98th and 99.6th percentiles, and low thyroxine levels were also included, as were 124 matched controls. The children of the 62 women with elevated TSH levels during pregnancy performed less well on all 15 neuropsychological tests carried out (in 2 of these the difference was significant), and children had more school difficulties and learning problems ($p=0.06$). In this study 77% of the women with hypothyroidism had high titres of TPO antibodies (6). These data further underline the notion that chronic autoimmune thyroiditis is the most frequent cause of low normal fT4 levels and raised TSH levels in these women. Taken together, the studies by Pop et al. and Haddow et al. provide evidence that not only overt but also relatively mild and hitherto unrecognised states of thyroid failure are associated with persistent and significant impairment in neuropsychological performance of the offspring.

Concluding remarks and consequences for clinical practice

Thyroid autoimmunity with its impaired thyroid reserve has important consequences on pregnancy outcome (table 1).

Table: key messages

Thyroid autoimmunity and recurrent pregnancy loss --> consider treatment with L-thyroxine
Thyroid autoimmune dysfunction during pregnancy --> hypothyroidism should be treated with full replacement dose immediately --> hyperthyroidism should be treated according to fT4 levels
Thyroid autoimmunity and offspring --> check for overt hypothyroidism and act accordingly. No data are available on the effects of LT4 in cases of mild/subclinical thyroid failure.

Thyroid autoimmunity and pregnancy loss: As there are now data to suggest that recurrent miscarriage in women with thyroid antibodies can be prevented by thyroxine administration (27), we are of the opinion that in women with recurrent miscarriage and thyroid antibodies treatment with L-thyroxine should be considered, though further controlled studies are essential.

Autoimmune thyroid dysfunction and obstetrical complications: As the risk to mother and child seems to be correlated with the severity of the thyroid dysfunction it is clear that - depending on the fT4 level at presentation - treatment should be instituted immediately. Thus, when a woman is diagnosed with hypothyroidism during pregnancy full replacement with thyroxine (1.6µg/kg ideal body weight) should be started immediately. In view of the expected increase in thyroxine requirement during gestation regular clinical and laboratory follow-up is essential, with periodic determinations of TSH and free T4 concentrations (30). Women diagnosed with hyperthyroidism during pregnancy should be treated with antithyroid drugs exclusively, aiming at a fT4 at - or slightly above - the upper limit of normal (33)

Thyroid autoimmunity and offspring: In a recent publication Morreale de Escobar et al. have summarized and discussed epidemiological and experimental evidence and - convincingly - argued that conditions resulting in first trimester hypothyroxinaemia (defined as a low for gestational age circulating maternal free T4, whether or not TSH is increased) pose an increased risk for poor neuropsychological development of the fetus (38). It is at present unknown if thyroxine replacement therapy will effectively prevent detrimental effects on the offspring in these cases. Clearly, double blind randomised trails are needed to clarify this issue (39).

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THYROID HORMONE AND DEPRESSION

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Introduction

One of the common symptoms of hypothyroidism is a depressed mood. However, most patients with major depression are biochemically euthyroid. On closer examination, a number of subtle abnormalities in the hypothalamus-pituitary-thyroid (HPT) axis occur in a large proportion of depressed patients, including alterations in serum concentrations of thyroid hormones and TSH. In addition, a number of clinical studies have suggested a therapeutic role for thyroid hormone co-medication in patients with depression who are biochemically euthyroid. The lack of insight into the pathogenesis of the neuroendocrine changes in the HPT axis, together with the uncertainty about a possible role for thyroid hormone treatment in depression have made the field somewhat controversial. In this paper, the need for larger and well-designed clinical studies on a possible role for T3 in the treatment of depression is discussed, while a hypothesis is put forward on the pathogenesis of HPT axis changes in major depression.

The hypothalamus-pituitary-thyroid (HPT) axis in depression.

There is abundant evidence that patients with major depression exhibit changes in the activity of both the hypothalamus-pituitary-thyroid (HPT) axis and the hypothalamus-pituitary-adrenal (HPA) axis. Serum T4 levels above the reference range have been reported in approximately 25% of the patients, with kinetic studies pointing to an increased daily production rate of T4 (1). Serum T3 is often normal, but may be decreased in a proportion of patients. Since the daily thyroid production rate of T3 was reported to be normal, a reduction in the deiodination of T4 into T3 in extrathyroidal compartments may be involved. The cause of increased T4 production is unknown at present. Serum TSH is low, but mostly within the normal range. The diurnal variation of serum TSH is attenuated, with a decreased nocturnal surge in untreated patients (1). Apparently, increased serum T4 is not the consequence of increased stimulation by TSH. Recently, Kalsbeek et al (2) reported sympathetic and parasympathetic innervation of the rat thyroid gland via multisynaptic autonomic pathways from the hypothalamus.

Some of these autonomic neurons in the PVN were TRH-immunoreactive. Therefore, autonomic activation which may occur in a subset of patients with depression is a possible mechanism for non-endocrine stimulation of the thyroid.

The attenuation of the diurnal TSH rhythm in major depression suggests changes in the hypothalamic regulation of TSH secretion, since the hypothalamic suprachiasmatic nucleus (SCN) generates the diurnal variations in serum thyroid hormones (2). Both increased and unaltered CSF levels of TRH have been reported in depression, with a lack of correlation between CSF TRH levels and TRH-stimulated serum TSH (3,4). This discrepancy may result from the fact that only a small proportion of TRH neurons is involved in the neuroendocrine regulation of serum thyroid hormones. In the hypothalamic PVN of patients with major depression we recently found decreased TRH mRNA as assessed by quantitative mRNA in situ hybridisation (5) which may contribute to decreased serum TSH in a subgroup of patients.

Apart from neuroendocrine changes in the HPT axis of patients with major depression, various authors have pointed to immunological changes related to the HPT axis in this patient group. Especially in patients with bipolar depression, the prevalence of thyroid peroxidase autoantibodies (TPO antibodies) is increased which may explain the association between bipolar disorder and hypothyroidism. Recently, the presence of TPO antibodies during gestation were found to be an independent marker for subsequent depression postpartum (15).

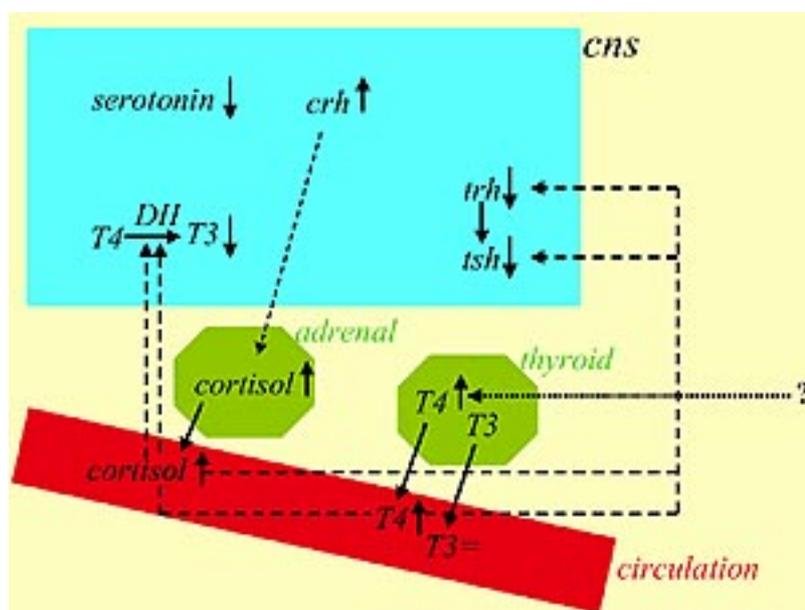
Thyroid hormones in the treatment of depression

Although major depression in itself is not associated with primary hypothyroidism as is apparent from decreased serum TSH and high serum T4, a number of studies have focussed on improving efficacy of treatment with antidepressants, mainly tricyclic antidepressants (TCA) by co-administration of thyroid hormone. The rationale for this type of research may have originated from the similarities between mental changes in hypothyroidism and depression. Early studies with TRH have been largely inconsistent and the pharmacokinetics of orally administered TRH-like peptides may have been an important source of variance. One double-blind crossover study reported strong and positive effects of intrathecal TRH in refractory depressed patients (6), but the number of patients was rather small.

The majority of studies addressing the use of thyroid hormones in depression have involved T3 and, less frequently, T4 (7). T3 monotherapy in depressed patients has been the subject of only two studies lacking a placebo group (1). Most studies have focussed on the question whether co-medication of antidepressants with thyroid hormone increases efficacy of TCA. A meta-analysis on the use of T3 co-

medication in the treatment of depression showed that T3 may indeed increase response rate and decrease depression scores in a subgroup of patients refractory to TCA (8). The authors concluded that there is a clear need for larger placebo-controlled studies involving not only TCA but also selective serotonin reuptake inhibitors (SSRI). Interestingly, a recent review and meta-analysis (9) supported the efficacy of T3 in accelerating clinical response to TCA in patients with nonrefractory depression. This might be a partial answer to the problem of the delayed onset of therapeutic response to antidepressants. If indeed T3 increases efficacy of treatment with SSRI, it would be very important to identify those patients that are likely to respond to T3 co-medication rather than to SSRI alone.

Pathogenesis



Legend to figure

A schematic representation of observed and hypothesized changes in HPT- and HPA axis regulation in major depression

Key events:

1) decreased serotonin and 2) increased CRH

Consequences: increased CRH results in hypercortisolism, leading to a) inhibition of D2, thereby decreasing intracerebral T3 which reinforces decreased serotonin, and to b) decreased hypothalamic TRH and inhibition of TSH release. The model links HPA and HPT axis changes to serotonin as a key player in the pathogenesis of depression

Criticism: increased T4 is unexplained. Activation of the autonomic innervation of the thyroid may possibly explain the dissociation between TSH and thyroid hormones

A number of authors have suggested that in major depression, the bioavailability in the central nervous system (CNS) of the biologically active thyroid hormone T3 may be decreased in the context of systemic euthyroidism (7). This is certainly an attractive idea since it may explain that T3 co-medication increases efficacy of TCA in some, but not all patients. Unfortunately, no data are present to directly support this hypothesis. One might assume an important role for the enzymes that play a key role in the regulation of the concentration of T3 in the CNS, i.e., the iodothyronine deiodinases. Specifically, type 2 deiodinase (D2) is important for deiodination of T4 to T3 in the brain, and therefore for the production of T3. Type 3 deiodinase (D3) is important for deiodination and, therefore, inactivation of T3 to T2. Interestingly, a number of different classes of antidepressants (lithium, TCA, and SSRI) enhance D2 activity and decrease D3 activity in rat brain, both resulting in increased local T3 concentration (for review see 1). For example, the SSRI fluoxetine was shown to enhance D2 activity and to inhibit D3 activity in rat cortex, limbic forebrain and striatum, matching areas with high 5-HT₂ receptor density (10). It should be noted, however, that various pharmacological and nonpharmacological treatments affect D2 and D3 activities in a highly treatment-specific and region-specific way (11). That a local increase of the concentration of T3 may affect serotonergic neurotransmission was strongly suggested by pharmacological experiments showing increased serotonin concentrations in the cerebral cortex of rats acutely or chronically treated with T3 (12). Therefore, T3 seems to enhance serotonergic neurotransmission and vice versa.

If indeed decreased D2 activity in the CNS of a subset of patients with major depression contributes to decreased bioavailability of T3 in the brain which may be reversed to some extent by antidepressants, the question is what the cause of decreased D2 activity in depression might be. One possible explanation is the mild hypercortisolism that occurs in some 40% of these patients (13), while increased serum T4 may also contribute. Glucocorticoids inhibit D2 activity in cultured human placental cells (14). No data are available on in vivo effects of cortisol on D2 activity in the hypothalamus.

Conclusion and hypothesis

Subtle changes occur in the hypothalamus-pituitary-thyroid axis in approximately 25% of patients with major depression. Consistent changes are high or increased serum T4, normal serum T3 and low, or decreased serum TSH with an attenuated diurnal variation. The pathogenesis of these changes is unclear, but may involve both neuroendocrine changes (activation of the hypothalamus-pituitary-adrenal axis) and neural mechanisms (activation of the autonomic innervation of the thyroid gland, functional changes in the hypothalamic SCN). At the level of the CNS, depression may involve

decreased bioavailability of T3 in a subset of patients, which may reinforce decreased cortical serotonin levels. SSRI may not only facilitate serotonergic neurotransmission but also enhance T3 production via an effect on D2 activity. Co-medication with T3 appears to increase efficacy and to accelerate response of TCA, but larger studies involving SSRI are needed. The identification of patients who are likely to benefit from thyroid hormone comedication would be important in view of the high prevalence of major depression and the relatively high rate of nonresponders to antidepressants (30-40%).

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ENDOCYTOSIS OF THYROGLOBULIN : STEPS TOWARDS AN INTEGRATED VIEW OF A COMPLEX PHENOMENON

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Over the last decade, there has been substantial improvement in the knowledge of the cellular and molecular mechanisms governing the first steps of the thyroid hormone secretory pathway, i.e. the internalization or endocytosis and intracellular transport of the prohormone, thyroglobulin (Tg). Advances lie in the progressive disappearance of dogmas and the input of convincing data either invalidating or supporting rather old hypotheses.

Thyroid hormone production from the precursor glycoprotein, Tg is based on the morpho-functional organization of polarized epithelial thyroid cells into follicle structures delimiting an internal compartment, the follicle lumen. Tg molecules secreted by thyrocytes into the lumen of thyroid follicles undergo, at the thyrocyte-lumen interface, unique post-translational modifications (iodination and iodotyrosyl residue coupling reactions) leading to the formation of hormone residues within their polypeptide chains.

Depending on numerous factors - including the supply of iodide as substrate, the activity of enzymes (thyroid peroxidase, TPO, and thyroid oxidase, ThOX) catalyzing hormone formation, the concentration and physico-chemical state of Tg - the hormone content of luminal Tg molecules varies to a rather large extent. Tg molecules newly arrived in the follicle lumen with no or a low hormone content would co-exist with "older" Tg exhibiting up to 6-8 hormone residues. The downstream processes responsible for the production of free thyroid hormones from these prohormonal molecules must adequately manage the use of these luminal heterogeneous Tg stores to provide appropriate amounts of hormones for peripheral utilization. One would expect to find control systems preventing excess hormone production that would result from the processing of excessive amounts of prohormonal Tg molecules and checking systems avoiding the use of Tg molecules with no or a low hormone content. The way the thyroid follicle proceeds to generate free hormones from stored hormone containing Tg molecules has been known for a long time. Tg molecules are taken up by polarized thyrocytes and then conveyed to lysosomal compartments for proteolytic changes that release T4 and T3 from their peptide linkages. There is an abundant literature on these two steps, especially on the first one, which represents the limiting point in the thyroid hormone secretory pathway. By contrast, the final step, i.e. the "transfer" of free T4 and free T3 from the intracellular compartments, in which they are generated, to the extracellular space, has never been really studied and at present, there is no satisfactory explanation for the transmembrane passage of these molecules that exhibit hydrophobic but also hydrophilic characteristics owing to their head and tail (alanine side chain) charges.

The recent evolution in the knowledge of Tg endocytosis has first been to consider that it could proceed via a mechanism different from phagocytosis, also named macropinocytosis, evidenced in rats under acute TSH stimulation (reviewed in 1). Results from studies performed in rats have been for a long time and are still in some instances extrapolated to the different animal species. Cellular events characterizing macropinocytosis i.e. apical membrane extensions or pseudopods and resorption vacuoles or colloid droplets are not or rarely observed in species other than rat. There is now a number of experimental data showing that in the thyroid of different species, internalization of Tg, mainly if not exclusively, occurs via vesicle-mediated endocytosis or micropinocytosis (reviewed in 2), an ubiquitous cellular process accounting for macromolecule internalization by all cell types.

A tentative explanation for the implication of macropinocytosis in rats

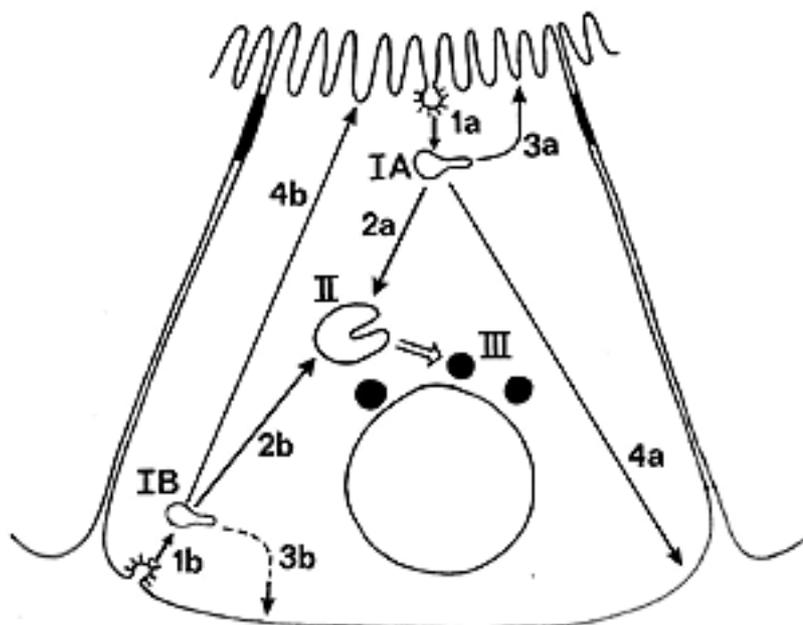
We have compared the Tg utilization rates required for a normal T4 production in rats and human to try to find an explanation for the involvement of phagocytosis / macropinocytosis in Tg endocytosis in rats. Considering that the T4 production rate is 85mg/day/15g thyroid tissue in human and about 3 λ g/day/15 mg tissue for a rat weighing 250g, it appears that the production rate of T4 in λ g/day/g tissue is about 5

in human but 200 in rats. As under normal iodide supply, the average hormone content of Tg should be similar in both species (about 2 T4 residues/molecule), it results that the amount of Tg degraded per day per g tissue should be close to 2 mg in human and 40-50 times higher in rats.

From the general literature on endocytic vesicles and data deriving from studies of thyroid micropinocytosis, one can estimate that endocytic vesicles with an internal diameter of 50nm would contain at least 10 Tg molecules if, as very likely (see below), Tg molecules enter these vesicles at a concentration of about 200 mg/ml, representing the intraluminal Tg concentration. Using the above mentioned values for the amount of Tg processed per day per g. tissue and assuming that the rat and human thyroids are composed of about 10^7 (3) and 10^{10} cells respectively, we can calculate that, in the human gland, the internalization of Tg would require a flux of about 200 vesicles per cell per min. This value is fully compatible with the established values in cell types as different as polarized kidney cells (4) or fibroblasts (5) which can form up to 1000 vesicles per min.. In contrast, the vesicle flux that would be needed in rats overrun by far this value. Thus, the vesicle-mediated endocytic process, probably also operative in rats, would not have the capacity to internalize Tg in sufficient amounts to sustain the T4 production required for homeostasis in rats. Although speculative, these calculations give groundings for the implication, in the rat, of an additional endocytic process with a high capacity such as phagocytosis.

Steps and cellular compartments involved in Tg endocytosis by micropinocytosis

The internalization process starts with the organization of microdomains at the apical plasma membrane of thyrocytes ; these microdomains or pits, resulting from the recruitment and assembly of proteins (clathrin , adaptins...) on the cytoplasmic side of the membrane, invaginate to finally generate coated vesicles after membrane fission. Luminal Tg molecules, either free or associated to membrane proteins acting as Tg receptors, enter the pits and are then sequestered into the newly-formed vesicles (6-8). The vesicles lose their coat and, through a complex fusion process, deliver their content into a first type of endocytic compartments, **the early apical endosomes** (see Figure). In these compartments, Tg molecules probably undergo sorting on the basis of recognition of different physico-chemical parameters either linked or independent such as the hormone content, exposed carbohydrates, conformation of peptide domains... A step of sorting appears as a prerequisite for subsequent differential cellular handling of Tg molecules. Indeed, it has been shown that internalized Tg molecules can follow different intracellular pathways. Part of Tg molecules are conveyed via a vesicle transport system to the second type of endocytic compartments, **late endosomes or prelysosomes** (see Figure).



Endocytosis and Transcytosis in the Thyroid Follicle.

IA : Early apical endosome	1-Initial phase of endocytosis
IB : Early basolateral endosome	2-Late phase of endocytosis
II : Late endosome	3-Recycling
III : Lysosome	4-Transcytosis
	a: processes initiated at the apical membrane
	b: processes initiated at the basolateral membrane

This route ending to lysosomes corresponds to the Tg degradation pathway for the generation of free thyroid hormones. It is reasonable to think that Tg molecules following this route are the more mature molecules (with a high hormone content) but, this has not been firmly demonstrated. The other Tg molecules present in early apical endosomes enter either of the following two routes; they are recycled back into the follicle lumen through a direct vesicular transport towards the apical plasma membrane (9) or via a two-step vesicular transport to the Golgi apparatus and then to the apical plasma membrane (10). Alternately, Tg molecules are transported and released at the basolateral membrane domain of thyrocytes via transcytotic vesicles (11); a process accounting for the presence of Tg in plasma (12-14). The orientation of Tg molecules towards one or the other of these three routes requires the presence of receptors. However, at least one intracellular pathway could simply convey Tg molecules that are not selected for entering other routes.

Receptors involved in Tg endocytosis

Receptors may operate at the apical plasma membrane for Tg internalization and downstream in apical early endosomes for Tg sorting. The requirement and/or the involvement of apical cell surface receptors has long been debated. Most investigators now recognize that receptors are not needed for internalization since Tg is present at a high concentration at the site of vesicle formation. So, Tg molecules are most likely internalized by fluid-phase endocytosis and not by receptor-mediated endocytosis. On the contrary, if apical membrane Tg receptors exist, their function would be to prevent the internalization of sub-classes of Tg molecules (15,16). As it is not conceivable that internalized Tg molecules could enter the different intracellular routes, described above, at random, Tg receptors must exist in early apical endosomes. A detailed review on potential Tg receptors has recently been made by Marino and Mc Cluskey (2).

The first candidate receptor, initially described by Consiglio et al.(17,18), was later identified as the asialoglycoprotein receptor composed of three subunits (RLH1,2 and 3). This receptor binds Tg at acidic pH and recognizes both sugar moieties and peptide determinants on Tg (19). As low-iodinated Tg molecules are known to have a low sialic acid content, this receptor could be involved in sorting immature Tg molecules for recycling to the follicle lumen. A second receptor, still not identified, named N-acetylglucosamine receptor (20), presumably located in sub-apical compartments, interacts with Tg at acidic pH; it could also act as a receptor for recycling immature Tg molecules back to the follicle lumen. A third receptor; Megalin, has recently been discovered in the thyroid and has been the subject of extensive studies yielding convincing data(2,21-23). Megalin is an ubiquitous membrane protein belonging to the LDL receptor family. It is located in the apical region of thyrocytes and its expression is regulated by TSH. Megalin, that binds multiple unrelated ligands, interacts with Tg with a high affinity. In vitro and in vivo data indicate that Megalin is involved in the transcellular transport or transcytosis of Tg molecules.

From the properties and subcellular location of these receptors, one can propose an integrated view of the sorting processes that would operate in early apical endosomes. The asialoglycoprotein receptor and the less defined N-acetylglucosamine receptor would recognize immature Tg for recycling and Megalin would interact with Tg subjected to apical to basolateral transcytosis. The remaining Tg molecules would enter, without sorting, the functionally important pathway i.e. the prelysosome-lysosome route.

Connections between Apical and Basolateral Endocytosis

The capacity of thyrocytes to internalize macromolecules is not restricted to the apical plasma membrane domain. Indeed, endocytosis of different proteins including Tg and serum albumin also occurs at the basolateral plasma membrane domain. Internalized molecules first enter early endocytic compartments, the basolateral early endosomes (24). It has been demonstrated that internalized proteins either reach late endosomes and lysosomes or undergo a transcellular transport into the follicle lumen by basolateral to apical transcytosis. As found in other cell types, thyroid late endosomes correspond to compartments connecting apical and basolateral endocytic pathways. Basolateral endocytosis and basolateral to apical transcytosis represent the route of entry of extrathyroidal proteins such as plasma proteins found into the lumen of thyroid follicles (25).

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HEREDITY AND ENVIRONMENT IN THE ETIOLOGY OF GRAVES' DISEASE.

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Introduction

Graves' disease (GD) is an organ-specific autoimmune thyroid disorder characterized (with rare exceptions) by hyperthyroidism, various degrees of diffuse goitre (30% lack goitre), ophthalmopathy (clinically evident in the minority) and rarely pretibial myxedema. From this, it is already clear that GD covers a wide range of phenotypes. The hyperthyroidism in GD is due to the presence of antibodies binding to and stimulating the thyrotropin receptor. The etiology of GD is multifactorial with clinical disease developing on the basis of genetic susceptibility interacting with environmental factors (1, 2, 3) (Fig. 1).

Affecting 0.5 to 1% of the population with a 5-10:1 female-to-male preponderance, GD is one of the most common autoimmune disorders. Despite a vast number of studies of the genetics of GD the reasons why Graves' disease develop in certain individuals are unknown. In fact, no single gene or genetic marker has been shown to be either necessary or sufficient for the development of clinically overt GD (3). The same is certainly true for any suggested environmental factor (4).

In the following we will briefly highlight the current perception regarding the relative importance of genetic and environmental factors in the etiology of GD.

Is there a genetic component λ

A family history of thyroid disease can be obtained in up to 50% of patients with GD and family studies have repeatedly demonstrated a familial aggregation of GD (2). By assuming that the population prevalence at large equals the cumulative lifetime risk, it is possible to make a rough estimate of the increased risk of disease for a relative given the presence of disease in the proband. With the background population frequency of 0.4-1.1% this implies that a sister has a 5.4-12.6 times increased risk of experiencing GD when her sibling already has the disease. For a brother the increased risk ranges from 1.2-7.4 times. This value for familial clustering is named lambda (λ). Whether this familial clustering is due to shared environment or a shared genetic predisposition can only be investigated in twins. Based on the assumption that the intrapair difference in environment is the same for MZ and DZ twins the fact that concordance rates have unanimously been found higher in MZ than in DZ twins can be taken as evidence of a genetic contribution to its etiology. In our two recent population based twin studies the probandwise concordance rates were 36% and 35% in MZ pairs versus 0% and 7% in DZ pairs, respectively, (5, 6). Our findings are supported by preliminary results from a population based twin study from California (7). The fact that the concordance rates for GD among MZ twins were well below 100%, clearly suggest that environmental factors are also important.

Estimation of the genetic contribution in GD

Heritability, which is independent of the disease prevalence, is considered the best indicator of the magnitude of the genetic influence. Using structural equation modelling, decomposing individual

specific and shared genetic and environmental factors, we have found that 79% (95% CI 38-90%) of the liability to the development of GD is attributable to additive genetic factors. Individual specific environmental factors not shared by the twins explained the remaining 21% (95% CI 10-37%) (6). The above suggests that search for susceptibility genes could be worthwhile but gives no information on the number of possible genes.

Candidate genes in Graves' disease

The major histocompatibility complex region

Consistent associations between GD and the class II HLA allele DR3 on chromosome 6p21 have been reported in Caucasian populations since the 1970s (2). Case-control studies have shown an increased frequency in GD of D3B1*0304, DQB1*0201, DQB1*0301/4 and DQA1*0501. Strong linkage disequilibrium exists across the HLA region, therefore, it has generally been difficult to ascertain which allele exerts an independent effect due to lack of power (too small data sets). However, DQA1*0501 seems to be an independent HLA susceptibility locus conferring a relative risk of 2.5-3.8 (2, 3, 8). Population based case-control studies are sensitive to the detection of susceptibility loci exerting small effects. However, they lack specificity and an enormous number of subjects need to be investigated. To detect a gene conferring a relative risk for the development of GD of 2.0 a population of around 800 cases is needed to have an 80% power to achieve a result with a significance of $p < 0.001$ (3). Therefore, it has been difficult to provide evidence for linkage between the HLA region and GD (3). Case-control studies of gene polymorphisms in the HLA class III region, which contains many genes encoding immune regulator proteins (including some of the cytokines) have yielded contradictory results. But neither tumor necrosis factor-b (TNF-b) nor TNF-a seem to be major susceptibility genes in GD. Neither does transporters associated with antigen processing (2, 3 8). With a probandwise concordance rate of 30-40% in MZ twins and a 7-10% risk of GD in HLA identical siblings with an affected proband (2) it can be calculated that HLA at best has a moderate effect (around a fourth of the total genetic effect) and suggests that other genes contribute.

CTLA-4 and other non-HLA markers

The cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4), on chromosome 2q33, is a critical molecule in the activation of T-cells by antigens. CTLA-4 activation leads to T-cell suppression and the possible augmentation of immune responses and development of autoimmunity (3, 9). Several studies on CTLA-4 polymorphisms have reported weak but positive associations with GD in various ethnic groups including Caucasians, Japanese and Hong-Kong Chinese (9). The risk, however, is weak, the relative risk being < 3 and clearly non-specific for GD since this polymorphism is also seen in autoimmune hypothyroidism, type 1 diabetes mellitus and Addison's disease (3, 9). It is currently debated whether CTLA-4 polymorphisms are specifically associated with TAO. The fact that linkage of CTLA-4 with GD has been reported, that this linkage becomes stronger when AITD families (not just GD) are included, as well as linkage of the CTLA-4 region with thyroid antibody production, suggests that CTLA-4 is an important susceptibility gene for the development of autoimmunity in general (3, 9). A vast number of non-HLA markers have been investigated in association studies. These include the immunoglobulin heavy chain region (IgH), the T-cell receptor, the interleukin-1-receptor antagonist (IL-1-RA), the TSH-receptor gene (TSH-R), the thyroid peroxidase gene (TPO), the thyroglobulin gene (Tg), the insulin-dependent diabetes mellitus 2 gene (IDDM2), the large multifunctional proteasome genes (LMP2 and LMP7), the IL-4 promotor region, IL-1a, IL-1b, IL-4 receptor, IL-6, IL-10, transforming growth factor-b (TGF-b), estrogen receptor b gene (ER-b), vitamin D receptor gene (VDR) and autoimmune regulator 1 gene (AIRE 1). The literature reports of conflicting findings most likely due to inadequate size and poorly matched cases and controls. More important, no confirmation from family based studies (e.g. linkage) is available.

Genetic markers showing evidence of linkage

Recently, Davies and colleagues have identified new loci including GD-1, GD-2 and GD-3. The GD-1 locus on chromosome 14q31 is located within 2cM of the multinodular goitre-1-gene (MNG-1) raising the possibility that they are in fact the same, conferring susceptibility to both disorders (10). This region is interesting since it also contains other thyroid autoimmunity candidate genes, including the genes for TSH-R, IgH, T-cell receptor α , IDDM11 and ER-b. However, no linkage has been demonstrated at any

of these loci.

Using the same 53 multiplex multigenerational families the same group have identified GD-2 (chromosome 20q11.2 (11). Interesting genes such as interleukin-6 nuclear factor (NF-IL6) map to this region. This gene encodes a transcription protein that binds to several regulatory regions of other cytokine genes.

Linkage has also been reported on the X-chromosome (Xq21.33-22) (GD-3) possibly explaining the female predisposition to GD (12). In recent UK studies GD-2 was linked to GD while GD-1 and GD-3 were not (13). The finding of a susceptibility locus on chromosome 18q21 awaits confirmation (14).

Is there an environmental component?

The following facts point towards the importance of environmental factors in the etiology of GD: 1) Even with more than 25 years of follow-up the probandwise concordance rates for GD are no higher than 30-40% in MZ twins (2, 6). 2) Specific candidate genes associated with GD are also present in a high proportion of healthy subjects (3). 3) There is a considerable regional variation in the prevalence of GD (15).

If so, how large is its effect?

In the only available study of heritability of GD, using two large population based twin cohorts, we have estimated that 79% of the liability to develop GD is attributable to additive genetic factors (heritability) whereas environmental factors explain the remaining 21% (6). It is, however, important to point out that none of the two components cause disease alone.

Can the environmental factors be identified?

A number of environmental factors have been associated with the development of GD. Much as is the case with genetic markers (4). Is it possible to distinguish causal from non-causal associations? Not with certainty. However, when attempting this, it is generally accepted that strength, consistency, specificity, temporality, dose-response, and biological plausibility of an association should be considered (16). In the following we will summarize our interpretation of the existing evidence for or against a causal relationship between specific environmental exposures and GD.

Iodine

Iodine is essential for the biosynthesis of thyroid hormone and influences thyroid growth and function. Perhaps the best evidence that we have for iodine affecting autoimmune thyroid disease comes from the study of animal models. Although the processes are complex, in general, iodine deficiency attenuates, while iodine excess accelerates autoimmunity in predisposed individuals (17). In humans, epidemiological surveys have over and over demonstrated that differences in prevalence and/or incidence of overt GD in different parts of the world closely mimic the magnitude of the iodine intake, with GD being more prevalent in areas with the highest iodine intake (15, 18). Furthermore, increases in iodine intake in iodine deficient regions generally increases the incidence of GD (19).

The course of GD is also affected by iodine. Thus patients given iodine supplementation after discontinuing antithyroid drug therapy (ATD) are more likely to relapse than those not given iodine. Also the response to ATD in GD is more rapid and the dose required to control the disease is smaller in iodine deficient areas than in iodine replete areas (20). Furthermore, remission rate in GD is inversely related to level of iodine intake as evidenced, at least in part, by the differences in remission rates between the US and Europe. Recurrence rate following thyroidectomy for GD is also related to level of iodine intake. The mechanisms are unclarified. However, the observations that iodine enhances the activity of lymphocytes that have been primed by thyroid-specific antigens, and the increase in TSHRab titres in relation to administration of excess iodine in GD patients are undoubtedly of importance.

Overall, the data - although the mechanisms remain to be clarified - show consistency, temporality, dose-response and the observations are biologically plausible. This suggests a causal relationship.

Smoking

It has long been recognized that smoking has a number of immunological effects involving both the humoral and cellular components of the immune response. As far as GD is concerned smoking has repeatedly been associated with an increased risk of developing GD and especially Graves' ophthalmopathy (TAO) (4). This is despite major differences in study designs, size of study populations, definitions of smokers and non-smokers, iodine intake, and methods for evaluating thyroid function and the degree of TAO. Since smoking, in a recent study (21), was - even after adjusting for stressful life events, daily hassles, social support and coping skills - still an independent risk factor for GD it is not likely that this association is due to confounding factors. It is worth noting that non-autoimmune hyperthyroidism has not been found associated with smoking (4).

Most studies including our own (4, 22) demonstrate a temporal relation with debut of GD 10 or more years after commencement of smoking. This goes for GD with or without TAO (23). A strong dose-response effect has been demonstrated with the relative risk for TAO increasing parallel to the current number of daily cigarettes smoked (23). In twins concordant for smoking but discordant for GD, the twins with GD smoked significantly more than their healthy co-twins (22) strengthening the evidence for a dose-response relationship. Whether giving up smoking is beneficial (in this context!) is still a matter of debate (23). As with iodine, smoking seems to affect treatment outcome in GD. The response to treatment of GD with or without TAO is better in non-smokers than in smokers (24).

The mechanisms by which smoking contribute to the development of GD, with or without TAO, in susceptible individuals is unclarified. However, the suggested mechanisms include: 1) A direct irritative effect of cigarette smoke. 2) Nicotine mediated stimulation of the sympathetic nervous system. 3) Alteration of the structure of the TSH receptor, making it more immunogenic and, 4) An influence on the cellular immune response, changing the profile of secreted cytokines.

From the above, it is evident that the association between smoking and GD (and especially TAO) is consistent, temporal, with a clear dose-response pattern and, finally, biologically plausible. These features strongly suggest a causal relationship.

Stressful life events

From the very first descriptions of GD stressful life events have been suggested in the etiology of GD (25). In a number of studies from e.g. Sweden, UK, Italy, Hong-Kong and Yugoslavia (25, 26), comparing cases and controls, largely similar findings of significantly higher negative life event scores in subjects with GD than in controls have been reported. Stressful life events were still strongly associated with GD in a recent Japanese study (21) where confounding factors such as daily hassles, social support, coping skills, smoking and drinking habits were adjusted for. When studied, the relative risk of GD increased as life event scores increased reflecting a dose-response relationship (21, 26). The major drawback of all the studies is that they are retrospective increasing their vulnerability especially to recall bias which to some extent is present in them all. Therefore, it is most difficult, if not impossible, to determine which came first the stress or the disease. The suggested mechanisms involve stress related stimulation of the hypothalamic-pituitary-adrenocortical axis ultimately leading to alterations in the profile of cytokine secretion.

Thus, while the association between stress and GD is consistent, strong, seemingly dose-dependent and biologically plausible, the lack of prospective data precludes firm conclusions as to the temporal sequence. Therefore, the question of causality remains unanswered.

Infections

It has long been recognized that infectious agents may induce thyroid autoimmunity by mechanisms such as inducing alterations/modifications of self-antigens, mimicking self-antigens, superantigen induced T-cell activation, and inducing expression of HLA molecules on thyroid cells, making the proposed causality biologically plausible (27)

Although Bech and colleagues found antibodies against *Yersinia enterocolitica* (serotype 3) significantly more often in subjects with GD than in controls (28) subsequent retrospective case-control studies have been contradictory (27). Thus, in spite of being biologically plausible the lack of

consistency, specificity and temporality, in addition to lack of prospective data suggests that there is no evidence for a causal relationship between *Yersinia enterocolitica* infection and GD.

Using the same arguments, no convincing evidence exists as to the vast number of other infectious agents, such as e.g. influenza B virus, retroviruses, human foamy virus, coxsackie B virus, and mycoplasma species, being of etiological importance in GD (27).

Other environmental factors

Certain drugs, in particular lithium (29) and amiodarone (30) have been suggested as possible environmental risk factors for GD. Based on the previous argumentation/criteria causality is not evidenced. Certainly, the same holds for alpha and beta interferon. Most recently, pulsed monoclonal antibody treatment of patients with multiple sclerosis has given exciting insight into the possibility of monoclonal antibodies against lymphocytes causing therapeutic modulation of the immune response, permitting the generation of antibody-mediated thyroid autoimmunity, in this case GD (31).

The possibility of an adverse intrauterine environment seems highly unlikely based on the findings in our recent population-based twin control study where no effect of any birth characteristic on the risk of developing GD could be demonstrated (32).

Conclusions

It is widely accepted that Graves' disease is a complex disease as is e.g. IDDM. That is, the clinical phenotype represents the net effect of all the contributing environmental, endogenous and genetic factors. It has been difficult to separate environmental influences from genetic susceptibility. Although the HLA and CTLA-4 gene region are well established susceptibility loci the magnitude of their contributions and the possible interaction with well established environmental factors such as dietary iodine intake and cigarette smoking is at large unclarified. Future studies should focus on adequate characterisation of phenotypes and control groups, better account of environmental factors and much larger cohorts. In addition to employing genome-wide linkage analysis and allelic association analysis of candidate genes (33, 34) it is imperative to study gene-environment interactions (35).

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ANIMAL MODELS OF GRAVES' AND THYROID EYE DISEASE.

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Graves' disease (GD) is a common autoimmune condition in which thyroid stimulating antibodies (TSAB) mimic the action of TSH (1). Since both the growth and function of the thyroid are controlled by TSH (2), TSAB lead to hyperthyroidism and diffuse goitre. The target of the autoimmune response in GD is the thyrotropin receptor (TSHR) (3). There are several lines of evidence suggesting that it may also provide a link between the thyroid and orbit resulting in thyroid eye disease (TED):- 1) Patients with TED tend to have the highest titres of TSAB (4); 2) TSHR transcripts have been demonstrated, using a variety of techniques, in the orbital adipose compartment (5,6); 3) Immunocytochemistry, with a spectrum of antibodies, suggests that the transcripts are translated into TSHR protein and that the receptors are also functional, albeit with high concentrations of TSH (7,8).

Despite recognising that TSAB cause GD and that GD and TED share a common autoantigen, very little progress has been made in understanding the mechanisms responsible for breaking immune tolerance. In other autoimmune diseases animal models have been invaluable in dissecting the immune mediators, even when the autoimmunity is induced rather than spontaneous. Models of GD and TED should be induced with the TSHR itself or TSHR primed T cells. Surprisingly, even though the science of autoimmunology began with the elegant demonstration that thyroiditis could be induced by injecting animals with autologous thyroid extracts and was subsequently found to be the result of autoimmune responses to thyroglobulin (9), TSHR induced models are a far more recent development. The delay was due to the scant TSHR expression in the thyroid, making it necessary to resort to recombinant methods to produce the quantities of receptor protein required by auto-immunization protocols. Most in vitro protein production methods, from synthetic peptides, through procaryotic expression in bacteria and eucaryotic expression in insect or mammalian cells, have been tried with varying degrees of success (reviewed in 10). I will review the models which come closest to reproducing the main features of GD and TED and begin by identifying what those might be:- 1) Elevated circulating thyroxine and/or suppressed TSH; 2) antibodies to the TSHR, at least thyrotropin binding inhibiting immunoglobulins (TBII) and preferably TSAB; 3) changes in thyroid architecture and size; 4) lymphocytic non-destructive thyroiditis; 5) clinical signs of hyperthyroidism such as weight loss; 6) female animals more susceptible than male and 7) orbital changes similar to those seen in TED including disordered structure of extra-ocular muscles, edema, infiltration by immune cells and fat accumulation.

The first clear induction of TSAB was achieved by immunising AKR/N mice with fibroblasts transfected so that they have stable expression of a class 2 MHC molecule, H-2k (homologous with the mice) and the full length human TSHR (11). When female mice were injected 6 times, at two weekly intervals with 107 fibroblasts by the intraperitoneal route, about 20% of them developed increased thyroxine levels and TSAB. In addition the thyroids of the animals were enlarged and displayed microscopic hypertrophy and hypercellularity but no lymphocytic infiltration. The same authors then repeated the protocol, but using fibroblasts expressing chimeras of the TSHR and luteinising hormone receptor (LHR), along with H-2k (12). The aim was to define regions of the TSHR which are required and/or sufficient to induce disease. TBII (but not TSAB) were induced in mice treated with cells expressing a chimera lacking the carboxyl part of the extra-cellular domain (ECD) of the TSHR but not when the fibroblasts expressed a receptor construct lacking the amino end of the ECD. The absence of TSAB and elevated thyroxine, in all except animals receiving cells expressing the full length human TSHR, confirms the requirement for numerous discontinuous residues in the ECD for complete autoreactivity to the TSHR. The model has been confirmed and extended by the group of Terry Davies (13). He modified the protocol to include Th1 (Freunds complete) or Th2 (alum) adjuvants, administered at the same time as the fibroblasts expressing H-2k and the human TSHR. Nine of 19 mice receiving alum had increased thyroxine levels and goitres and with an earlier onset than in mice not receiving the alum (9 weeks versus 11). In contrast, induction of hyperthyroidism was slower in the mice treated with complete Freunds (14 weeks). No difference in susceptibility was noted between male and female animals. These experiments confirm the Th2 nature of the autoimmune response to the TSHR which

results in a Graves'-like illness in mice, as reported previously by others (please see below). The most recent variation to this approach used cells expressing H-2d and the full length murine TSHR to induce TSAB and hyperthyroxinemia in the majority of treated BALBc mice examined at 26 weeks (14). The animals also exhibited weight loss and their thyroids displayed focal necrosis and lymphocytic infiltration.

Some success in modelling GD and TED has been achieved by transferring TSHR primed T cells to naive syngeneic recipients. We have used unfractionated T cells and a CD4+ enriched population with the in vivo TSHR priming step performed using the receptor produced in bacteria (ECD-MBP) or genetic immunization (please see below). In both cases in vivo priming was followed by an in vitro priming period using ECD-MBP. In our first study (15), BALBc and NOD recipients were examined 16 days after transfer of syngeneic receptor primed T cells and both strains of mice displayed thyroiditis but of very different phenotype. In the BALBc mice, B cells and immunoreactivity for IL-4 and IL-10 were found but in the NOD mice there were very few B cells and immunoreactivity for INF γ , indicating the Th2 and Th1 nature of the induced disease respectively. Neither strain had developed antibodies to the receptor in the recipient animals at this early stage although these were present in the donor mice. Whilst these animal studies were in progress, we and others were able to demonstrate TSHR transcripts and protein in the orbit, particularly in the adipose compartment (5-8). Consequently in more recent experiments (16), to determine the kinetics of disease induced using unfractionated T cells and a CD4+ enriched population, the mouse orbits were also examined. In both BALBc and NOD recipients the Th2 and Th1 nature of induced thyroiditis respectively was confirmed and found to persist for the 12 week duration of the experiment. At 4 weeks, TSHR antibodies, including TBII, had been induced in both strains and these too persisted throughout the experiment. Changes in thyroid hormone levels were more difficult to evaluate, especially in the BALBc. In NOD recipients of TSHR primed T cells, thyroxine levels were reduced, as might be expected from the destructive thyroiditis induced in this strain. 4 Four weeks after transfer, BALBc recipients of TSHR primed and control non-primed T cells had reduced thyroxine levels which slowly recovered in the latter. At 8 and 12 weeks, some BALBc recipients of receptor primed T cells had increased thyroxine, relative to the control non-primed recipients.

When examining the orbits, all of the NOD recipients of primed and non-primed cells, displayed normal histology with intact well organised muscle fibre architecture. BALBc orbits of primed (but not non-primed) T cells appeared strikingly different. The muscle fibres were disorganised and separated by periodic acid Schiff positive oedema. There was accumulation of adipose tissue and infiltration by immune cells, especially mast cells. These changes were observed in 17 out of 25 (68%) BALBc recipients of receptor primed cells and did not correlate with TBII or T4 levels. However, orbital changes were observed only in mice having the most severe thyroiditis with 25-30% of the gland occupied by interstitium which also correlated with the most skewed Th2 response, B:T cell ratio 1.6-1.9 and IL-4:INF γ ratio >2.5.

One of the most ingenious protocols involves immunization with the cDNA for the full length human TSHR cloned into a eucaryotic expression vector. (17). We must assume that the cDNA is taken up into the myocytes at the site of injection (usually the anterior tibialis) and subsequently expressed at the surface of these cells. Myocytes do not express MHC-class II or the co-stimulatory molecules necessary to activate T cells. Consequently there must be a phase, perhaps triggered by inflammation of the muscle, in which professional antigen presenting cells become involved, maybe by phagocytosing fragmented receptor released from myocytes.

Some success was achieved since 14/15 female BALBc mice treated with receptor cDNA developed antibodies to the TSHR measured by FACS and the majority contained TBII activity. One serum contained TSAB resulting in 800% increase in cAMP production and which persisted for 18 weeks. Thyroid hormone levels remained normal throughout the experiment. All mice displayed severe thyroiditis with many infiltrating B cells but no thyroid destruction, quite the opposite with signs of epithelial thickening and budding.

The method was then applied to the NMRI outbred strain of mice with very exciting results (18). 30 male and 30 female mice underwent the genetic immunization protocol and virtually all developed receptor antibodies detectable by flow cytometry. 9/30 males displayed signs of hypothyroidism with reduced T4. Five out of 29 females developed stable hyperthyroidism with circulating TSAB accompanied by increased thyroxine but undetectable TSH. In addition Th2 thyroiditis and orbital

changes, including infiltration by mast cells and macrophages, were induced. Analysis of the MHC haplotype of the mice revealed that they were predominantly H2q, irrespective of whether disease had been induced or not. This highlights the importance of non-MHC genes in the development of GD and also TED and will be a focus for future studies using this model, which in the words of the authors 'provides the most convincing murine model of GD available to date'.

A number of further conclusions can be derived from these models. The induction of a TED-like disease using TSHR cDNA or primed T cells is further support for this antigen being an important target in TED as well as in GD and that a Th2 autoimmune response to the receptor can result in TED. One feature of Th2 reactivity, the participation of mast cells, has been shown to induce prostaglandin synthesis and GAG production in human orbital fibroblasts, at least in vivo (19). Mast cells have been reported in human TED biopsies (20) but their precise role warrants further investigation. Furthermore increases in circulating IgE, which could activate mast cells (21) and stem cell factor, a mast cell growth factor (22), have been reported in GD. We have been able to demonstrate IgE antibodies binding directly to the TSHR in a small number of GD patients with TED (ms submitted) using flow cytometry.

Perhaps the most convincing evidence for the Th2 nature of GD and TED, is a human model happened on by chance. In patients with multiple sclerosis (MS) treated in vivo with a monoclonal antibody to CD52, >95% of their circulating T lymphocytes were eliminated and there was considerable amelioration of their disease. Eighteen months after this treatment, T cell numbers had returned to 35% and B cells to 180% of pretreatment values but 12/34 patients had developed GD with TSAB (23). The deviation from Th1 to Th2, although beneficial for MS, was permissive for GD and stresses the importance of balance in maintaining appropriate immune responsiveness.

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PENDRIN AND ITS FUNCTION IN THYROCYTES, THE KIDNEY AND THE INNER EAR

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Figure. Model of iodide transport in thyroid follicular cells.

Iodide is actively transported into thyroid follicular cells by the sodium-iodide symporter (NIS) at the basolateral membrane. At the apical membrane, pendrin (PDS) is thought to facilitate iodide efflux into the follicular lumen. Thyroperoxidase (TPO) oxidizes iodide and subsequently iodates tyrosyl residues of thyroglobulin (TG) in presence of hydrogen peroxide (H₂O₂) (organification). The iodotyrosines, mono- and diiodotyrosyl (MIT, DIT) are coupled to T₄ or T₃, a reaction that is also catalyzed by TPO (coupling). Thyroglobulin is internalized into the follicular cell, hydrolized in lysosomes, and the thyronines T₄ and T₃ are released into the blood stream.

Pendred's syndrome and mutations in the PDS/SCL26A4 gene

After linking Pendred's syndrome to chromosome 7q22-31.1, the PDS gene was cloned in 1997, a key step in unraveling the molecular cause of the curious association of sensorineural deafness, goiter and partially impaired iodide organification (1). Mutational analyses in numerous patients indicate that mutations in the PDS gene are very diverse and more than fifty alterations have already been published (1-5). The majority of the reported PDS mutations are simple missense mutations, a smaller subset consists of nonsense mutations with or without preceding frameshift, and there are several mutations affecting splice donor or acceptor sites (6). Recessive mutations in the PDS gene have not only been found in patients with the classic Pendred's syndrome, but also in families classified as having non-syndromic autosomal recessive deafness DFNB4, or hearing loss associated with non-syndromic (familial) Enlarged Vestibular Aqueduct (7, 8). PDS gene mutations may thus account for a significant proportion of congenital and progressive hearing loss.

Elucidation of the functional roles of pendrin

The ongoing functional characterization of pendrin, the protein encoded by the PDS gene, is particularly fascinating and has led to several surprising findings. Pendrin is a highly hydrophobic membrane protein (1). It belongs to the solute carrier family 26A and is also referred to as SCL26A4. The SCL26A family contains several transporters of sulfate or other anions. An additional, functionally distinct SCL26A family member is prestin, a recently cloned motor protein found in outer hair cells (9). Prestin has no known anion transport capability, but intracellular anions are thought to be essential for its electromotility (10). Remarkably, the genes encoding pendrin, prestin and down-regulated in adenoma (DRA) are located in close vicinity on chromosome 7q21-31 and have a very similar genomic structure suggesting a common ancestral gene.

Initial functional studies of pendrin in *Xenopus* oocytes and Sf9 insect cells, as well as functional studies using cultivated thyrocytes from patients with documented Pendred's syndrome, revealed that pendrin is unable to transport sulfate (11, 12). However, pendrin was shown to mediate uptake of chloride and iodide (11), and to act as a chloride/formate exchanger in *Xenopus* oocytes (13).

Pendrin in the thyroid

In thyroid follicular cells, pendrin is inserted into the apical membrane (14, 15).

This, together with the impaired iodide organification and its ability to transport iodide in oocytes, suggests a possible role in iodide transport into the follicle (14, 16). To further characterize the iodide transport properties of pendrin, we recently compared the functional consequences of two naturally occurring mutations found in a patient with Pendred's syndrome to wild type pendrin (17). Transfected cells expressing only pendrin are unable to accumulate iodide at physiological doses. In contrast, cells expressing the sodium iodide symporter (NIS) alone show a significant uptake of iodide. Cotransfection of NIS and pendrin leads to a pronounced decrease in intracellular iodide content, a phenomenon that is absent in coexpression experiments of naturally occurring pendrin mutations with NIS (17). These

results support the concept that pendrin mediates iodide efflux from mammalian cells loaded with iodide. The kinetics of pendrin-mediated iodide transport remain to be determined. Despite the partial organification defect of iodide, most individuals with Pendred's syndrome are clinically and biochemically euthyroid, at least under conditions of normal nutritional iodide intake (6). Furthermore, the prevalence of goiters may be lower in patients with PDS mutations living in iodine-replete regions (18). Moreover, Pds knockout mice do not display an enlarged thyroid or abnormal thyroid hormone levels (19). These observations indicate that iodide may traverse into the follicular lumen independent of pendrin, either by means of the electrochemical gradient between the thyrocyte and the lumen and/or the presence of other iodide-transporting channels.

Pendrin in the kidney

In addition to its expression in the thyroid and the inner ear, PDS mRNA was readily found in the kidney [Everett, 1997 #4]. (1) Subsequently, PDS mRNA expression was found to be abundant in the renal cortex, and nephron segment RT-PCR detected the transcripts in the proximal tubule and cortical collecting duct (CCD) cells (20). Immunoblot analyses localized pendrin to the cortical brush-border membrane of CCD cells (20). Functional studies in transfected human embryonic kidney (HEK-293) cells then demonstrated that pendrin is an exchanger of chloride with bicarbonate, hydroxyde and formate (20). Using immunohistochemistry, pendrin could be identified in certain intercalated cells of the CCD, specifically in a subpopulation of cells thought to mediate bicarbonate secretion (21). In contrast, pendrin was undetectable in kidneys from the Pds $-/-$ mouse (21). Perfused tubules isolated from alkali-loaded wild type mice secreted bicarbonate, whereas tubules from alkali-loaded Pds $-/-$ mice failed to secrete bicarbonate (21). In summary, there is increasing evidence that pendrin can function as an apical exchanger of chloride with bases such as bicarbonate in a subset of intercalated cells of the cortical collecting duct (20, 21).

Patients with Pendred's syndrome have no apparent abnormalities in acid-base metabolism, possibly due to the presence of other chloride/base exchangers. The possibility that subtle alterations may be present under conditions of metabolic alkalosis has not been formally addressed.

Pendrin in the inner ear

The most prominent and obligatory clinical sign in patients with Pendred's syndrome is profound sensorineural hearing impairment (6). The majority, if not all patients with deafness associated with mutations in the PDS gene appears to have an enlargement of the endolymphatic sac and duct (22, 23). The so-called Mondini cochlea, the replacement of the cochlea by a single cavity or a rudimentary structure, is a less frequently observed malformation (4, 5, 22). Consistent with the human phenotype, mice with targeted disruption of the Pds gene are completely deaf and also display signs of vestibular dysfunction (19). The inner ear appears to develop normally until embryonic day 15, after which time severe endolymphatic dilatation occurs, reminiscent of that seen radiologically in deaf individuals with pendrin mutations. Moreover, severe degeneration of sensory cells and malformation of otoconia and otoconial membranes is detectable after the second postnatal week (19). In situ-hybridization studies in the developing mouse localized PDS mRNA to the endolymphatic duct and sac, in areas of the utricle and saccule, and in the external cochlear sulcus region (24). This expression pattern involves several regions thought to be important for endolymphatic fluid resorption. Although the exact role of pendrin in the inner ear remains to be defined, its role in other tissues and the phenotypes associated with pendrin mutations suggest that the enlargement of the endolymphatic system is caused by a defect in anion and fluid transport. Limited functional studies in oocytes suggest that mutants found in Pendred's syndrome are associated with a complete loss of function for chloride and iodide transport, whereas alterations found in non-syndromic hearing loss display only partial inactivation (25).

Pendrin in other tissues

Aside from its expression in the inner ear, the thyroid and the kidney, pendrin expression has been reported in other tissues. Pendrin and the sodium iodide symporter appear to be expressed in the placenta (26). Immunohistochemical analysis revealed that NIS protein was present on the entire membrane of the cytotrophoblast, whereas pendrin was mainly located at the brush border membrane of syncytiotrophoblast cells facing the maternal side (26). The functional significance of these two anion transporters in the placenta is currently unknown.

Very low levels of PDS mRNA expression have been reported in tissues such as lung, breast,

endometrium, prostate and testis (27). Whether these transcripts are of physiological importance is currently unknown. Pendrin protein expression was also documented in Sertoli cells (27). This interesting finding awaits confirmation particularly in light of the fact that a novel SCL26A member has recently been identified in the testis (28).

Summary and perspective

Mutational analysis of the PDS gene has revealed that it is not only the molecular cause of the classic Pendred's triad, but also of two forms of non-syndromic autosomal recessive deafness. PDS gene mutations may thus be an even more frequent cause of congenital deafness or progressive hearing loss. Molecular analysis of the PDS gene, in combination with radiologic evaluation of the inner ear, can be useful for making a definitive diagnosis.

Functionally, pendrin appears to play a role as chloride/base exchanger in the kidney. In the thyroid, it appears to facilitate iodide efflux into the follicular lumen. In the auditory system, pendrin is probably also involved in anion and fluid transport given that mutations result in morphologic alterations during development leading to an enlarged endolymphatic system and subsequent destruction of the sensory cells.

The elucidation of the functional role of pendrin has already provided many unanticipated surprises and as we discover more about its structure and function we may encounter further fascinating facets.

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SUBCLINICAL THYROID DISEASE: TO TREAT OR NOT TO TREAT

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Subclinical thyroid disease is defined by an abnormally high (subclinical hypothyroidism) or low (subclinical hyperthyroidism) serum TSH with peripheral thyroid hormone concentrations within the laboratory reference ranges.

Such abnormalities in thyroid function tests are very common in the population and have been extensively dealt with in textbooks and reviews. At the time of writing a search (limited to title and review) of Pub Med lists 19 papers on subclinical hypothyroidism during the period 1986-2001 and 8 on subclinical hyperthyroidism (and many more if all field search is used) (1). The present publication primarily deals with the controversial issue: Should patients with this abnormality be treated or not.

Final conclusion

Yes, if a patient with subclinical thyroid disease prefers to be treated after being adequately investigated and after being properly informed about the disease and the therapy, therapy should be offered. Whether or not the patient chooses therapy the importance of regular control should be substantiated.

Practical procedure

Below is given a list of points to follow when dealing with a patient with subclinical thyroid disease. In the following some comments and details on specific points are given.

Checklist when dealing with a patient with subclinical thyroid disease:

1. Verification of the diagnosis by repeated testing after for example 3 months.
2. The subtype of disease (nosological type) should be established.
3. Status on clinical symptoms and signs of the disorder.
4. Status on other risk factors and diseases and evaluation of long-term risks and prognosis.
5. Information of the patient about the disease, and on the possibility of therapy or wait and see control.
6. Follow the patient's decision.
7. Follow-up control

Subclinical thyroid disease and severe illness

Transient serum TSH abnormalities are common in hospitalized and especially in severely ill patients, and in the period after recovery (2,3). This may be induced by the disease or by the medication given. Subclinical thyroid disease should not be diagnosed and therapy not initiated under such circumstances.

Transient subclinical thyroid disease

In general the causes of subclinical thyroid disease are the same as for overt thyroid disease, and also the types of therapy are the same. As in overt disease it is important to evaluate the possibility of a transient state before initiating lifelong medication or before giving destructive therapy. Subacute or silent thyroiditis should be excluded and the possibility of excessive iodine intake or medication as the cause for the disorder should be evaluated. During the first year after radioiodine therapy or thyroid surgery, subclinical thyroid abnormalities are common, and lifelong or permanent therapy should not be given.

The common causes of subclinical hypothyroidism

Spontaneous subclinical hypothyroidism is common in all populations but more so the higher the iodine intake (4). The patients can be split in two main subtypes: Those with measurable thyroid antibodies (TPO-Ab and/or Tg-Ab) in serum and those without.

Antibody positive patients presumably suffer from autoimmune thyroiditis. They have the highest risk for progressive thyroid failure with in the order of 5-10% of patients progressing from subclinical to overt hypothyroidism per year (5,6).

The mechanism behind the frequent abnormality of elevated serum TSH without detectable thyroid antibodies is at present not clarified. These patients may suffer from a different subtype of autoimmunity with predominant T-cell response. Autopsy studies (7) have demonstrated a considerably higher frequency of histological thyroiditis than found by investigation of serum samples in population studies. However other mechanisms may be involved as well. This disorder is associated with a lower risk of progression to overt thyroid disease (around 2% per year, (5)).

Knowledge on the risk of progression is important for the decision on control interval, especially if therapy is not given. On the other hand we do not agree that a higher risk of progression supports early initiation of therapy. The current state should be the dominant basis for the decision on therapy. If the current state is not sufficiently severe to initiate therapy, this can be initiated when/if progression is observed. Whether or not therapy is given now, follow-up has to be performed. If early therapy could prevent progression of thyroid failure and stabilize a state of partial physiological regulation of thyroid function, this would be an important argument for early therapy. T4 substitution is often followed by a fall in thyroid antibody levels (8) but controlled studies on the clinical relevance of this are lacking.

The common causes of subclinical hyperthyroidism

The importance and dominant cause of subclinical hyperthyroidism depends fundamentally on the iodine intake level of the population. In a population where the iodine intake is mild to moderately low (median urinary iodine excretion 25-120 µg/24 h) or has been so within recent decades, the dominant cause of subclinical hyperthyroidism is autonomous thyroid nodules (4,9). In such areas subclinical hyperthyroidism is even more frequent than subclinical hypothyroidism, affecting 5-10% of subjects above 60 years of age (4,9). On the other hand, spontaneous subclinical hyperthyroidism is not common in high iodine intake areas, where the abnormality is dominated by patients receiving T4 in high doses (10) as excessive substitution for hypothyroidism or in an attempt to reduce the size of thyroid nodules, or to prevent recurrence of previous thyroid cancer.

Arguments for and against therapy of subclinical thyroid disease

Major arguments in favour of therapy

1. Thyroid function is not fully compensated by the altered serum TSH in subclinical thyroid disease, and peripheral thyroid hormone action is not normal in the individual subject.
2. Subclinical thyroid disease may be associated with impaired function of various organs, which is reversible by therapy of the thyroid disorder.
3. Subclinical thyroid disease may increase the long term risk of developing various serious disorders.
4. Therapy of subclinical thyroid disease is often uncomplicated and inexpensive with a burden of control not different from observational control.
5. The likelihood of spontaneous resolution is low with most types of subclinical thyroid disease.
6. In pregnancy impaired thyroid function in the mother may impose a risk to the foetus (11-14).

Major arguments against therapy

1. A considerable proportion of patients will not feel more healthy if treated.
2. Therapy will often involve lifelong intake of medication (subclinical hypothyroidism).
3. Therapy may involve a significant risk of side effects (subclinical hyperthyroidism).
4. The long term-risk from subclinical thyroid disease may depend on other risk factors - and it may be low in subgroups of patients.
5. More studies are needed to determine precisely the magnitude of clinical abnormalities and risks associated with the abnormality - and the long-term benefits of therapy.
6. Subclinical thyroid disease is very common. For such common disorders more documentation is needed before therapy is legalized.

Many of the arguments are similar for subclinical hyper- and hypothyroidism. It is evident that even if

peripheral thyroid hormone values are by definition within laboratory reference ranges the altered serum TSH signals abnormally low or high thyroid function. When groups of patients are compared with healthy controls (15), or thyroid hormones in serum are measured before and after normalisation of serum TSH by therapy (16), the average concentrations of T4 and T3 are significantly altered. Variation of serum thyroid hormone concentrations in the individual is much more narrow than the broad laboratory reference ranges (17). Thus, there is ample space for T4 and T3 concentration values being abnormal for the individual within the laboratory reference ranges. The time of progression of thyroid dysfunction from subclinical to overt (with T4 or T3 leaving the reference range) may vary widely between individuals depending on their normal level of T4 and T3 being high or low in the reference range (17). Serum TSH is much more (10 times) sensitive to alterations in thyroid function than serum T4 (18). Thus, TSH will leave the reference range much earlier than T4 or T3, and give the pattern of subclinical thyroid disease (19).

Clinical consequences of mild thyroid disease

The slightly to moderately abnormal thyroid hormone levels (for the individual) manifest as small aberrations in peripheral thyroid hormone effects (20-22). Subclinical thyroid disease is by nature not different from overt thyroid disease but on average a milder abnormality. Consequently the clinical findings are not different but milder. Careful monitoring of heart function has revealed functional abnormalities in both subclinical hyper- and hypothyroidism which are reversible upon therapy (16, 23-25). An uncontrolled study suggests that subclinical hypothyroid patients have a high frequency of increased intraocular pressure, which is reversible upon therapy (26).

Another important hormone effect is exerted on brain and neuromuscular function. In a blinded placebo controlled study Jaeschke et al (27) reduced average serum TSH from 12.1 to 4.1 mU/l by T4 administration for more than 6 months in 15 elderly subjects (16 controls). Most of the series of questionnaires and cognitive function tests applied in the study developed in favour of therapy, but this was only statistically significant ($p = 0.01$) for a composite psychometric memory score. The improvement was equivalent to a difference of 8.7 points in an IQ-test. This was deemed of limited importance by the authors, who advocated a watchful waiting in middle-aged and older subclinically hypothyroid patients. Such watchful waiting will normally include as much control and blood tests as therapy. Considering the relatively insufficient dose of T4 administered in this study, the physiological nature and low price of T4 substitution therapy and the observed effect on memory, it is hard to agree on their conclusion. In our opinion a decision on watchful waiting should be taken by the informed patient, not by the doctor or the healthcare system.

Long-term risks associated with subclinical thyroid disease

A major concern in subclinical hypothyroidism is the risk of atherosclerosis. Subclinical hypothyroidism is associated with an atherogenic lipid profile in serum and T4 substitution is followed by a less atherogenic profile including a small decrease in serum total cholesterol (0.2 - 0.4 mmol) and a decrease in LDL-cholesterol (28). No large intervention studies on the effect of substitution therapy on vascular disease and mortality are available. In the Wickham follow-up study no significant association between cardiovascular disease and subclinical hypothyroidism at the investigation 20 years earlier was found (29). On the other hand, a recent Dutch study of a cohort of elderly women, found subclinical hypothyroidism to be associated with the same proportion of myocardial infarctions as other known risk factors such as hypercholesterolaemia, hypertension, smoking and diabetes mellitus (30).

In subclinical hyperthyroidism the major concern is the long term risk of atrial fibrillation and of osteoporosis. Analysis of the Framingham data revealed a 3 fold increase in incidence of atrial fibrillation in participants with serum TSH < 0.1 mU/l (31). Thyroid hormone increases bone turnover and many studies have evaluated the association between subclinical hyperthyroidism and osteoporosis. In a meta-analysis long term TSH suppression by T4 administration was found to have no effect on bone mass in premenopausal women, whereas postmenopausal women had an excess bone loss of 0.91% per year (32). Two controlled intervention studies have shown that therapy of subclinical hyperthyroidism due to multinodular goitre in postmenopausal women ameliorate bone mineral loss which was around 2% per year in untreated patients (33,34).

The evidence that subclinical hyperthyroidism may have deleterious effects has been obtained in patients with suppressed serum TSH (< 0.1 mU/l). Borderline low TSH (0.1-0.4 mU/l) has not been associated with disease.

Therapy of subclinical hypothyroidism

Thyroxine substitution therapy is straight forward following the guidelines for overt hypothyroidism. The T4 dose is adjusted to normalize serum TSH. TSH reference ranges may vary depending on the assay method and reference population. In a Danish population study the 95% reference range for subjects with no thyroid disease by history, ultrasound and measurement of TPO-Ab was 0.4-3.6 mU/l (35). Often laboratory references are broader and a somewhat arbitrary limit of 4 or 5 mU/l for diagnosing subclinical hypothyroidism is often employed. During therapy small dose adjustments can stabilize serum TSH around 0.8-2.5 mU/l, followed by yearly lifelong control of thyroid function.

Therapy of subclinical hyperthyroidism

The therapy of choice in patients with multinodular goitre or a solitary hot adenoma is radioiodine. The risk of complication is higher than for T4 therapy of subclinical hypothyroidism. Around 10-20% of this type of patients develop hypothyroidism over 5 years after radioiodine (36) and 1-2% may develop Graves' Disease due to activation of autoimmunity against the TSH receptor (37). A beneficial effect of radioiodine is reduction of goitre volume (around 50% after 1-2 years). If low dose antithyroid medication is given, this has to continue lifelong (38).

Conclusion

Subclinical hypo- and hyperthyroidism are not well-defined disorders, but mild to moderate thyroid failure or thyroid hyperactivity in continuum with overt thyroid disease.

Many patients with subclinical hypothyroidism have no clear symptoms or signs of the disease, and will not be able to feel the difference between T4 therapy or not. However 1/4-1/2 of patients will feel a general improvement in health after normalization of serum TSH (39,40) and improved memory function can be demonstrated by objective tests (27,41). Risk factors for atherosclerosis will improve slightly upon therapy and a recent population study in elderly females suggests that subclinical hypothyroidism may be an independent risk factor for myocardial infarction of similar magnitude as hypertension, hyperlipidaemia, smoking and diabetes mellitus. Substitution therapy with T4 is straight forward with few side effects.

Subclinical hyperthyroidism may affect well-being (21) and supranormal supraventricular pacing of the heart has been demonstrated. This may be the reason for the 3 fold increase in risk of atrial fibrillation. Postmenopausal women are at increased risk for osteoporosis and bone mineral density responds favourably to therapy. Patients with suppressed serum TSH due to autonomous nodules should be offered radioiodine therapy. T4 substitution therapy of hypothyroidism should be properly controlled to avoid overtreatment. T4 therapy to fully suppress serum TSH should be limited to patients with a high risk of recurrent thyroid cancer.

Long-term follow-up after therapy is necessary. Some patients are reluctant to receive therapy and in such cases control of the biochemical and clinical thyroid state as well as other risk factors is appropriate depending on the state.

It has been argued that labelling, polypharmacia and expenses are prohibiting problems (27). However, many elderly subjects (in Denmark at least) are now taking series of ill-defined, undocumented, costly, over the counter 'natural' products to improve their health. If such a person has an abnormally elevated serum TSH it is certainly more appropriate to substitute with T4 to normalize thyroid function.

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