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TYROSINE KINASE INHIBITORS IN THE TREATMENT OF THYROID CANCER

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ABSTRACT

Thyroid cancers are the most common endocrine cancers and constitute around 1% of all human malignancies. Papillary thyroid carcinoma (PTC), follicular thyroid carcinoma (FTC) and anaplastic thyroid carcinoma (ATC) are derived from follicular epithelium. Among these PTC and FTC are coined under the term differentiated thyroid cancer (DTC) and comprises 90% of all thyroid cancers. The prognosis of DTC are in general favourable with an initial treatment of total thyroidectomy and radioactive iodine ablation, and TSH suppression in the follow-up period. This combined treatment regimen called the conventional treatment is highly successful in patients without metastases. Less than 10% of patients with DTC present with or ultimately develop distant metastases and around 40-50% of these subjects are unresponsive to conventional treatment. In radioiodine refractory subjects 10-year survival is around 20%. Conventional chemotherapeutics have also been tried in these subjects with success rates less than 25%. On the other hand, for medullary thyroid cancer (MTC), which originates from the parafollicular C cells, the only curable treatment is surgery. However, 60-80% of these subjects have metastases on admission and 5-year survival rates are less than 50%. ATC has the worst prognosis among thyroid cancers, with an expected survival less than 6 months after diagnosis. Thus for radioiodine treatment refractory DTCs, locally advanced and metastatic MTCs and ATCs new treatment modalities are needed. This review will focus on the use of tyrosine kinase inhibitors (TKIs) in thyroid cancers and a summary of the results of clinical trials to date will be presented.

Key-words: *thyroid cancer; tyrosine kinase inhibitor; targeted therapy; thyroid.*

INTRODUCTION

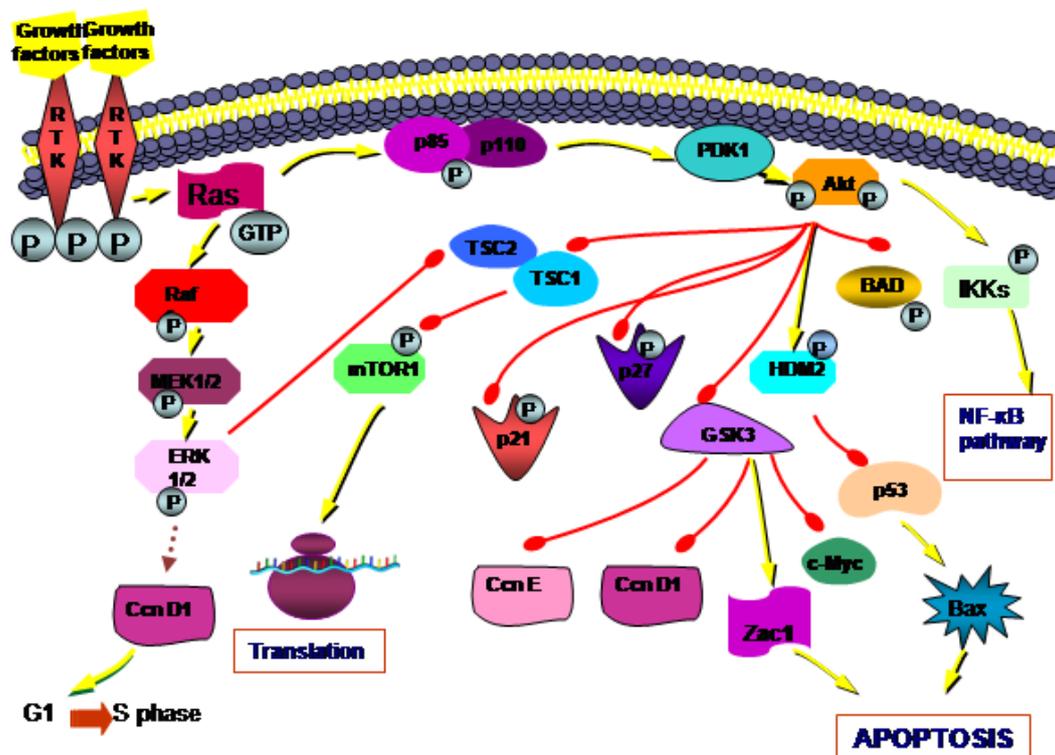
The management of patients with thyroid cancer depends on the histological subtype and stage of the tumour. Among thyroid cancers, a great majority of DTC patients can be cured with the conventional treatment regimen composed of surgery, radioactive iodine and thyroid hormone therapy. Less than 10% of DTC patients present with or ultimately develop distant metastases (1-3). Around 40-50% of these subjects are refractory to radioactive iodine treatment (1,4) and have a 10-year survival rate of 10-20%. In radioactive iodine resistant subjects, conventional chemotherapy response rates are low and long term cure is rare (5). For patients with MTC, which comprises around 5% of thyroid cancers, the only curable treatment choice is surgery. For the treatment of ATC, which is luckily the least common histologic type of thyroid cancer (~1%), surgery, radiotherapy and chemotherapy have been used with disappointing results. Treatment choices for radioactive iodine refractory DTC, locally advanced and metastatic MTC and ATC are limited. In recent years, use of TKIs in clinical trials of thyroid cancer patients have shown promising results. Considering the role of Ras/Raf/ mitogen-activated ERK kinase (MEK)/ extracellular signal-regulated protein kinase (ERK) 1/2 signalling pathway in the pathogenesis of thyroid cancers, TKIs targeting the upstream of this pathway, seem a rational choice of treatment. This review will be a summary of the mechanism of action of TKIs, their expected role in the treatment of thyroid cancers and results of clinical trials.

RECEPTOR TYROSINE KINASES and DOWNSTREAM PATHWAYS

Receptor tyrosine kinases (RTKs) transmit signals that regulate cell proliferation and differentiation, promote cell migration and survival, and modulate cellular metabolism (expert). Epidermal growth factor receptor (EGFR), vascular endothelial growth factor receptor (VEGFR), fibroblast growth factor receptor (FGFR), glial cell line-derived neurotrophic factor receptor (RET), platelet-derived growth factor receptor (PDGFR), hepatocyte growth factor receptor (MET) are among the several RTKs. Growth factors activate these RTKs, which then activate two key signal-transduction components: the lipid kinase phosphoinositide 3-kinase (PI3K)/Akt and the Ras/Raf/MEK/ERK 1/2 signalling pathways (Figure 1, Figure 2).

Any of Ras/ERK 1/2 and PI3K/Akt elements, when converted to a constitutively active state, is sufficient to drive 'susceptible' but otherwise normal cells into a state that exhibits at least some properties of an oncogenically transformed cell (6). The Ras/ERK 1/2 pathway is a key signaling pathway that is involved in the regulation of normal cell proliferation, survival, growth and differentiation (6). Ras is the most frequently mutated oncogene in human tumours (7). The ERK 1/2 pathway is dysregulated in approximately one-third of all human cancers. PI3Ks are a family of proteins involved in the regulation of cell growth, metabolism, proliferation, glucose homeostasis and vesicle trafficking. Mutation in one or another PI3K pathway component accounts for up to 30% of all human cancers.

Figure 1- A simplified diagram of the Ras/ERK 1/2 and PI3K/Akt pathway. Yellow lines show activation, red lines show inhibition of the corresponding protein.

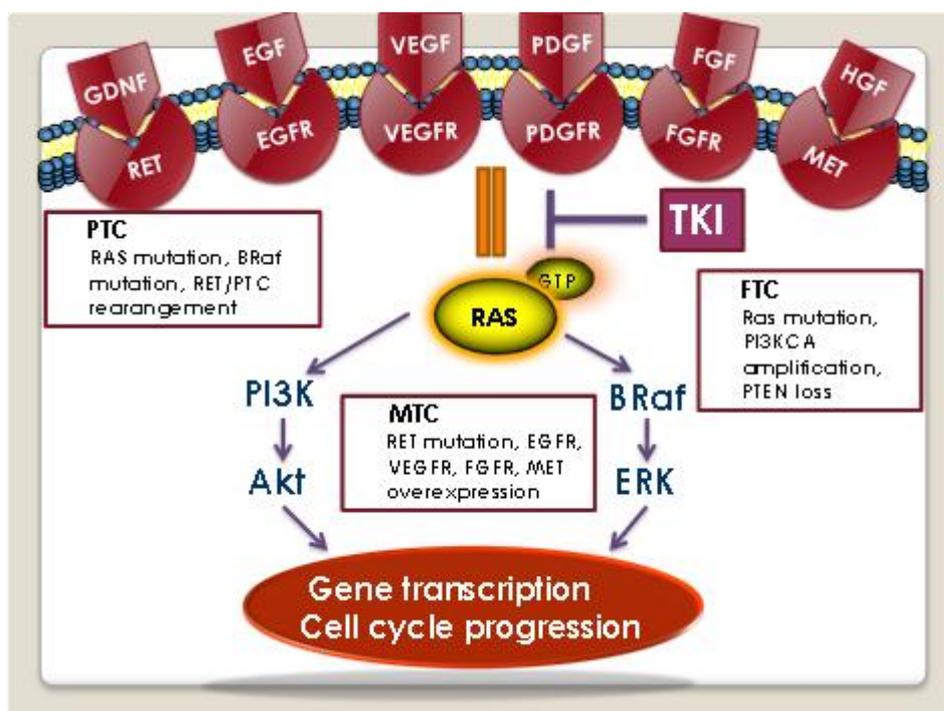


ABBREVIATIONS: BAD: BCL2-antagonist of death; CcnD1: Cyclin D1; CcnE: Cyclin E; ERK: Extracellular signal-regulated protein kinases; FOXO: Forkhead box O; GSK3: Glycogen synthase kinase-3; HDM2: Human homolog of murine double minute ubiquitin ligase; IKK: IκB kinase; MEK: Mitogen-activated protein/extracellular signal-regulated kinase (ERK) kinase; mTOR: Mammalian target of Rapamycin; p21: Cyclin dependent kinase inhibitor p21^{Cip1/WAF1}; p27: Cyclin dependent kinase inhibitor p27 Kip1; PDK1: Phosphoinositide-dependent kinase 1; RTK: Receptor tyrosine kinase; TSC: Tuberous sclerosis.

Regarding thyroid cancers, several mechanisms including overexpression or mutation of RTKs, rearrangements producing chimeric oncogenes, activating mutations of oncogenes, inactivation of tumour suppressor genes due to mutations, deletions and epigenetic changes, have all been reported with changing rates (Figure 2). Figure 2 shows the demonstrated genetic changes that have been detected in PTC, FTC and MTC. To start with receptors, the role of RET is very well established in MTC (8). In addition to its well-known role in hereditary MTC, it is now known that approximately 50% of sporadic MTCs harbor activating RET mutations (9,10). Overexpression of EGFR (11), MET (12) and FGFR4 (13) were also reported in MTCs. On the other hand, RET/PTC represents a recombinant protein product from a chromosomal rearrangement with the combination of the 3' portion of the RET gene and the 5' portion of a partner gene (14). This recombination results in constitutive activation of the tyrosine kinase in RET. Among the more than 10 types of RET/PTC, which are mainly found in thyroid cancer, the most common and important types are RET/PTC1 and RET/PTC3 (14). RET/PTC occurs in about 15-20% of adult PTC patients (15). As RET/PTC was also

reported to occur with high prevalences in benign thyroid tumours and Hashimoto's thyroiditis, it appears that, RET/PTC alone, under physiological expression, may not be sufficiently oncogenic and additional genetic alterations may be required for thyroid cell transformation (14). Ras mutations are common particularly in the follicular variant of PTC, FTC and poorly-differentiated thyroid cancer (PDTC) with a prevalence around 20-40% in most series (14). B-Raf which is an important component of RTK downstream pathway, is mutant in an average of 44% of PTC and its mutation was also frequently associated with the absence of radioiodine sensitivity (16). PI3KCA gene, which encodes for the catalytic subunit of PI3K, was found to be amplified in 24% of FTC (17) and 42% of ATC (18). An epigenetic inactivating mechanism through aberrant methylation of the PTEN gene was also reported in FTC and particularly in ATC (19,20). Activation of PI3K/Akt pathway in thyroid tumours was also noted with PPAR γ /Pax8 rearrangement (14). As a result, in general PI3K/Akt pathway is commonly activated in FTC, while Raf/ERK 1/2 pathway is commonly activated in PTC (14).

Figure 2- A simple diagram showing RTKs, their ligands and downstream pathways. Some of the genetic changes that have been demonstrated to date in PTC, FTC and MTC are listed in rectangles.



ABBREVIATIONS: EGF(R): Epidermal growth factor (receptor); ERK: extracellular signal-regulated protein kinase; FGF(R): fibroblast growth factor (receptor); FTC: Follicular thyroid cancer; GDNF: Glial cell derived neurotrophic factor; HGF: hepatocyte growth factor; MET: hepatocyte growth factor receptor; MTC: Medullary thyroid cancer; PDGF(R): platelet-derived growth factor (receptor); PI3K: phosphoinositide 3-kinase; PTC: Papillary thyroid cancer; PTEN: Phosphatase and tensin homolog deleted on chromosome 10; RET: Glial cell line-derived neurotrophic factor receptor; TKI: Tyrosine kinase inhibitors; VEGF(R): vascular endothelial growth factor (receptor).

TYROSINE KINASE INHIBITORS

Generally, RTKs are activated through ligand-induced oligomerisation, typically dimerisation, which juxtaposes the cytoplasmic tyrosine kinase domains (21). For most RTKs, this juxtaposition facilitates autophosphorylation in trans of tyrosine residues in the kinase activation loop or juxtamembrane region, inducing conformational changes that serve to stabilise the active state of the kinase (21). These and other phosphotyrosine residues serve as recruitment sites for a host of downstream signalling proteins. TKIs are a group of small molecules that interfere with the interaction between the kinase domain and ATP, thereby inhibiting phosphorylation of the kinase and downstream substrates (8). TKIs used in the treatment of thyroid cancers and the corresponding RTKs inhibited by are shown in Table 1.

TABLE 1- Tyrosine kinase inhibitors and the corresponding tyrosine kinases inhibited by the drug (8,34,38).

TKI	RET	VEGFR	EGFR	C-KIT	PDGFR	FGFR	Bcr-ABL	BRAF	MET
Vandetanib	√	√	√						
Sunitinib	√	√		√	√				
Sorafenib	√	√		√		√		√	
Motesanib	√	√		√	√				
Pazopanib		√		√	√				
Aksitinib		√							
XL 184	√	√							√
Imatinib				√	√		√		
Gefitinib			√						

ABBREVIATIONS: C-KIT: Stem cell factor receptor; EGFR: Epidermal growth factor receptor; FGFR: fibroblast growth factor receptor; MET: hepatocyte growth factor receptor; PDGFR: platelet-derived growth factor receptor; RET: Glial cell line-derived neurotrophic factor receptor; TKI: Tyrosine kinase inhibitors; VEGFR: vascular endothelial growth factor receptor.

CLINICAL TRIALS

Table 2 shows a list of clinical trials performed with TKIs to date. Case reports and phase I trials have not been included in this list.

TABLE 2- Clinical trials of tyrosine kinase inhibitors performed in thyroid cancers.

Reff.	Type and length of study	Initial/Assessable patient number (n)	Partial response*	Stable disease	Median progress-free survival (PFS)
Gross DJ et al., 2006, (24)	PHASE 2, imatinib 1X600-800 mg, 12 months	MTC (6/6)	-	-	n=4 progression, n=2 toxicity
De Groot JWB et al., 2007, (25)	PHASE 2, imatinib 1x600-800 mg, 12 months	MTC (15/15)	-	26% (n=4) > 24 months	Severe toxicity
Frank-Raue K et al., 2007, (26)	Open-label, imatinib 1x600 mg, 8 months	MTC (9/5)	-	(n=5) 6 months (n=1) 12 months	6 months
Ha HT et al., 2010, (27)	PHASE 2, imatinib 2X400 mg	ATC (11/8) (all have overexpression of PDGFR)	25% (n=2/8) Evaluation at 8 weeks	50% (n=4/8) Evaluation at 8 weeks	Estimated 6 months PFS 36%
Schlumberger MJ et al., 2009, (28)	PHASE 2, motesanib 1X125 mg, Up to 48 weeks	MTC (91/83)	%2 (n=2) (duration 32 weeks and 21 weeks)	%81 (n=74) (in %48 ≥24 weeks)	48 weeks
Sherman S et al., 2008, (29)	PHASE 2, motesanib 1X125 mg	DTC (93/82)	%14 (n=13)	%67 (n=62) (in 35% ≥24 weeks or longer)	40 weeks
Lam ET et al., 2010, (30)	PHASE 2, sorafenib 2X400 mg	MTC (16/15) (hereditary)	6.3% (n=1) 20.7 months	87.5% (n=14) (in 8 patients ≥15 months)	17.9 months
Gupta-Abramson V et al., 2008, (31)	PHASE 2, sorafenib 2X400 mg	PTC (18/15), FTC (9/7), MTC (1/1), ATC/poorly differentiated (2/2)	23% (n=7) 18+ to 84 weeks	53% (n=16) 14-89+ weeks	79 weeks
Kloos RT et al., 2009, (32)	PHASE 2, sorafenib 2X400 mg, ARM A-chemotherapy naive PTC ARM B- prior chemotherapy +ve PTC and other thyroid cancers	PTC (41/36)	15% (n=6) 6-14 months	56% (n=23) >6 months	n=28 chemotherapy (-) 16 months, n=8 chemotherapy (+) 10 months
		FTC/ Hürthle (11/10)	0	82% (n=9)	4.5 months
		ATC (4/4)	0	25% (n=1)	-

Reff.	Type and length of study	Initial/Assessable patient number (n)	Partial response*	Stable disease	Median progress-free survival (PFS)
<i>Table 2 continued</i>					
Hoftijzer H et al., 2009, (33)	PHASE 2, sorafenib 2X400 mg, 26 weeks	DTC (32/26)	25% (n=8)	34% (n=11)	58 weeks
Cohen EEW et al., 2008, (34)	PHASE 2, Axitinib 2x5 mg	PTC (30/22), FTC (15/14), MTC (11/5), ATC (2/2), other (2/2)	8/30 PTC, 6/15 FTC, 2/11 MTC, 1/2 ATC, 1/1 other	38% (n=23) \geq 16 weeks	18.1 months
Pennell NA et al., 2008, (35)	PHASE 2, Gefitinib 1x250 mg	PTC (n=11), FTC (n=6), Hürthle (n=1), ATC (n=5), MTC (n=4) (n=25 cases evaluable in total)	-	12%	3.7 months
Carr L et al., 2010, (36)	PHASE 2, Sunitinib 37.5 mg/day	Thyroid cancer (35/33) (7 MTC) (28 DTC)	3% complete response (n=1) 28% PR (n=10)	46% (n=16)	12.8 months
De Souza JA et al. (ASCO Meeting 2010-5504), (37)	PHASE 2, Sunitinib 50 mg, 4/2 week	MTC (24/23)	35% (n=8)-median 37 weeks	57% (n=13)-median 32 weeks	
Bible KC et al., 2010, (38)	PHASE 2, Pazopanib, 800 mg/day 4 week cycles	DTC (39/37)	49% (n=18)		11.7 months
Wells SA et al., 2010, (39)	PHASE 2, vandetanib 1x300 mg	Hereditary MTC (30/29)	20% (n=6) median 10.2 months	53% (n=16) \geq 24 weeks	27.9 months
Robinson BG et al., 2010, (40)	Vandetanib, 1X100 mg	Hereditary MTC (19/18)	%16 (n=3) Median 6 months	%53 (n=10) \geq 24 weeks (n=2) \geq 2 months and <6 months	Grade 1-2 toxicity
Leboulleux S et al. (ITC 2010-OC-023), (41)	Vandetanib 1x300 mg	DTC (n=72)	8.3%		11 months
	Placebo (randomised)	DTC (n=73)	5.5%		5.8 months
Wells SA et al. (ASCO Meeting 2010-5503), (42)	Vandetanib (PHASE 3, double-blind)	MTC (n=231) (hereditary or sporadic)	48% continue drug, 37% progress, 15% death		Not reached
	Placebo	MTC (n=100)			19.8 months

*Rates given according to the initial patient numbers of the study.

ABBREVIATIONS: ATC: Anaplastic thyroid cancer; DTC: Differentiated thyroid cancer; MTC: Medullary thyroid cancer; PDGFR: Platelet-derived growth factor receptor; PTC: Papillary thyroid cancer.

Importantly in all these studies patient accrual, follow-up and treatment response were performed according to Response Evaluation Criteria in Solid Tumours (RECIST) 1.0 guidelines (22). According to these criteria, only patients with measurable lesions should be included in the studies to evaluate objective drug response. All measurable lesions up to a maximum of five lesions per organ and 10 lesions in total, representative of all involved organs, should be identified as target lesions and recorded and measured at baseline. The evaluation of target lesions should be as follows; complete response—the disappearance of all target lesions; partial response—at least a 30% decrease in the sum of the longest diameter of target lesions, taking as reference the baseline sum longest diameter; progressive disease—at least a 20% increase in the sum of the longest diameter of target lesions, taking as reference the smallest sum longest diameter recorded since the treatment started or the appearance of one or more new lesions; stable disease—neither sufficient shrinkage to qualify for partial response nor sufficient increase to qualify for progressive disease, taking as reference the smallest sum longest diameter since the treatment started (22). The subjects included in these studies are patients with refractory DTC, locally advanced or metastatic MTC and ATC. Patients with radioiodine refractory disease may be defined as patients who have at least one lesion without radioiodine uptake or that has progressed within 1 year after radioiodine treatment (23).

Imatinib was the first TKI to be used in thyroid cancers. It was used in MTC patients in 3 studies (24-26) without any objective response and considerable toxicity reported in two of these studies (24,25). Additionally, imatinib was used in ATC in a very recent study with 25% partial response at the end of 8 weeks and was well-tolerated (27). The authors stated that, due to the difficulty of accruing patients with this rare malignancy at a single institution, further investigation of imatinib in ATC should better be in a multi-institutional setting.

Motesanib has been used both in MTC and DTC patients (28,29). In the MTC study only a 2% partial response was noted (28). Among patients with tumor marker analysis, 69 (83%) of 83 and 63 (75%) of 84 had decreased serum calcitonin and carcinoembryonic antigen during treatment, respectively, compared with baseline. Although the objective response rate was low, a significant proportion of MTC patients (81%) achieved stable disease while receiving motesanib. In the study of DTC patients, in which 67% of patients had PTC, the objective response rate was 14% (29). Among the 75 patients in whom thyroglobulin analysis was performed, 81% had decreased serum thyroglobulin concentrations during treatment, as compared with baseline levels.

Sorafenib, the only TKI which also inhibits B-Raf, was studied in hereditary MTC and DTC and a small number of ATC patients (30-33). In the study of 15 hereditary MTC patients, 1 partial response and 1 fatal toxicity was reported (30). In a study performed in a mixed population of patients with mainly PTC and FTC and 1 MTC and 2 ATC/PDTC, an overall clinical benefit rate (partial response + stable disease) of 77%, median progress-free survival of 79 weeks, and an overall acceptable safety profile was observed (31). However a single patient died of liver failure that was likely treatment related (31). In a similar study, study arm A included 33 chemotherapy naïve PTC

patients while arm B included 8 prior chemotherapy positive PTC patients in addition to FTC and ATC patients (32). A partial response of 15% was observed in the assessable 36 PTC patients (32). In 14 (78%) of 18 Tg-assessable PTC patients, Tg declined more than 25%. B-Raf mutation was detected in 17 (77%) of 22 PTCs analysed (32). Four of 10 paired tumor biopsies from PTC patients showed a reduction in levels of VEGFR phosphorylation, ERK phosphorylation, and in VEGF expression during sorafenib therapy (32). No objective response was noted for other tumours. In another sorafenib study in DTC patients, the primary endpoint was reinduction of radioactive iodine uptake at 26 weeks of treatment (33). A 25% partial response was noted however no reinduction of radioiodine uptake at metastatic sites was observed (33).

With regard to axitinib (34) and gefitinib (35), there is one published phase II trial for each, which included all histological types of thyroid cancers. In the axitinib study, there was a partial response of 30% in the whole study group and the most significant side effect was hypertension. In the study with gefitinib which included 25 thyroid cancers with different histologic types there was no objective response and only 12% stable disease (35).

Sunitinib has been used for the treatment of thyroid cancer in two phase II studies to date (36,37). In this study by Carr and colleagues, both MTC and DTC patients were included (36). One complete response and 28% partial response were observed. The other study included only MTC patients, with a partial response rate of 35% (37).

Among TKI, one of the most promising drug seems to be pazopanib which produced in DTC patients a 49% partial response (38). The two patients who died during treatment were reported to have pre-existing contributory disorders.

Probably the most commonly studied TKI in thyroid cancers is vandetanib (39-42). Wells and colleagues reported 20% partial response with 300 mg/day (39) while Robinson and colleagues reported 16% partial response with 100 mg. daily vandetanib (40). Moreover in the former study, in 24 patients, serum calcitonin levels showed a 50% or greater decrease from baseline that was maintained for at least 4 weeks; 16 patients showed a similar reduction in serum carcinoembryonic antigen levels (39). In International Thyroid Congress, September 2010, Leboulleux and colleagues presented a randomised placebo controlled study of vandetanib in DTC patients (41). Statistically significant progress-free survival prolongation was observed for vandetanib vs. placebo (41). However, the objective response rate (8.3% vs. 5.5%) did not reach statistical significance. The only double-blind phase III trial with a TKI was performed with vandetanib in 331 MTC patients (42). Also called ZETA, the results of this study showed significantly increased progress-free survival in the vandetanib group vs. placebo [hazard ratio 0.45, 95% confidence interval (CI) 0.30–0.69, $p=0.0001$] (42).

In subjects given TKI treatment, moderate to severe toxicity has been noted in 30-40% of the subjects. In their elegant review, Le and colleagues have given a list of side effects reported in more than 20% of patients treated with TKIs (8). According to this list, fatigue, weight loss, anorexia,

headache, rash, hand-foot syndrome, diarrhoea, nausea/vomiting, abdominal pain, dyspnea, hypertension are the side effects seen in several of TKIs reported with different rates (8).

CONCLUSIONS

Cytotoxic systemic chemotherapies for advanced, metastatic thyroid carcinomas have limited effectiveness. With significant advances in our understanding regarding the molecular basis of thyroid cancers it seems logical to propose the use of TKIs in thyroid cancers. To summarise the published trials mentioned in this review, highest partial response rates in DTC patients have been observed with pazopanib (38), in MTC patients with vandetanib (42) and in ATC patients with imatinib, although this latter study was able to recruit a small number of patients (27). On the other hand, even in the same pathologic group of tumours, there may be considerable differences between the mutations and molecular changes which may alter the drug response. In a study by Bass and colleagues, biomarkers were studied as predictors of response to treatment of metastatic MTC and DTC with motesanib (43). A certain rate of increase in serum placental growth factor (PIGF) and decrease in soluble VEGF receptor 2 levels after initiation of therapy predicted response to motesanib in patients with advanced DTC or metastatic MTC. Lower baseline VEGF levels were associated with longer progress-free survival. In a recent update of the phase II study with sorafenib there was a correlation between longer progress-free survival and the presence of B-Raf mutations (44). Specifically, for patients with PTC/FTC, the progress-free survival for those with wild-type B-Raf was 54 weeks compared to 84+ weeks for patients with B-Raf^{V600E} ($p = 0.028$). Thus, maybe in the future the use of TKIs in thyroid cancers may come along with certain molecular analyses beforehand to ensure treatment response.

As a conclusion, TKIs may open a new era in the treatment of radioactive iodine refractory DTC, advanced MTC and ATC patients in near future. However, the published clinical trials are relatively sparse compared to other malignancies and there is only one published phase III trial yet in thyroid cancers. A possible reason for this is, the difficulty in accrual of enough number of patients to these clinical trials. It may be possible to overcome this difficulty by multi-institutional trials recruiting patients from several centers. On the other hand, given that there is no proof yet TKIs improve overall survival and they have quite significant undesirable effects, patients must be selected carefully before initiation of therapy. Randomised clinical trials for several agents are underway that may lead to eventual approval of TKIs for thyroid cancer.

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MECHANISM OF CHEMICAL DISRUPTORS OF THYROID FUNCTION

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ABSTRACT

Endocrine disrupting chemicals (EDCs) are synthetic or natural substances in the environment, food, and consumer products. They disrupt reproductive, developmental and other homeostatic systems by interfering with synthesis, secretion, transport, metabolism and action of endogenous hormones. Many such chemicals affect the thyroid hormone (TH) system at multiple levels. They may alter the hypothalamo-pituitary-thyroid feedback system, TH synthesis, TH transport and metabolism, and TH receptor (TR) action. TH mainly regulates cellular homeostasis by controlling basal metabolic rate in adults, whereas, during development, it plays a critical role in functional organization and cellular differentiation in many organs, particularly in the brain. Thus, the consequence of exposure to EDCs in the fetus or infant may be more serious than in the adult. Perinatal exposure to certain environmental pollutants may cause abnormal brain development similar to that seen in perinatal hypothyroidism. Such substances may not always share structural similarity and act on different molecular target of the TH system. In this article, after a general overview of EDC action, previous studies showing the effect of representative EDCs on the TH system are summarized. Then, the molecular mechanisms of polychlorinated biphenyl (PCB) action on the TH system are discussed further. PCB is one of the most well-known EDC that widely exists in the environment. It disrupts TR-mediated transcription, but may not act as competitive inhibitor of TH. Our recent studies may provide a novel idea regarding the effect of EDCs on the TH system.

Key-words: Thyroid hormone, brain development, critical period, environment

GENERAL OVERVIEW OF ENDOCRINE DISRUPTING CHEMICALS

Endocrine disrupting chemicals (EDCs) are synthetic or natural substances in the environment, food, and consumer products. According to the Environmental Protection Agency (EPA) of the United States, an ECD is defined as “an exogenous agent that interferes with synthesis, secretion, transport, metabolism, binding action, or elimination of natural blood-borne hormones that are present in the body and are responsible for homeostasis, reproduction, and developmental process”. EDCs comprise highly heterogeneous substances, including synthetic chemicals and their byproducts. Furthermore, some natural substances, such as isoflavones, in food may also disrupt our hormone system. Initially, it has been thought that these EDCs may act by binding to hormone receptors, particularly nuclear hormone receptors such as steroid/thyroid hormone receptors. However, it has turned out that their mechanisms of action are much broader (see ref. 1, for review). They may not only act through nuclear receptors, but also membrane-associated receptors. Some may affect hormone synthesis/secretion in endocrine cells, transport in plasma, or breakdown and excretion. Because of such diversity of action, their molecular structure is also diverse. Even some heavy metals such as cadmium, mercury and lead may disrupt hormonal systems.

During the evolutionary process, it is only recently that living organisms were exposed to synthetic chemicals. Thus, many EDCs have long half-lives and accumulate in the environment, wildlife and humans, even if the production of such chemicals has been banned decades ago. Other chemicals such as bisphenol A may not be less persistent but, since they are widely used, they can nonetheless cause significant exposure. Socio-economical factor also influence the exposure. Contamination of industrial chemicals in soil and ground water is of great concern in industrialized areas, whereas exposure to pesticides and fungicides may be of greater concern in agricultural areas. Furthermore, the regulation policy of chemical compounds differs greatly among countries. Thus, exposure level of each EDC is greatly different around the world.

Exposure to EDC in adulthood and during development has different consequences. During development, various developmental-stage specific molecular and cellular changes are induced with critical control of gene expression (2). Particularly, in developing brain, proliferation of precursor cells, differentiation, migration, dendritogenesis and synaptogenesis are sequentially

induced in a distinct pattern to undergo remodeling until establishment of adult functional properties. Many hormones such as estrogen and thyroid hormone (TH) play critical roles in controlling such processes. Since most processes are irreversible, their disruption may lead to serious consequences. Thus, exposure to EDC during development may induce more adverse effects.

Another important nature of EDCs is the so called "low dose effect", which is defined as doses below the range typically used in toxicological studies. If some EDCs act directly on receptors, low levels of exposure, particularly during developmental critical period, can disrupt hormone actions (3). If the concentration of EDC is higher, it may inhibit receptor-mediated action by down-regulation of receptor levels. As a result, dose-response relationships show non-monotonic, i.e. "inverted-U-shaped", curves. Thus, traditional toxicological assumptions based on the monotonic dose-response curve, in which more of the chemical leads to a greater effect, may not be applicable to assess the toxicity of EDCs (4).

THYROID HORMONE ACTION ON DEVELOPING BRAIN

Perinatal exposure to certain EDCs such as polychlorinated biphenyl (PCB) and polybrominated diphenyl ether (PBDE) may cause abnormal brain development similar to that seen in perinatal hypothyroidism (5). Thus, before discussing on the effect of EDCs on TH-mediated action, the role of TH on brain development is briefly summarized.

The TH (L-triiodothyronine: T3, thyroxine: T4) plays an important role in development and functional maintenance of the central nervous system (6). Deficiency of TH during pre- and early postnatal period results in abnormal brain development known as cretinism in humans. Screening for neonatal hypothyroidism has been introduced in most developed countries, which greatly contributed to prevent sporadic cretinism induced by congenital abnormalities. Although controversies still exist, a previous study has documented an increase in congenital hypothyroidism in the United States (7). At present, no definitive causes for such increase have been determined. The involvement of environmental factors including EDCs has been considered. On the other hand, endemic cretinism induced by iodine deficiency remains a serious health and socio-economic problem in many underdeveloped countries (8). While treatment of sporadic

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cretinism with TH beginning in the neonatal period permits normal neurological development, such treatment at birth does not completely prevent neurological abnormalities in endemic cretinism, which is caused by a combination of maternal and fetal hypothyroxinemia during gestation (9). Even if the maternal hypothyroidism during pregnancy is mild and no clinical or biochemical manifestations except for elevated plasma TSH concentration are observed, it may result in lower IQ scores of the offspring (10). These results indicate that there is a distinct “critical period” of TH action involved in neurological development.

T₃, the active compound of TH, is produced locally in the brain by 5'-deiodination of T₄, which enters the developing brain more easily than T₃ (11). This is probably because the brain-specific organic anion transporter (Oatp)1c1 at blood-brain barrier (BBB), a major TH transporter at BBB, preferentially binds T₄ (12). After crossing the BBB, T₄ is taken up by astrocytes to be deiodinated by type 2 iodothyronine deiodinase (13). Then, T₃ is transferred to neurons or oligodendrocytes possibly through the monocarboxylate transporter (MCT) 8 (14). Then it binds to nuclear TH receptor (TR) expressed in these cell types to regulate gene expression of its target genes.

It is generally believed that TH action in the brain is mainly exerted through binding to nuclear TRs (15), although non-genomic actions of TH have been reported (16). Nuclear TR is a ligand-regulated transcription factor that binds to a specific nucleotide sequence termed as TH-responsive element (TRE) as a homodimer or heterodimer with retinoid X receptor (RXR) (15, 16). Then, it binds to a series of proteins termed coactivator or corepressor in a ligand-dependent manner to regulate transcription of target genes (17). At least three subtypes of TR (α 1, β 1 and β 2) are expressed in humans (18). These TRs are widely distributed in the developing brain (19,20). Each TR subtype is expressed in a region- and developmental stage-specific manner. It should be noted that, although TH action in the brain is greater during development, the TR level is greater in adult brain (15). Thus, TH sensitivity may be controlled by other unknown mechanisms. Most likely, epigenetic mechanisms such as DNA methylation and histone modification are involved.

ENVIRONMENTAL CHEMICALS THAT MAY AFFECT TH SYSTEM

Table 1 shows a list of environmental chemicals that may affect TH system. It should be noted that this list shows only representative chemicals with representative references. Many additional chemicals may be involved and many additional excellent studies have been done. As shown in this table, various kinds of chemicals may behave as EDCs for the TH system. Regarding chemicals mainly used for therapeutic purposes, in addition to TH and anti-thyroid drugs such as methimazole (MMI) and propylthiouracil (PTU), several drugs used for non-thyroidal illness may disrupt the TH system. Such chemicals include amiodarone (anti-arrhythmic) (21), diethylstilbestrol (synthetic estrogen) (22), fenamate (anti-inflammatory drug) (23), phenobarbital (anticonvulsant) (24), and phenytoin (anti-epileptic) (25). Several pesticides including carbamate (26), dichloro-diphenyl- trichloroethane (DDT) (27), endosulfan (24), and fipronil (29) also affect the TH system. Furthermore, many industrial chemicals and their derivatives are also known to disrupt the TH system. Such chemical includes benzophenone 2 (30), dioxin (31), methylcholanthrene (32), PBDE (33,34), PCB and its hydroxylated form (OH-PCB) (35-38), perchlorate (39), and thiocyanate (39). In addition to synthetic chemicals, natural substances such as polyphenols (catechin and isoflavones) that are found in plants also affect the TH system (40-43). Not only organic chemicals but also inorganic heavy metals such as cadmium (46) and lead (47), and organic metals such as methyl-mercury (48) may also disrupt the TH system. Since the chemical structures of these chemicals vary greatly, their mechanism of action is also variable.

Table 1. Representative endocrine disrupting chemicals affecting mammalian thyroid hormone system

1. Pharmaceuticals
Thyroid hormone (T3 and T4), anti-thyroid drugs (MMI, PTU), amiodarone (21) DES (22), fenamate (23), phenobarbital (24), phenytoin (25)
2. Pesticides
Carbamate (26), DDT (27), endosulfan (28), fipronil (29), etc.
3. Industrial chemicals and byproducts
Benzophenone 2 (30), dioxin (31), methylcholanthrene (32), PBDE (33,34), PCB (35-38), perchlorate (39), thiocyanate (39)
4. Polyphenols
Catechin (40), isoflavones (41-43)
5. Products associated with plastics
Bisphenol A (44), phthalates (45)
6. Heavy metals
Cadmium (46, 47), lead (47), methyl-mercury (46)

A diagram for TH synthesis, transport, site of action and metabolism is shown in Fig. 1A. Essentially, environmental chemicals can modify all pathways. Fig. 1B shows possible mechanisms of each chemical action. Several chemicals may modify the hypothalamo-pituitary-thyroid axis. For example, thyrotropin-releasing hormone (TRH) action is disrupted by phenytoin (25). Thyrotropin (TSH) synthesis and secretion are suppressed by endosulfan, possibly due to oxidative stress (28). DDT disrupts TSH action on the thyroid by preventing internalization of the TSH receptor (27). Iodine uptake and TH synthesis may be modified by other chemicals. Perchlorate and thiocyanate are well-known potent inhibitors of the Na^+/I^- symporter (NIS) at the basolateral membrane of thyroid epithelial cells (39), and thus are of great health concern particularly in the United States (48). Amiodarone-induced thyroid dysfunction is also well recognized (49). It also inhibits iodine transport, but the action is exerted without involvement of NIS and TSH receptor (21). TH biosynthesis may be also modified by several chemicals. A recent study has shown that benzophenone 2, a chemical used as an ultraviolet filter, inhibits TH synthesis catalyzed by the thyroid peroxidase (TPO) (30). In addition, high doses of polyphenols such as catechin (40) and isoflavones (42) also inhibit TPO activity. However, since the half-lives of such natural substances are much shorter than those of other synthetic chemicals, and since the remaining TPO activity is sufficient to catalyze normal TH synthesis (42), their influence may not be serious except when a tremendously large amount is consumed.

After secretion, TH binds to plasma proteins such as thyroxine-binding globulin (TBG), transthyretin (TTR), or albumin. Some OH-PCB congeners may inhibit TH-TTR binding (36). However, the effect of such inhibition on thyroid function is not clear, which is discussed more in detail in the next chapter. T3 is mainly produced locally from T4 by type 1 and 2 iodothyronine deiodinases (D1 and D2, respectively). Then T3 is further deiodinated by type 3 deiodinase (D3). These deiodinases play a critical role for the physiological effects of TH (50). The activities of such deiodinases are modified by heavy metals, such as cadmium (46,47), lead (47) and methyl mercury (46). However, further studies are required to clarify the toxicities of these heavy metals. Polyphenols may also affect deiodinase activities. Genistein suppresses both D1 and D2 activity *in vitro* (41), whereas a large dose of catechin suppresses D1 activity in the rat thyroid (40). However,

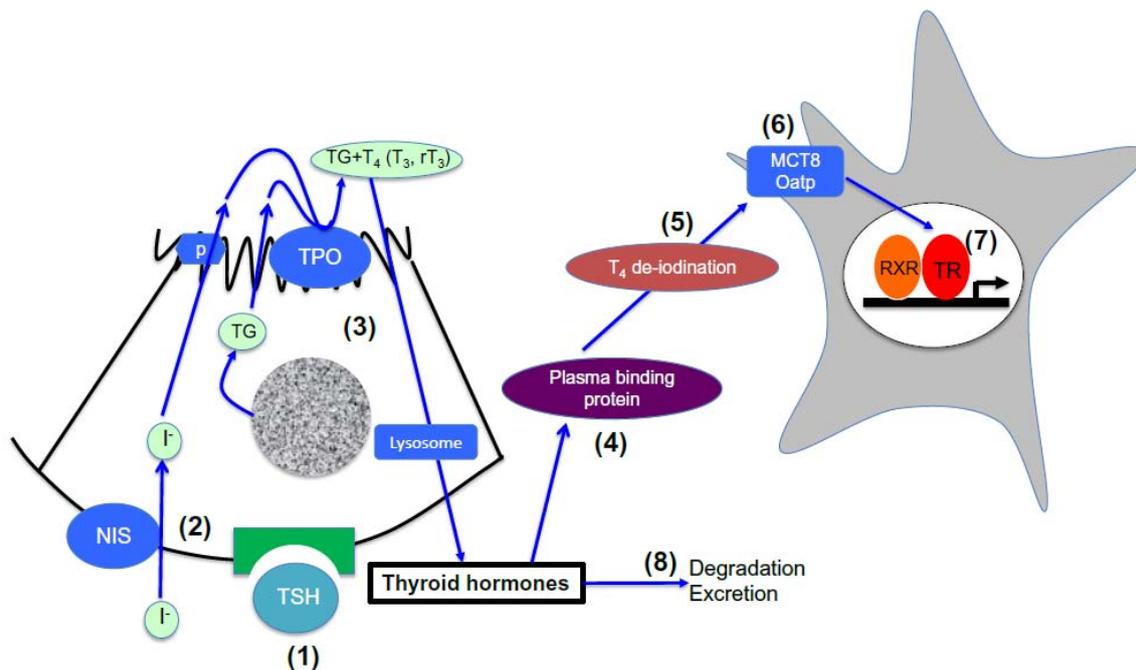
as discussed above, the impact on human health of the consumption of polyphenols may not be serious. PCB/PBDEs also influence deiodinase activities (34,35), which are also discussed later.

In the target organs of TH, TH crosses the plasma membrane through its specific transporters such as MCT8, MCT10 and Oatp1c1 (51). A recent study has shown that an anti-inflammatory drug, *i.e.* fenamate, competitively inhibit Oatp 1c1-mediated T4 transport (23). Since research on TH transporters has become particularly active recently, more chemical that interferes with TH transport through MCT or Oatp will likely be identified. After entering the cell, TH binds to TR. High doses of Isoflavones inhibit TR α expression in rat liver (43). On the other hand, soy protein extract inhibits TR β expression, although distinct protein species responsible for such inhibition have not yet been identified (43). TR-mediated transcription may be modified by several chemicals. Bisphenol A suppresses TR-mediated transcription by recruiting corepressor proteins (44). Phthalates such as dibutyl phthalate, mono-n-butyl phthalate, and de-2-ethylhexyl phthalate suppress TR-mediated transcription, although the mechanism of suppression has not yet been clarified (45). PCB, OH-PCB and PBDE also activate/suppress TR-mediated transcription, which will be discussed later.

TH is eliminated from plasma after chemical modulation such as glucuronidation and sulfation. Conjugation with glucuronic acid is one of the main metabolic pathways of TH metabolism. Many chemicals induce UDP-glucuronosyltransferase activities to increase the clearance of TH. Such chemicals include carbamate (26), dioxin (31), fipronil (29), methylcholanthrene (24,32), PCB (37), and phenobarbital (24).

In addition to the chemicals mentioned above, many other chemicals may possess thyroidal or anti-thyroidal action. These include perfluoroalkyl acids (PFAAs), which are a family of synthetic, highly stable perfluorinated compounds with a wide range of uses in industrial and consumer products. Their presence in the environment and toxicity in animal models have raised concern over low-level chronic exposure effects on human health (52). Although trials to examine the association between PFAAs and thyroid-related diseases have started (53), further efforts are required to clarify their toxicity on the TH system.

A



B

Mechanisms	Chemicals	References
(1) Modulation of TSH secretion, or TSH action.	DDT endosulfan phenytoin	27 28 25
(2) Inhibition of iodine transport	amiodarone perchlorate, thiocyanate	21 39
(3) Inhibition of synthesis and secretion of TH	benzophenone 2 catechin isoflavone (MMI, PTU)	30 40 42
(4) Inhibition of TH-serum binding protein binding	OH-PCB	36
(5) Modulation of iodothyronine deiodination	cadmium, methylmercury chatechin isoflavone lead PBDE PCB	46, 47 40 41 47 34 35
(6) Inhibition of TH transport across the membrane	fenamate	23
(7) Modulation of TR-mediated transcription	bisphenol A isoflavone PBDE PCB, OH-PCB phthalate	44 43 33 38 45
(8) Modulation of breakdown or excretion.	carbamate dioxin fipronil methylchoranthrene PCB phenobaribital	26 31 29 32 37 24

Figure 1. A. Thyroid hormone synthesis, secretion, action and degradation pathway. Chemicals that may affect each step shown as (1)-(8) are shown in Fig. 2B. Abbreviation: NIS, Na⁺/I⁻ symporter; p, pendrin; TPO, thyroid peroxidase; TG, thyroglobulin; MCT8, monocarboxylate transporter 8; Oatp, organic anion transporter; RXR, retinoid X receptor; TR, thyroid hormone receptor. B. List of chemicals that may affect the TH-system as shown in A.

THE EFFECT OF POLYCHLORINATED BIPHENYLS ON DEVELOPING BRAIN

Grandjean and Landrian (2006) have performed a systematic Internet search covering several data banks (54). Even by excluding drugs, food, additives, microbial toxins, and snake venoms and similar biogenic substances, still more than 1,000 chemicals can cause neurotoxicity according to laboratory studies. However, only 5 chemicals are marked as having actually been shown to cause developmental neurotoxicity in humans. These are arsenic, lead, methyl-mercury, PCBs and toluene. Among them, PCB has been considered as a potent EDC. In this chapter, therefore, the effect of PCB on the TH system is further discussed.

Many of the endocrine disrupting chemicals are organohalogenated compounds. Such chemical includes dioxins, PCBs and organochlorine pesticides such as DDT. Among these compounds, PCB is one of the best known substances that may affect brain development. Because its chemical nature is similar to that of oil (lubricative, low electric conductivity), but inflammable, PCB was widely used in industrial and household appliances, such as electrical fluid in transformers and capacitors, hydraulic lubricants, paints, and copy paper. Due to its toxicity, however, its production was banned in 1972. Most PCBs are still widely spread in the environment because of their chemical stability, and use of PCB-containing appliances is still allowed in many countries. Probably because of such reasons, the levels of PCB in various human tissues have not greatly altered until recently (55), and nano-picomolar concentrations of PCB are still contained in blood, breast milk and almost all natural products. PCBs contain 206 congeners due to differences in chlorination. Perinatal exposure to PCB results in abnormal brain development similar to that seen in perinatal hypothyroidism (5). The intellectual development of child is partly retarded with increased PCB concentration in maternal milk, which reflects the perinatal exposure level (56). Initially, it was thought that the effect of PCB was exerted through inhibition of TH secretion from the thyroid gland, or competitive inhibition of TH binding to TH-binding proteins such as transthyretin in plasma (36). However, although PCB treatment decreases plasma TH levels in experimental animals, the PCB exposure level and the changes in plasma TH level are not correlated with one another in humans (57). Even in the rat, although perinatal PCB exposure induces a decrease in the total T4 levels in plasma, the growth rate of PCB-treated animals is sometimes identical to that of control animals, indicating that the animals are generally euthyroid

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(58). Thus, the major action of PCB to induce abnormal brain development may not be through the decrease in plasma TH, but rather, PCB may act directly on TR to modulate its action.

Using conventional reporter assays, several groups have examined the effect of PCB and OH-PCB on TR-mediated transcription (38,59,60). PCB and OH-PCB either suppress or activate TR-mediated transcription in a congener- and cell- specific manner. As a typical example, the effect of OH-PCB (4-OH- 2',3,3',4',5'-penta- chlorobiphenyl) on TR-mediated transcription on F2-TRE in CV-1 cells is shown in Fig. 2. PCB suppresses TR-mediated transcription at low dose (100 pM) (61). Interestingly, the dose response relationship is not so evident, and the magnitude of suppression at 100 pM and 5 μ M is similar, indicating that the suppression may not be due to competitive inhibition of T3 binding to TR. We have examined several congeners including a PCB mixture commercially used for various purposes (Aroclor 1254), and obtained similar results (38).

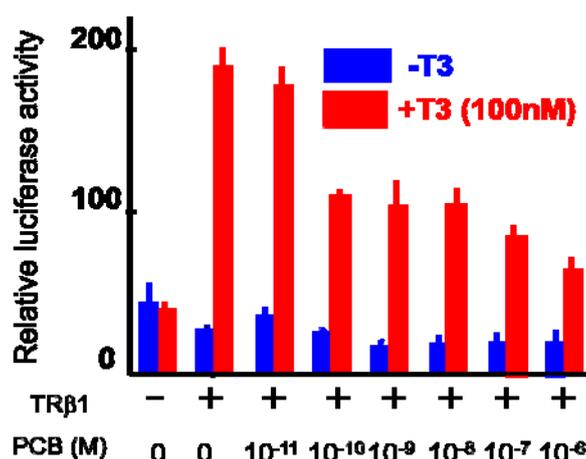


Figure 2. Effect of OH-PCB on TR-mediated transcription, studied by a reporter assay. Expression vector encoding human TR β 1 was transiently transfected with luciferase reporter vector containing F2-TRE into CV-1 cells. OH-PCB (4-OH-2',3,3',4',5'-penta CB) was added to the culture medium with T3 and incubated for 24 hrs.

Furthermore, the effect of PCB is greater in neuron-derived clonal cells than other clonal cells of different tissue origin (61). These results indicate that PCB may affect TR-mediated transcription in a tissue specific manner. Taken together with previous results showing that PCB exposure may induce abnormal development particularly in the neuronal function, these data suggest that the developing brain may be more vulnerable to PCB exposure than other organs. PCB does not dissociate coactivators from TR in the presence of T3 nor does it recruit corepressors to TR. Instead, it dissociates TR from TRE (Fig. 3) (38).

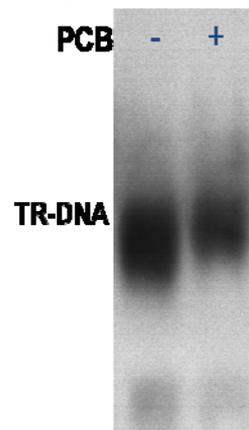


Figure 3. Dissociation of TR-TRE binding by OH-PCB, studied by an electromobility shift assay. Binding between ^{32}P -labeled F2-TRE and TR β 1/RXR protein complex was examined in the presence of T3 with/without OH-PCB.

Furthermore, by using chimeric proteins generated from TR and glucocorticoid receptor (GR) that do not respond to PCB treatment, we have confirmed that the DNA binding domain of TR is responsible for suppression by OH-PCB (Fig. 4) (62). These results indicate that the site of action of PCB in TR molecule may not be the ligand-binding domain, but rather the DNA-binding domain. Finally, our current study suggests that several PBDE congeners also act on the DNA-binding domain to suppress TR-mediated transcription (63).

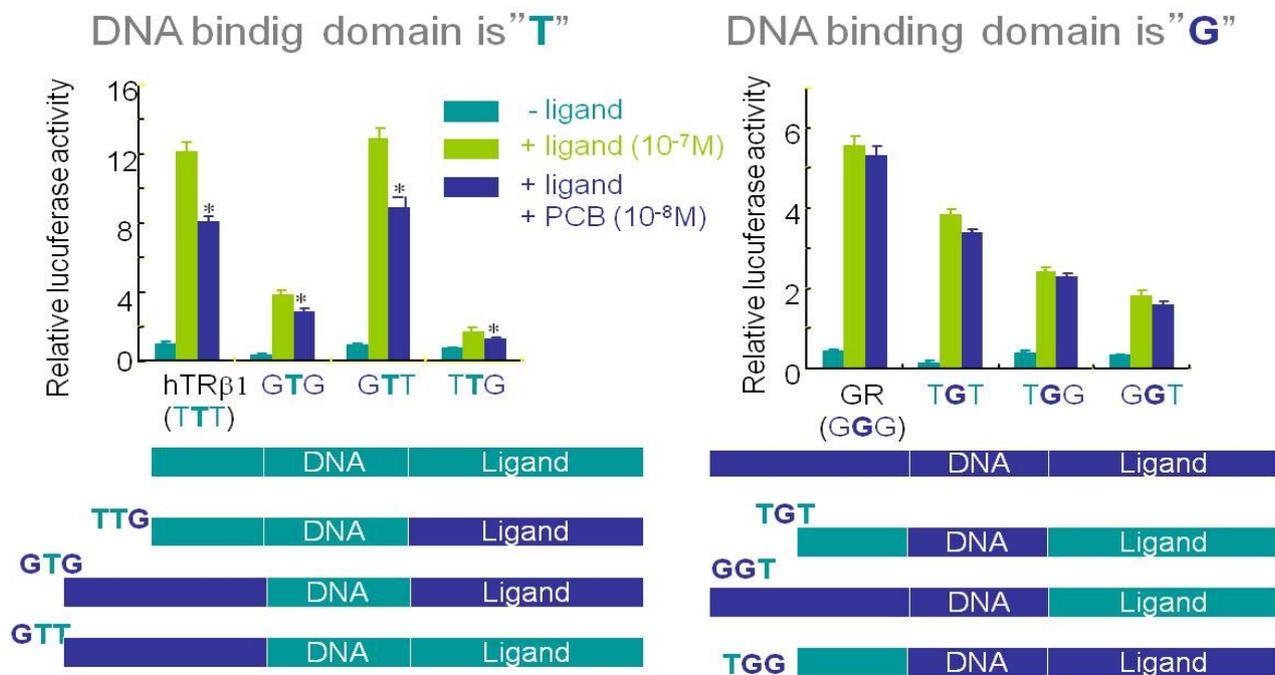


Figure 4. Chimeric proteins generated from TR β 1 (green) and GR (blue). Reporter assays were performed using either F2-TRE or GRE, depending on the molecular structure of the DNA binding domain (DBD). Note that all chimeras harboring TR-derived DBD showed suppression of transcription by PCB.

CONCLUSION

TH plays a major role in brain development. Disruption of the TH system may alter the differentiation and functional development of the central nervous system. Thus, EDCs are of great concern for human health. In the present article, a list of chemicals that may act as EDC of TH system is reported. As discussed above, pathways involved in TH synthesis, secretion, action and degradation can be modulated by environmental chemicals. It should be noted, however, that results of animal model or in vitro studies do not always correlate with those of epidemiological studies. By considering the diversity of action of EDCs, the current clinical strategy to evaluate thyroid function and status (i.e., free and bound THs and TSH in plasma, and antibodies) may not be sufficient to evaluate their toxicity. Clear endpoints to access the toxicity of EDCs still need to be identified.

Chemicals shown in this article are not endogenous substances, and thus their site of action may be not always specific. For example, PCBs and their metabolites act at multiple site of the TH system such as TH-plasma binding protein interaction (36), deiodinase activity (35), TR-mediated transcription (38), and activation of UDP-glucuronidase (37). Furthermore, PCB induces apoptosis through Bcl-2, Bax and caspase-3 proteins (64), oxidative stress (65), and calcium oscillations (66) in neuronal cultures, indicating that non-thyroidal pathways are also involved in the neurotoxicity of PCBs.

By considering such diversity, it is easy to explain the current difficulty in clarifying the outcome of exposure to EDCs during brain development. Due to the insufficiency of available data, the possible involvement of EDCs on the increased incidence of congenital hypothyroidism and neurodevelopmental diseases such as autism and attention-deficit-hyperactive disorder is still hypothetical. Whether or not further studies will better clarify the interaction between EDCs and the TH system, and raise public awareness on EDCs may have a significant impact on the future of the next generations.

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Maternal Thyroid Hormone Action during Embryo-Fetal Development

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Abstract

Tetraiodothyronine (T4) is secreted by the thyroid gland as a prohormone which is converted in the tissues to triiodothyronine (T3), the active hormone that binds to nuclear receptors and initiates thyroid hormone (TH) action. Maternal TH is necessary for differentiation, growth and metabolism in mammals as well as in lower organisms. The biological action of TH can be mediated by TH receptors (TRs) binding to TH-responsive elements (TREs) in the regions of target genes which regulate gene transcription by chromatin remodeling during brain development. Hormonal changes and metabolic demands during pregnancy result in alterations in the biochemical parameters of thyroid function. The main events occurring during pregnancy are: (i) a marked increase in serum thyroxine-binding globulin levels; (ii) a marginal decrease in free thyroid hormone concentrations; (iii) a frequent trend toward a slight rise in basal thyrotropin (TSH) values between the first trimester and term; (iv) a transient stimulation of the maternal thyroid gland by elevated levels of human chorionic gonadotropin (hCG), resulting in a rise in free TH and a decrease in serum TSH concentrations during the first trimester; and (v) altered peripheral metabolism of maternal TH. The changes that occur in T4 and T3 concentrations during pregnancy might generate clinical challenges during brain development. If so, T4 replacement would be needed in those women with hypothyroidism. We will review the most recent advances in: (i) molecular mechanisms of TH during embryo-fetal brain development, and (ii) morphological changes in the brain due to maternal hypothyroidism.

Key-words: maternal thyroid hormones, neuron, cortex, differentiation, hypothyroidism, iodine, mouse models

Introduction

During development of the central nervous system (CNS), neurons are born, differentiate, and migrate to appropriate positions while emitting axons and dendrites. Not only must developing neurons migrate correctly, but their processes must make appropriate contacts with their targets and form synapses with other neurons; for axons, these targets are frequently distant from the neuronal cell body. These “progressive” events in neural development are followed by “regressive events”, including naturally occurring developmental cell death and neuritic and synaptic pruning, which eliminate erroneous or superfluous connections. Orchestrating the formation of these specific connections is a highly complex process, controlled by multiple genetic and epigenetic factors, in which thyroid hormones (THs) play a major role (1).

Thyroid hormone action during embryo-fetal development

Thyroid hormone function in the embryo: activity and source

A causal relationship between inappropriate maternal thyroid function and damage to the developing brain has been hypothesized since the early 20th century, mostly on the basis of reports from localities in which iodine deficiency occurs. During the last five decades, this connection has been supported epidemiological studies (2). A series of studies showed that, in regions where endemic goiter with cretinism occurs, the onset of pregnancy was not accompanied by an increase in circulating thyroxine (T₄), a normal physiological response to the onset of pregnancy in women with an adequate iodine intake (2).

The availability of THs is crucial for brain development. A growing body of clinical and experimental evidence indicates that even slight decreases in serum levels of THs can have significant consequences on brain development. The onset of fetal thyroid function (FTF) occurs at 16-20 weeks post-conception in humans, corresponding to embryonic day 17-18 in rodents (earlier in mice, later in rats), which are commonly used for experimental studies. Before this, the mother is the only source of THs for the developing fetal brain. An inadequate supply of iodine during gestation results in damage to the fetal brain that becomes irreversible by mid-gestation, the onset of fetal

thyroid function. The fetal cerebral cortex depends on maternal T4 for the production of T3 for nuclear receptor-binding and biological effectiveness (2). Iodine deficiency during pregnancy may induce mild maternal hypothyroxinemia and cause delayed neurobehavioral development in the progeny (2-6). Recent studies have shown that even modest maternal hypothyroxinemia (i.e., low circulating free T4) before onset of fetal thyroid function at mid-gestation may lead to irreversible neurological damage. Children exposed to maternal hypothyroxinemia present reduced IQ scores, subtle deficits in cognition, memory, and visuospatial ability (7), and delayed mental and motor function (8, 9).

In 2000, Gabriella Morreale de Escobar et al. (10) questioned whether defects in neuropsychological development are related to maternal hypothyroidism or to maternal hypothyroxinemia. Permanent alterations in the cytoarchitecture of the cerebral cortex appear in the progeny of hypothyroxinemic rats (11). Hypothyroxinemia seems to be much more frequent in pregnant women than either clinical or subclinical hypothyroidism or autoimmune thyroid disease, especially in regions where the iodine intake of the pregnant woman is inadequate to meet her increased requirement for T4 (10, 11).

THs (both T4 and T3) have been detected in the rat embryo and in fetal brain before the onset of FTF (12, 13). T4 has been found in human coelomic and amniotic fluid in the first trimester of pregnancy, suggesting that maternal T4 crosses the placenta (14, 15). Significant levels of total T4 were found in umbilical cord sera of term neonates with congenital hypothyroidism, unable to produce any T4 due to total iodide organification defects. After birth, T4 serum levels gradually decreased, and became undetectable within 2 weeks, indicating that substantial amounts of T4 are transferred from mother to fetus during late gestation (16).

Recent evidence shows that maternal T4 is necessary for early neurogenesis (11, 17). THs are necessary for differentiation, growth and metabolism in mammals as well as lower organisms (18). The molecular actions of THs are mediated by thyroid hormone receptors (TRs) binding to TH-responsive elements (TREs) in the regulatory regions of target genes that regulate gene transcription by chromatin remodeling (19, 20)(*Figure 1*). TRs concentration increases rapidly in the human fetus during the second trimester, a period of high sensitivity of the brain to thyroid hormones. In the rat, the

equivalent period is the last quarter of pregnancy. In the absence of TH, TRs bind a complex of inhibitory proteins that promote deacetylation and inhibit gene transcription (21) (Figure 1).

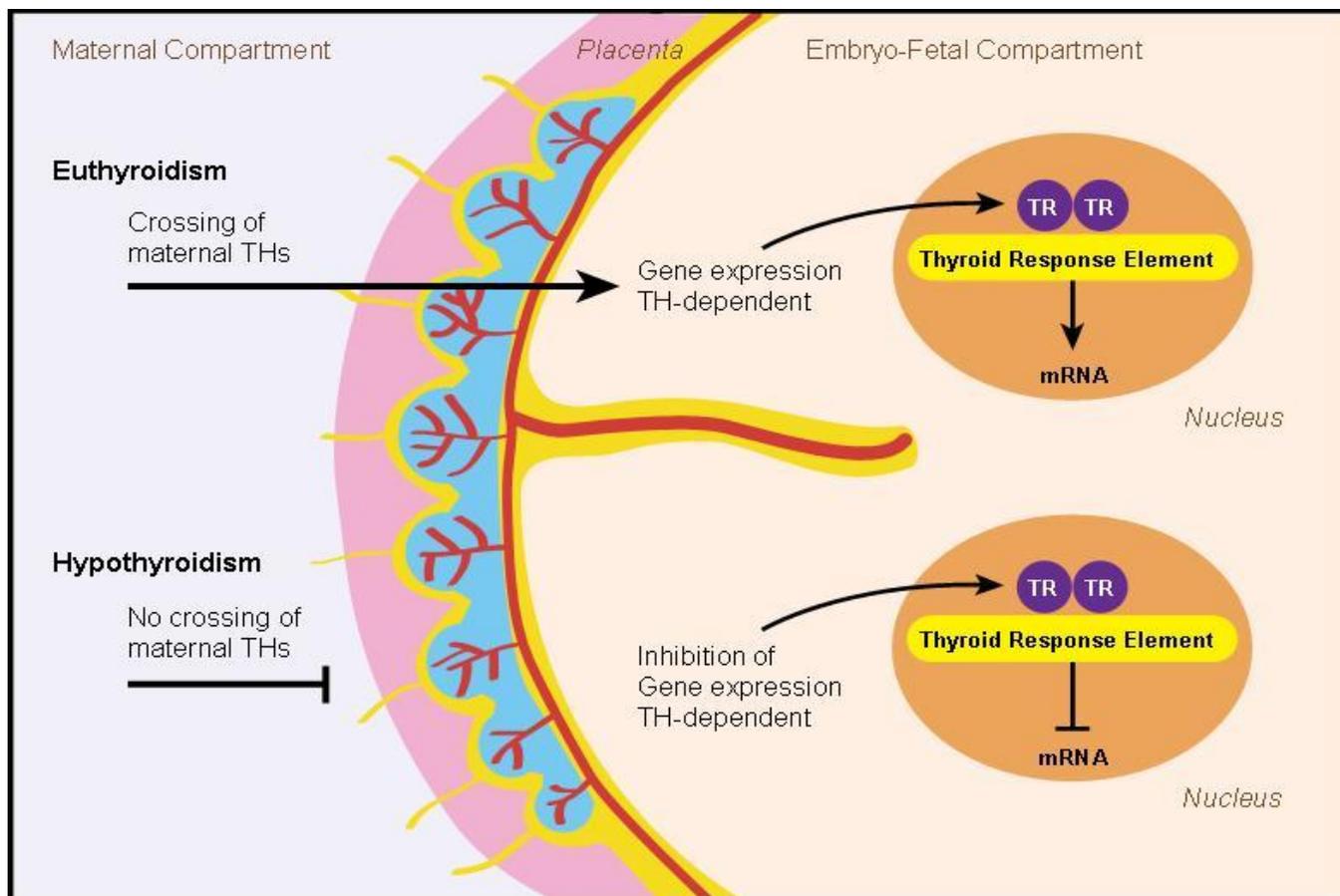


Figure 1. Maternal thyroid hormone action during embryo-fetal development. Maternal thyroid hormones (THs) cross the placental barrier as early as E11.5 (embryonic day 11), before fetal thyroid function (FTF) is active, and transactivate gene expression through embryonic thyroid receptors (TRs). By contrast, when function of the maternal thyroid gland is blocked (hypothyroidism: low T3 and T4 and high TSH), transplacental transfer of THs is inhibited, and TH-dependent gene expression is not transactivated by TRs.

The diverse effects of TR might be achieved via combinatorial complexes of TR with various cellular proteins. Many TR-interacting proteins have been reported to be involved in TH action by modulating expression of positive TRs targets and regulating the response to set levels of T3 (22).

Thyroid hormone action, embryo-fetal development, and mouse models

Obregon et al. (12) showed that maternal THs cross the placental barrier at an early stage (E11.5) of rat embryonic development, prior to the onset of FTF. Recently, we confirmed and extending this finding, showing (23) *in vivo* (a transgenic mouse model) that maternal THs cross the

placental barrier, bind embryonic TRs, and activate transcription in different primordia as early as E11.5, a crucial stage of central nervous system (CNS) development before the onset of FTF (E15.5-E17.5) (*Figure 1*). Previous studies detected T4 and T3 by RIA (radioimmunoassay) in E10-E12 rat trophoblasts and E13-E20 embryos and placentas, as well as in amniotic fluid (12). In contrast, Quignodon et al. demonstrated T3 signaling at E15.5 (late stage of CNS development) in the midbrain roof by the use of a chimeric yeast Gal4 system (24).

Our mouse model (23) allows us to trace TH action during early and late development by following the expression of β -galactosidase (beta-gal) driven by the TH-dependent murine thyroid hormone response element (TRE2 \times) from the myelin basic protein gene. Results of *in vitro* experiments show that TRE2 \times transactivation is strongly responsive to T3 action by TR β 1, TR β 2 or TR α 1, but not to other nuclear receptors and ligands such as estrogens or retinoic acid (23). To the best of our knowledge, this is the first transgenic mouse model that can trace the action of THs during diencephalon differentiation (prosencephalic regionalization) (*Figure 2*)(23). The diencephalon includes the thalamus, hypothalamus, and epithalamus, and is involved in the physiological regulation of several biological processes. It serves as a relay for sensory input and plays an important role in the regulation of several motivated behaviors. In addition, it is an important part of other motor and sensory pathways and other pathways that link the cortex to the cerebellum and other subcortical structures. It exerts its effects in part by regulating the release of hormones from the pituitary gland.

We found β -gal expression in the diencephalon primordium beginning at E11.5-E12.5 and continuing until E13.5, which was lost in the embryos of hypothyroid pregnant mice (23). Importantly, T3 treatment of hypothyroid pregnant transgenic mice from E9.5 to E12.5 rescued β -gal expression in the diencephalon primordium of E12.5 transgenic embryos, reproducing the pattern observed in embryos of euthyroid transgenic mice (23). These results showed that TRE2 \times transactivation was specifically driven by TH (23). In addition, our data reveal β -gal expression in the small intestine primordia at E17.5 (late fetal development) (*Figure 2*) (23). This result highlights that THs are involved in mammalian intestinal epithelial development (23).

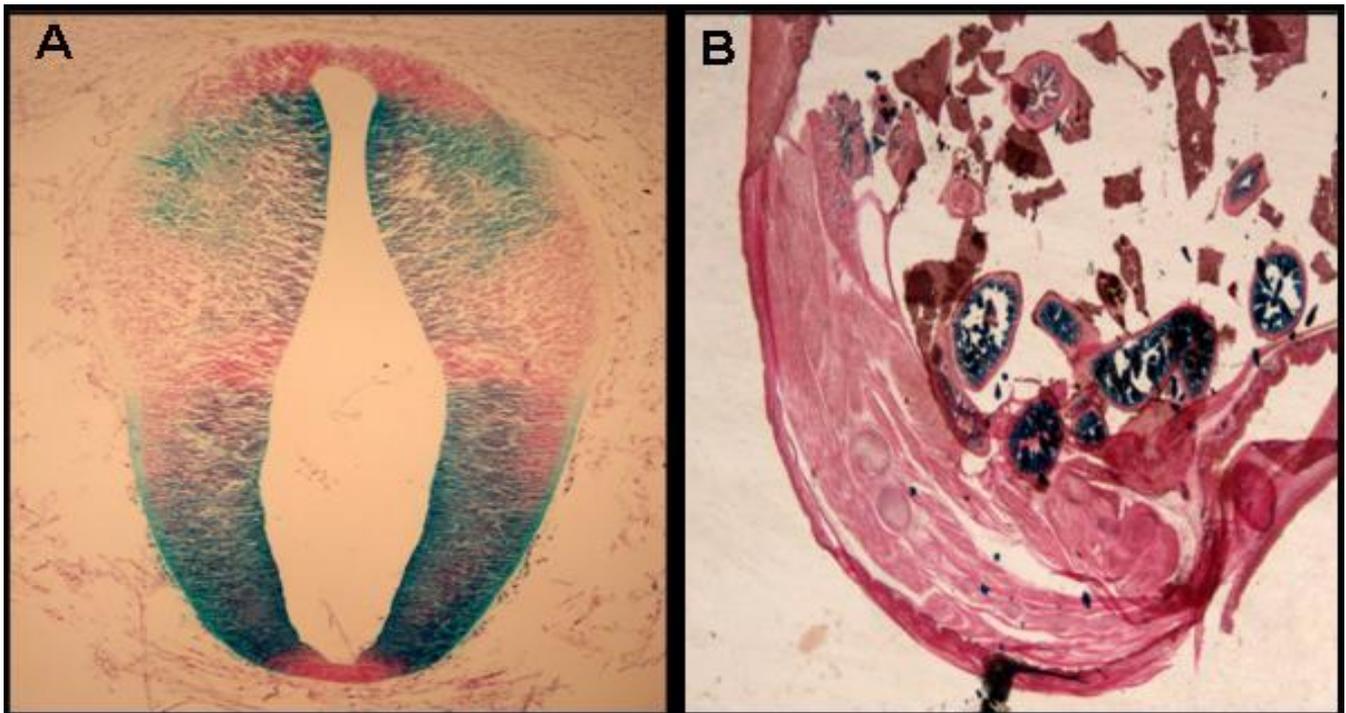


Figure 2 Early and late embryo-fetal development: results from the $TRE2x$ transgenic mouse model that ubiquitously expresses a reporter gene ($LacZ$) tracing thyroid hormone action. (A) 10x magnification of the diencephalon of E12.5 $TRE2x$ positive transient transgenic mouse: β -gal expression (blue, counterstained with neutral red) in the lateral walls of the III ventricle. (B) Low magnification of the abdomen of E17.5 $TRE2x$ positive transgenic mouse: β -gal expression (blue, counterstained with neutral red) in the ileum.

Other transgenic mouse models have been used to study TH action during embryo-fetal development. Quignodon et al. used the yeast UAShsp68/Gal4/TR α 1/LacZ system and found β -gal expression in the roof of the midbrain at late stages (E15.5) when FTF is already active (24). Nagasawa et al. used TR β 1/LacZ system and described β -gal expression in the midbrain, auditory vesicles, limbs and face at early stages (E9.5-E12.5), and in the root of whisker follicles and the intestine primordia at E17.5 (25).

Molecular mechanisms of thyroid hormone function and thyroid hormone-dependent gene expression

The molecular mechanisms by which TH impacts brain development are becoming better understood. TH appears to regulate fate specification of early cortical neurons (26), migration of cortical (17) and cerebellar neurons (27), synaptogenesis (28, 29) and apoptosis (30, 31). However, the specific genes that are directly regulated by THs through the TRs, and which account for TH effects on specific developmental events, are poorly characterized. In rodents, most efforts to identify

TH-regulated genes have focused on the postnatal period. Moreover, we know little about the DNA regulatory elements through which TRs exert their actions on gene regulation. In extra-thyroidal tissues, T3 concentration in the intracellular and nuclear compartments is dependent on (i) the circulating levels of THs, (ii) their rates of entry and exit in and out of the cell and the nucleus, (iii) the rate of T4 to T3 conversion, and (iv) T3 degradation in the cell. The formation and degradation of T3 in tissues are dependent primarily on the activities of three seleno-deiodinases - type 1 (D1), type 2 (D2) and type 3 (D3) - that catalyze the selective removal of iodine from iodothyronines and convert the precursor T4 into the active hormone T3 and the inactive hormones reverse T3 and T2 (32, 33). Cellular uptake and release of THs are mediated by several transporters. Among these, OATP1C1 (Na-independent organic anion transporting polypeptide) is expressed almost exclusively in brain (microvessels and the choroid plexus) and mediates intracellular transport of T4; while monocarboxylate transporter 8 (MCT8) mediates intracellular transport of different iodothyronines. Remarkably, MCT8 is localized at the basolateral membrane of thyrocytes and is particularly important in the secretion of TH from the thyroid gland (34) and in the transport of T3 into the brain (35-37). MCT8 was recently identified by Friesema et al. (38) and found to be expressed in normal human placenta at 6 weeks of gestation, with increasing expression during advanced gestation (39). In addition, human placenta expresses high levels of D3 which appears to be important in maintaining circulating and tissue levels of fetal THs at a level that is exceedingly low relative to maternal or adult levels. This mechanism suggested that TH levels during development are tightly regulated within narrow limits and that D3 and D2 are critical for this specific regulation (40). Overall, the concentration of active T3 in the brain depends on T3 transport through the blood brain barrier, which is mediated by MCT8, and on the activity of D2 generating T3 from T4. In addition, Galton et al. have shown that in the brains of mice with disrupted D2 gene, indicating that the T3 generated by D2 may not be equivalent to the T3 reaching the brain from the circulation (41).

However, few studies (30, 42-44) have attempted to identify TH-responsive genes in the developing brain using functional genomics approaches. Dong et al. recently used a large-scale approach to identify direct gene targets of TH action in the mouse brain during the post-neonatal

period (45). They identified TR β 1 binding fragments corresponding to 91 genes in the cerebellum of postnatal day 19 male mice. One of the genes identified in the study is SMS (spermine synthase), which showed TR binding sites in the promoter region. SMS is essential for transforming spermidine to spermine, a ubiquitous cellular polyamine that plays critical roles in many cellular processes, including transcription and translation (46). Importantly, SMS deficiency in mice is associated with deafness, inner ear abnormalities, and hyperactivity (47). All of these phenotypes are common manifestations of developmental hypothyroidism.

Morte et al. identified genes differentially expressed in the cerebral cortex of hypothyroid rat fetuses on day 21 after conception compared to normal fetuses. Functional analysis revealed genes involved in the biogenesis of the cytoskeleton, neuronal migration and growth, and branching of neurites. Twenty percent of the differentially expressed genes were related to each other and centered on the Ca²⁺ and calmodulin-activated kinase (*Camk4*) pathway. In primary cultures of fetal cortical neurons, the *Camk4* gene was regulated directly by T3 (48).

Recent results confirm the critical role of adequate maternal TH levels in proper fetal neocortical cyto-architecture and underscore the importance of early thyroxine replacement for hypothyroid mothers (49). The decreased number and length of radial glia, loss of neuronal bipolarity, and impaired neuronal migration seen in offspring of animals deficient in thyroid function were prevented by early TH replacement. The authors found that reelin is regulated by TH. Reelin down-regulation in hypothyroidism is not due to enhanced apoptosis in Cajal-Retzius cells nor is it mediated through brain-derived neurotrophic factor-tyrosine receptor kinase B alterations. Hypothyroidism significantly increased TR α 1 while decreasing expression of reelin, apolipoprotein E receptor 2, very low-density lipoprotein receptor expression, and thereby decreasing activation of cytosolic adapter protein disabled 1, compromising reelin signaling. In addition, integrins α 5 and β 1 were significantly decreased without alteration of α 3, showing intact neuroglial recognition but disrupted adhesion and glial end-feet attachment (49).

The action of TR β 2 requires T3 at appropriate developmental stages (23, 50). For example, during retinogenesis, TR β 2 coordinates cone opsin patterning in response to the increasing TH levels

that accompany development (50). In mice, the concentration of circulating TH rises progressively during postnatal development, providing a temporal signal that induces the expression of M opsin in the second postnatal week. In TR β 2-deficient mice, cones lack M opsin and express only S opsin, indicating a crucial role for TH signaling in opsin patterning and maturation of the color visual system. In mice with congenital hypothyroidism, induction of M opsin expression is impaired; conversely, treatment with excessive T₃ suppresses the induction of S opsin expression at early stages. Exposure to excessive T₃ at inappropriate neonatal stages promotes cone-specific cell death.

Morphological changes in the fetal brain due to maternal hypothyroidism or hypothyroxinemia

THs have effects in three different phases of brain development: early gestation, late gestation and early postnatal. TH influence on brain development depends first of all on availability, which in turn depends on maternal/fetal TH production and on the presence of transporters and deiodinases in neural cells. Moreover, the expression of different TH receptors in neurons varies through time and space, making different brain areas and cell types sensitive to TH at precise times during development. Perturbing the normal occurrence of these events can have a wide range of consequences on neural development.

The precise timing of TH deficits correlates with the time course of the events occurring during brain development. The developing brain is most sensitive to THs during the first 2-3 weeks in rats, corresponding to the first 2 years of age in humans. Maternal TH economy is strongly influenced by pregnancy, and requires a doubling of T₄ production, necessitating a corresponding increase in iodine availability. In some cases, the mother cannot meet these requirements, and TH levels decline (2).

Endemic cretinism is classically defined as a condition that includes: a) endemic goiter and severe iodine deficiency; b) clinical manifestations with a predominant neurological syndrome; and c) correction of the syndrome by providing adequate iodine.

Neurogenesis, myelination, dendrite outgrowth, and synaptogenesis occur during late embryonic and postnatal development of the brain, especially in cerebral cortex, and insufficient TH concentration in

plasma alters cortical development. Subtle decreases in maternal THs have dramatic consequences on fetal brain development: clinically, impairments in neuropsychological development and cognitive function have been observed in offspring of women with hypothyroidism. Maternal hypothyroidism is also associated with intelligence quotient decrement in offspring (2-5). Experimental rodent models of maternal hypothyroidism exhibit both morphological changes, including altered cortical lamination, and functional changes, including hearing loss, delayed eye opening, poor performance on maze tests and impaired motor development. TH deficiency can also alter synaptic transmission in the hippocampal formation and affect long-term potentiation (LTP). Rovet and Simic (51) show that a transient decrease in maternal THs in the fetus affects the development of visual abilities. TH defects during pregnancy have been also associated with autism (52).

Although the details of the mechanisms underlying TH action during early embryo-fetal development remain to be elucidated, recent studies have identified some potential gene targets of TH action during late development. Microarray analysis comparing hypothyroid E21 fetuses to controls identified several genes which are differentially expressed in cerebral cortex, including genes involved in the biogenesis of the cytoskeleton, neuronal migration and growth, and neurite branching (48). One of these, the Ca^{++} and calmodulin-activated kinase (Camk4) gene, is directly regulated by T3 in primary cultures from fetal cortex. Therefore, the Camk4-cAMP-responsive element binding protein 1 (Creb1) pathway is thought to play a relevant role in TH-dependent cortical development.

THs are widely believed to regulate genes that control formation of the corpus callosum and neuronal migration. In fact, maternal hypothyroidism alters the development of radial glial cells and leads to permanent structural alterations in the neocortex and hippocampus. Recently, heterotopias were reported in the corpus callosum of developmentally hypothyroid rats, indicating that slight TH insufficiencies can induce cortical dysplasia (53). Maternal hypothyroxinemia in mice clearly alters neuronal migration during neocortico-genesis (54). In the developing cerebral cortex, cortical progenitors migrate to form the upper layers with an inside-out gradient using the scaffold of radial glia and influenced by secretion of reelin from Cajal-Retzius cells in the marginal layer (55). Reelin binds to apolipoprotein E receptor 2 (ApoER2) and very low-density lipoprotein receptor (VLDLR),

inducing phosphorylation of cytosolic adapter protein disabled 1 (Dab1); both reelin and Dab1 have been identified as targets of TH action (56). The reelin gene has specific intronic TH-responsive elements (TRE), thus, TH regulation of reelin expression is likely involved in regulation of neuronal migration (49). Maternal hypothyroidism down-regulates reelin levels and causes morphological changes in neuroglia (49).

During development, THs can affect both progressive and regressive events. One of the major players in the control of neurogenesis and apoptosis is the gaseous neurotransmitter Nitric Oxide (NO), produced by neuronal NO synthase (NOS) expressed in newly generated neurons. Maternal THs repress neuronal NOS expression, affecting neurogenesis and neuronal survival in the rat embryo (57).

Deficient cellular maturation has been found in the cerebral cortex of hypothyroid rats (58). Defects in the myelination of the cerebral cortex and the maturation of neuronal circuits lead to permanent brain dysfunction (59). Most studies have focused primarily on cerebral cortex, due to the finding of cognitive impairment in cretinism, other brain structures are affected by low TH levels as well. For example, THs increase the rate of neuronal proliferation in the cerebellum (60), and act as the “time clock” to end neuronal proliferation and stimulate differentiation (60, 61). THs can influence the orderly pattern of migration to appropriate regions in the brain, particularly in the cerebral and cerebellar cortex (62), and stimulate the outgrowth and development of axons and dendrites (63). Intriguingly, maternal hypothyroidism affects maturation of cerebellum and leads to defects in granule cell migration, Purkinje cell arborization, timing of apoptosis, and neuronal integration (64, 65). In addition, a deficiency of THs in the neonatal rat has been shown to play an important role in the disorganization of the cerebellar cortex (28, 66, 67). In hypothyroid rats the external germinal layer is reduced at P35 and granule cells remain in a proliferative phase longer than in controls, resulting in decreased cell differentiation (68). Rat hypothyroidism also delays migration of cerebellar granule cells from the external germinal layer to the internal granular layer (69). Cerebellar development is delayed compared to cerebral cortex, thus these effects occur mostly after FTF onset. Other studies

confirm that reduction or absence of THs during brain development results in molecular, morphological, and functional alterations in the cerebellum (70, 71).

Finally, along with morphological alterations in brain development and functional impairment, TH imbalances can also have profound biochemical effects on neurotransmitter expression due to their action on the expression of key molecules in neurotransmitter production and catabolism. TH defects affect cholinergic neurons in a regionally selective way (72), and type II deiodinase (D2) affects the local production of serotonin (73). In hypothyroidism, monoamines (serotonin and dopamine) are decreased in the rat brain (74). In addition, the expression of GABA is significantly increased (75) and choline acetyl transferase (ChAT), a key enzyme in the production of Ach, significantly decreases (76) because TH serves as positive regulator for the ChAT gene expression (77).

Conclusions

In the past 15 years relevant discoveries have been made in the field of TH action and development. In humans, maternal THs are crucial for the normal development of the human CNS. TRs show a tissue-specific ontogenetic expression during embryo-fetal development. Maternal THs and embryonic TRs act synergistically to regulate transcription of target genes involved in the proliferation, migration and differentiation of different cytotypes of the central nervous system, intestine, and other embryo-fetal primordia before fetal thyroid function is active. Existing mouse models that identify TH and TR action during embryo-fetal development may have clinical relevance, facilitating the design of endpoint assays and the testing of new molecules that modulate or mimic TH action.

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CLINICAL ASPECTS OF *BRAF* MUTATION IN THYROID CANCER

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ABSTRACT

The *BRAF*^{T1799A} mutation has been extensively studied in recent years in thyroid cancer, particularly for its aggressive role and clinical potential in papillary thyroid cancer (PTC). This mutation is the most common known genetic alteration in PTC, seen in 45% of cases on average, with a lower prevalence in low-stage disease. The mutation causes a change in amino acid at position 600 from valine to glutamic acid in the *BRAF* protein kinase, constitutively activating it and resulting in aberrant activation of the MAP kinase signaling pathway. Most of the studies have shown a strong association of *BRAF*^{T1799A} mutation with aggressive clinicopathological outcomes of PTC, including tumor extra-thyroidal extension, lymph node metastasis, advanced TNM stages, loss of radioiodine avidity, disease persistence/recurrence, and even patient death. The aggressive role of *BRAF*^{T1799A} mutation has also been demonstrated in low-stage and micro-PTC. This mutation is therefore generally viewed as a strong prognostic molecular marker for poorer prognosis of PTC. The *BRAF*^{T1799A} mutation can be tested for on thyroid fine needle aspiration biopsy specimens to assist diagnostic evaluation of thyroid nodules and preoperative risk stratification of PTC. Information from such testing can be helpful in guiding early decision making on surgical and medical treatments of thyroid nodules and PTC. The *BRAF*^{V600E} mutant has also been demonstrated to be an effective therapeutic target in thyroid cancer. Use of *BRAF* mutation as a diagnostic and prognostic molecular marker as well as a therapeutic target has shown an increasing impact on our current management of thyroid cancer.

Key Words: *BRAF* mutation, thyroid cancer, molecular marker, diagnostic marker, prognostic marker, therapeutic target.

Introduction

As the most common endocrine malignancy, thyroid cancer has caught considerable attention globally. An important, and often controversial, issue related to this cancer is how to optimally manage it. Giving the rapidly rising incidence of thyroid cancer in recent years (1-3), this issue becomes even more challenging. The controversy starts at the initial evaluation of thyroid nodule, the clinical “precursor” of thyroid cancer, where diagnostic and therapeutic dilemmas are a subject of frequent debate (4,5). It continues at surgical decision making on how to optimize the type and extent of surgery and subsequently at the medical decision making on how to optimize postoperative treatments, such as radioiodine ablation and follow-up surveillance. The fundamental goal in making these efforts in the management of thyroid cancer, from which controversies can arise, is to optimally balance the benefit against the harm of treatments. To this end, clinicopathological criteria are currently the main, and often the only, “gold standard” that is universally used for risk stratification and guidance of the management of thyroid cancer. This practice has been improved significantly in recent years by better understating of clinicopathological behaviors of thyroid cancers from numerous recent studies and the increasing use of certain imaging modalities, such as ultrasonography. Expert opinion- and evidence-based practice guidelines have also been greatly helpful in optimizing the management of thyroid cancer as exemplified by the stellar practice guidelines from the European Thyroid Association (6) and American Thyroid Association (7).

Nevertheless, there is still great room for further improvement in the efficiency and efficiency of today’s practice of thyroid cancer medicine, particularly in those on-going controversial areas. It is unlikely that this can happen only based on optimizing the use of classical clinicopathological criteria. New promises lie in molecular medicine. Specifically, thyroid cancer molecular markers may have the best potential in helping improve the diagnostic, prognostic, and therapeutic efficiencies for thyroid cancer. In this regard, the widely investigated *BRAF*^{T1799A} mutation, as a unique diagnostic and prognostic genetic marker and effective therapeutic target for thyroid cancer, has shown great promises. Its clinical potential has recently drawn considerable attention from clinicians around the world who manage patients with thyroid cancer. Summarized here are several major clinical aspects of *BRAF*^{T1799A} mutation in thyroid cancer, which reflects how this marker can be practically useful in the management of this cancer.

BRAF Mutation in Thyroid Cancer

BRAF is one, and often the most potent one, of the three Raf kinases (A, B, C) that relay the signaling of the Ras → Raf → MEK → MAP kinase/ERK pathway (MAPK pathway) (8,9). The diagram in Figure 1 illustrates this pathway. Numerous mutations have been discovered in the *BRAF* gene in human cancers, but the most common one is the *BRAF*^{T1799A} point mutation that causes an amino acid change in codon 600 from valine to glutamic acid in BRAF. The resultant BRAF^{V600E} is a

constitutively activated serine and threonine protein kinase, causing oncogenic over-activation of the MAPK pathway (10).

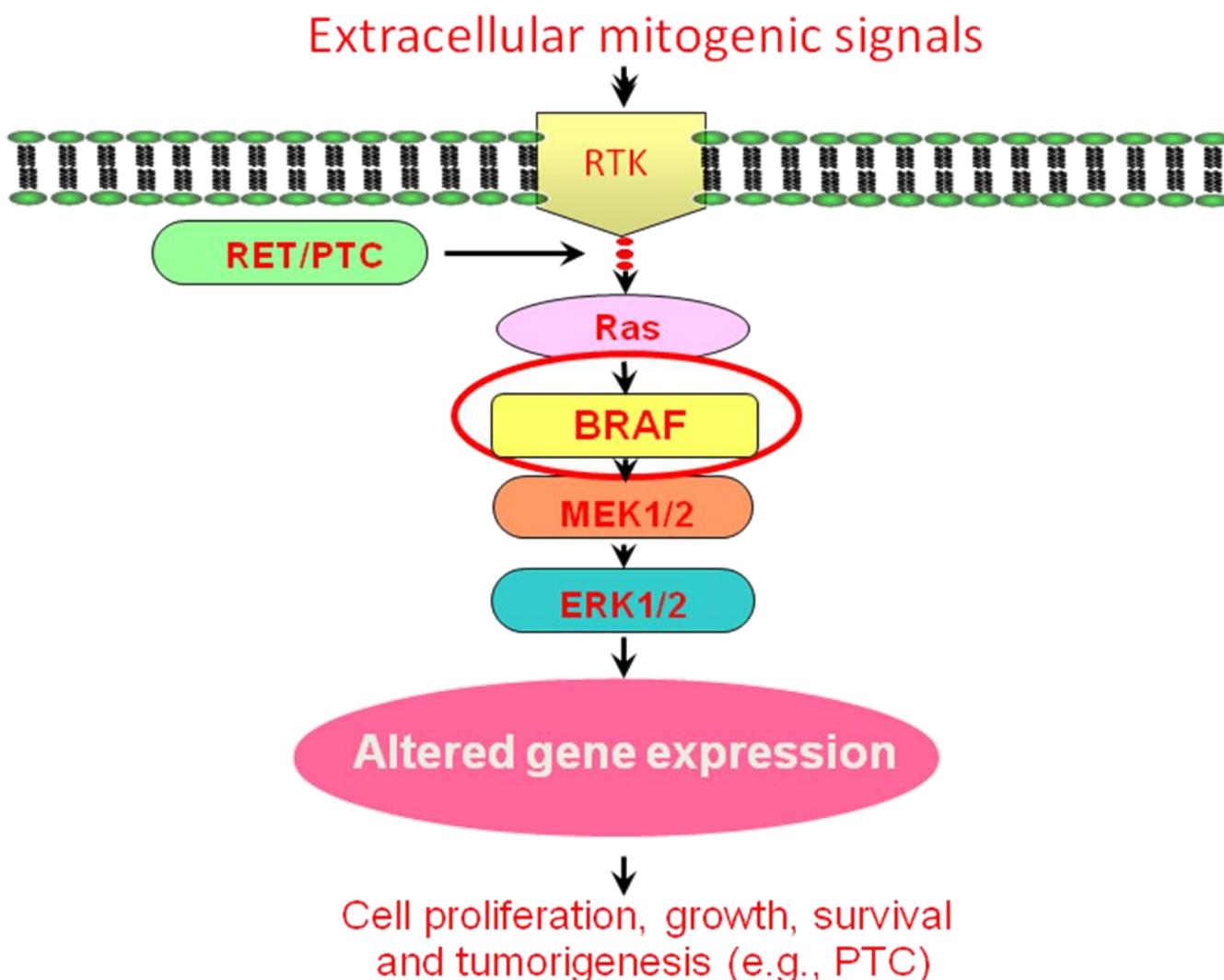


Figure 1. Illustration of the MAP kinase signalling pathway. This figure is adapted from reference 13. RTK, receptor tyrosine kinase.

This is now a well-known mechanism involved in the tumorigenesis of papillary thyroid cancer (PTC). Since the initial submission and acceptance of the first manuscript of a Johns Hopkins group on the discovery of the $BRAF^{T1799A}$ mutation in thyroid cancer seven years ago (11), numerous studies have been published on this mutation in thyroid cancer. Among different thyroid tumors, the $BRAF^{T1799A}$ mutation occurs exclusively in PTC and what appears to be PTC-derived anaplastic thyroid cancer (ATC), in 45% of the cases of the former and 25% the latter on average (12). The prevalence of $BRAF^{T1799A}$ mutation in small thyroid cancers or cancers with low stages, such as TNM stages I and II, was generally lower, with a prevalence of about 35% (13). No $BRAF^{T1799A}$ mutation has been found in benign thyroid tumors and follicular thyroid cancer. A Greek study reported a high prevalence of

BRAF^{T1799A} mutation in medullary thyroid cancer (14); no other studies demonstrated the presence of this mutation in this type of thyroid cancer. Although several other types of *BRAF* mutation have been discovered in PTC, they are uncommon and the *BRAF*^{T1799A} mutation is most important in the tumorigenesis of PTC (15-17). The clinical utility of *BRAF* mutation in PTC has therefore been investigated virtually exclusively for the *BRAF*^{T1799A} mutation, which, for simplicity, is termed *BRAF* mutation hereafter in this review.

Diagnostic Value of *BRAF* Mutation for Thyroid Cancer

Many investigators have been interested in the diagnostic value of *BRAF* mutation testing on fine needle aspiration biopsy (FNAB) specimens in the evaluation of thyroid nodules. As can be expected, the specificity of *BRAF* mutation as a diagnostic marker on FNAB specimens for PTC was exceptional as it uniformly achieved 100% in several early studies (12). However, the diagnostic sensitivity of *BRAF* mutation was poor when used in the general evaluation of thyroid nodules. Although about 45% of PTC harbored this mutation, only a few percent of thyroid nodules in general are PTC. Therefore, if applied to the general diagnostic evaluation of thyroid nodules, very few percents of thyroid nodule patients may be found to be positive for this mutation, giving a low diagnostic sensitivity. This diagnostic sensitivity was increased to around 8% on FNAB specimens that fall into the classical indeterminate category (12). This is still a low sensitivity that is unlikely to be generally applicable, if used alone, in most areas of the world where the *BRAF* mutation rate is around 45%. However, the diagnostic sensitivity of *BRAF* mutation was significantly increased when used in combination with cytological characteristics of a thyroid nodule, particularly when suspicious for PTC (18,19).

Vitale's group demonstrated that when combined with galectin-3, *BRAF* mutation detected on FNAB specimens showed a significantly increased diagnostic sensitivity (20). Nikiforov's and Pacini's groups have recently tested the diagnostic value of combinational use of *BRAF* mutation with *Ras* mutations, *RET/PTC* rearrangement, and *PPAR γ /Pax8* rearrangement in assisting the evaluation of thyroid nodules and reported an increased diagnostic sensitivity (21,22). It remains to be investigated how these strategies could alter the current practice. Unlike *BRAF* mutation, which is a cancer-specific marker, the other three genetic alterations have been widely found also in benign thyroid conditions, including adenomas and even Hashimoto's thyroiditis, with relatively high prevalences (23). Therefore, the diagnostic specificity of using *BRAF* mutation in combination with these additional mutation markers could be potentially problematic although its diagnostic sensitivity is increased compared with the use of *BRAF* mutation alone. Good diagnostic utility of a molecular marker combination approach will likely rely on the discovery of new molecular markers that, like the *BRAF* mutation, are highly specific for thyroid cancer.

It should be noted that the diagnostic utility of testing for *BRAF* mutation alone on FNAB may have a special clinical place in regions that have a high prevalence of *BRAF* mutation, such as South

Korea, where PTC accounts for >90% of thyroid cancers and *BRAF* mutation is exceedingly prevalent, up to >80% in some series (24-26). The diagnostic sensitivity of *BRAF* mutation on FNAB in Korea is therefore exceedingly high, unlike most of the regions in the world where a modest prevalence of *BRAF* mutation in PTC has been generally reported (12,13). Aside from the diagnostic value, testing for *BRAF* mutation on FNAB has a special and more important clinical place for its high prognostic value in preoperative risk stratification of thyroid cancer as will be further discussed.

Prognostic Value of *BRAF* Mutation in Papillary Thyroid Cancer

There are few molecular markers that have been so extensively studied as *BRAF* mutation for its prognostic value. Previous meta analyses on a large number of studies clearly showed an association of *BRAF* mutation with high-risk clinic-pathological characteristics of PTC, including extrathyroidal extension, lymph node metastasis, and advanced TNM stages III and IV (13, 27). There have been also a few individual studies that failed to show significant association of *BRAF* mutation with high risk pathological characteristics (28, 29). Of these studies, the one by Fugazzola et al was a pooled multicenter analysis from Italy that failed to show a significant prognostic role of *BRAF* mutation (29). However, many other Italian investigators, including some co-authors of the Fugazzola et al paper, later were able to show a significant prognostic role of *BRAF* mutation for poor clinico-pathological outcomes of PTC when examining individual series from their institutions (13, 30, 31, 35). The reason for this discrepancy is not clear. Possible variation in pathological characterization of tumors and extent of thyroidectomy and neck dissection could potentially be among the explanations. Variation in diagnostic criteria of clinical disease progression, such as tumor persistence and recurrence, could be another contributing factor for the inconsistent findings on the prognostic value of *BRAF* mutations in these studies.

A recent updated meta analysis on numerous studies continued to show a strong relationship of *BRAF* mutation with aggressive clinicopathological characteristics of PTC (30). This relationship was even seen in papillary thyroid microcarcinomas (PTMC) in several studies (31-34). Importantly, *BRAF* mutation is also demonstrated to be associated with PTC persistence/recurrence in many studies (27,13,30) and even increased patient mortality (35). The findings from these numerous studies on the clinicopathological association for the *BRAF* mutation in PTC have confirmed, in virtually all aspects, the results of an early report of Xing et al on a comprehensive examination of the association of *BRAF* mutation with extrathyroidal extension, lymph node metastasis, advanced stages III and IV, and recurrence/persistence of PTC (36). The Xing et al study for the first time reported a strong association of *BRAF* mutation with persistence/recurrence of PTC, which was subsequently confirmed in a number of studies around the world (Fig. 2). The association of *BRAF* mutation with PTC persistence/recurrence was seen even in PTC of low TNM stages (I and II) in the Xing et al study, which was confirmed subsequently by Kebebew et al (37). In the Xing et al study, patients with

BRAF mutation in PTC required more aggressive treatments for recurrent diseases, including repeated surgical intervention and external beam radiation therapies (36).

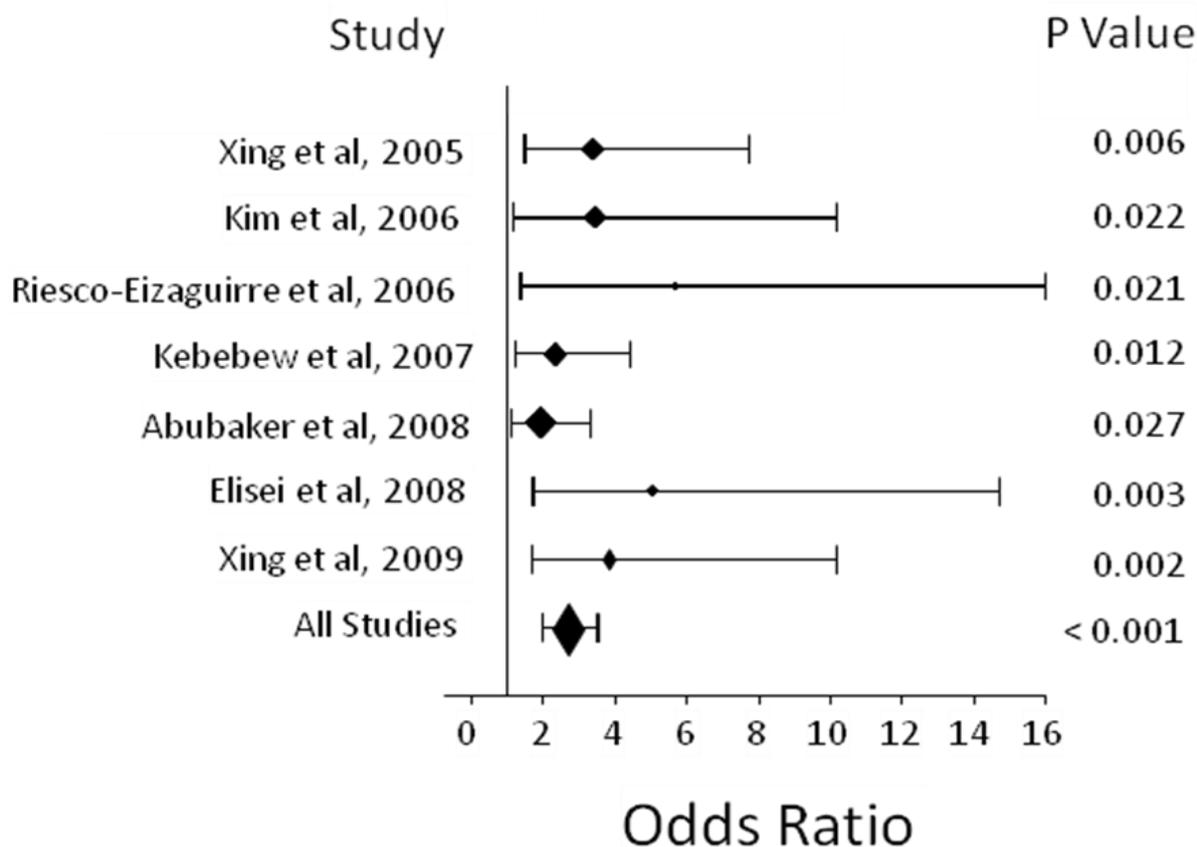


Figure 2. Meta-analysis of the association of *BRAF* mutation with the recurrence of papillary thyroid cancer. Shown are odds ratios for tumor recurrence with *BRAF* mutation in the indicated studies. These studies reported specific information on how to define tumor recurrence, including the criteria used and the follow-up time to monitor the recurrence. Citations of these studies are detailed in reference 30, from which this figure is adapted.

This finding has been confirmed in a recent study by Yip et al (38). Also an interesting and initial observation in the Xing et al study was the association of *BRAF* mutation with decreased or absent radioiodine avidity of recurrent PTC (36), which was confirmed in several subsequent studies (39-41). This phenomenon is an important cause of the failure of *BRAF* mutation-harboring PTC to respond to radioiodine treatment and hence disease persistence/recurrence. An underlying molecular mechanism is the aberrant silencing of the thyroidal iodide-handling genes, including those for the sodium-iodide symporter (NIS), thyroid-stimulating hormone receptor, thyroperoxidase and thyroglobulin as shown in numerous studies (13,30). An interesting recent study by the Santisteban's group demonstrated that the *BRAF*^{V600E} promoted silencing of NIS through an autocrine loop that involved and required the release of transforming growth factor (TGF) β and the activation of the TGF β signalling system (42). It remains to be investigated whether this is a mechanism generally involved in

$BRAF^{V600E}$ -promoted silencing of thyroid iodide-handling genes in PTC. Other important molecular mechanisms underlying the aggressive role of *BRAF* mutation in PTC include over-expression of various tumor-promoting molecules, including proto-oncogenes, and aberrant silencing of various tumor suppressors promoted by the *BRAF* mutation (13, 30). Thus, the powerful prognostic value of *BRAF* mutation for poorer prognosis of PTC, as reflected by an odds ratio of 3-5 for disease persistence/recurrence (30), has both strong clinicopathological and molecular bases.

Use of *BRAF* Mutation in Risk Stratification for Conventionally Low-risk Papillary Thyroid Cancer

Given the strong association of *BRAF* mutation with aggressive clinicopathological outcomes in most studies, it has become increasingly recognized and anticipated that this mutation can be a useful prognostic genetic marker in the risk stratification of PTC. In fact, *BRAF* mutation is already used in many institutions, including the author's, as a prognostic marker to assist the management of patients with PTC. There are several clinical areas that could be particularly helped with this prognostic use of *BRAF* mutation. Among these is the management of conventionally low-risk PTC patients, such as those with TNM stage I disease and PTMC. Although patients with these cancers generally have a low recurrence and mortality rate, many of them seem to be bound to recurrence and some even to death. These patients may lack obvious conventional high-risk clinicopathological characteristics. Consequently, it is often impossible, based on the current risk criteria, to predict the clinical course of the disease in these patients. Dilemma then often exists with respect on how to optimally treat these patients to prevent disease progression and at the same time balance against the increased risk of aggressive treatment-associated adverse effects. For example, whether to pursue total thyroidectomy vs lobectomy, neck dissection vs no neck dissection, and radioiodine treatment vs no radioiodine treatment has been particularly controversial in the management of these patients. Given the relatively low risk of progression in these patients, the recent ATA guideline on the management of differentiated thyroid cancer holds a conservative stance on these patients; for example, it recommends lobectomy with no radioiodine treatment for PTMC in the absence of classical clinicopathological risk factors, such as lymph node metastasis and extrathyroidal extension (7). As *BRAF* mutation is a strong and independent predictor of PTC persistence/recurrence (35-37) with a power higher or at least equal to some of the conventional clinicopathological risk factors of PTC, such as extrathyroidal extension and lymph node metastasis (13, 30), it should be reasonable to consider total thyroidectomy for patients with *BRAF* mutation-positive PTMC and perhaps even prophylactic central neck dissection in selected patients by experienced surgeons. This seems to be justifiable particularly given the association of *BRAF* mutation with the loss of radioiodine avidity of PTC, which may make it difficult to eradicate residual disease or lymph metastases with radioiodine, rendering initial complete surgical eradication of the disease particularly important in *BRAF* mutation-positive patients. The current ATA guideline also recommends sparing radioiodine treatment for

PTMC in patients without classical clinicopathological risk factors (7). Given the established aggressive role of *BRAF* mutation in low-stage PTC and PTMC (13,34), it is reasonable in this clinical setting to regard *BRAF* mutation as an equivalent to a conventional high-risk factor and therefore treat these patients with radioiodine ablation if *BRAF* mutation is positive. This may help maximize the chance of eliminating thyroid cancer cells to prevent future recurrence. Importantly, and more realistically, radioiodine ablation treatment will facilitate the convenience and reliability of the use of serum thyroglobulin testing in these *BRAF* mutation-positive patients who have a higher risk for recurrence and may therefore need particularly vigilant and reliable surveillance during follow-up. It should be noted that an important rationale in this clinical practice using *BRAF* mutation as a risk factor is that its value is at least equal to some of the conventional risk factors that normally prompt relatively aggressive treatments of thyroid cancer (7).

Use of *BRAF* Mutation Testing on FNAB Specimens to Assist Preoperative Risk Stratification of Papillary Thyroid Cancer

BRAF mutation can be easily tested for on thyroid FNAB specimens as shown in numerous studies (12, 18-22, 25, 26, 43, 44). Xing et al recently demonstrated that preoperative testing for *BRAF* mutation on FNAB specimens had a high power in prospectively predicting lymph node metastasis and extrathyroidal extension of PTC as well as a higher recurrence rate when analyzed retrospectively (45). Therefore, although testing for *BRAF* mutation alone on FNAB specimens may have a limited diagnostic value in general evaluation of thyroid nodules, preoperative testing for *BRAF* mutation on FNAB specimens has a unique prognostic value in assisting risk stratification to guide early decision making for the appropriate treatment of PTC. Testing for *BRAF* mutation in conjunction with certain conventional imaging studies, such as ultrasonography, may be expected to be even more helpful in preoperatively assessing the risk level of PTC and guiding decision making for its optimal managements. *BRAF* mutation is the only molecular marker that has been demonstrated to have a significant preoperative prognostic value for PTC. Pathological risk factors, which are currently the main criteria for risk stratification of thyroid cancer, usually become known only postoperatively and therefore have limited value in preoperative surgical planning for thyroid cancer. Preoperative knowledge of *BRAF* mutation status of PTC would be particularly useful to surgeons in defining the initial type and extent of thyroid surgeries, such as total thyroidectomy vs. lobectomy and neck dissection vs. no dissection as discussed above. This knowledge of *BRAF* mutation will also be useful in helping physicians decide the need and extent of radioiodine treatment and the vigilance level of subsequent surveillance and follow up of the patient. Appropriate planning can thus be made at an early stage of the treatment course of the PTC patient. As a prognostic marker, *BRAF* mutation may not be of much value in guiding the management of advanced cases of thyroid cancer as aggressive treatments, including, for example, total thyroidectomy and neck dissection as well as radioiodine ablation, have been well shown to be beneficial and therefore their indication for aggressive treatment

is generally clear. However, it remains to be investigated whether use of *BRAF* mutation can better tailor the management of certain specific issues that may be affected by the presence of *BRAF* mutation in such patients. For example, since *BRAF* mutation is associated with increased resistance of PTC to radioiodine ablation treatment, a relatively high dose of radioiodine might be required to treat these patients. In contrast, a relatively low dose may be reasonable for *BRAF* mutation-negative cases since they are usually sensitive to radioiodine. In the same context, in a right clinical setting, a non-radioiodine treatment, such as external radiation therapy, and a non-radioiodine diagnostic testing, such as PET scan, should be preferred to radioiodine-based measures for PTC. In these clinical settings of PTC patients, the *BRAF* mutation status that has been documented early in the disease course of the patient, such as one obtained through preoperative testing on FNAB specimens, could be very helpful.

Therapeutic Potential of Targeting *BRAF* Mutation in Thyroid Cancer

Another area related to *BRAF* mutation in thyroid cancer that holds great clinical potential is *BRAF* mutation-based therapeutic targeting of this cancer. Using a large number of what were believed to be authentic thyroid cancer-derived cell lines, several studies demonstrated a clear *BRAF* mutation dependence of inhibition of cell proliferation by MEK inhibitors (46-49). Some earlier studies also demonstrated a *BRAF* mutation dependence of cell inhibition by MEK inhibitors although the cells used in these studies were later shown to be mostly non-thyroid cancer-derived cells (50, 51). A new class of recently developed *BRAF*^{V600E} mutant-selective inhibitors, as represented by PLX4720, could potently and selectively inhibit melanoma cells that harbored *BRAF* mutation (52). Recently, Salerno et al (53) tested PLX4720 and another *BRAF*^{V600E} inhibitor, PLX4032, in thyroid cancer cells and, as expected, *BRAF* mutation-selective sensitivity to these inhibitors was clearly demonstrated in these cells. Recent phase I clinical trials showed remarkable therapeutic effects of PLX4032 in melanoma (54,55). These exciting results suggest that *BRAF* mutant-selective inhibitors may be clinically effective for *BRAF* mutation-positive thyroid cancers. Interestingly, in a recent clinical trial, even a non-MAPK pathway-specific inhibitor, motesanib, which is a classical VEGF receptor tyrosine kinase inhibitor, could inhibit thyroid cancer tumor growth preferentially in patients that harbored the *BRAF* mutation (56). It is possible that the *BRAF* mutation-promoted MAPK pathway could enhance or synergize receptor tyrosine kinase-coupled signaling pathways and therefore potentiate the cell sensitivity to their inhibitors.

Combination of a MEK inhibitor with a PI3K inhibitor showed synergistic effects on the inhibition of thyroid cancer cell proliferation (47). Recent studies also demonstrated a synergistic inhibition of cell proliferation and xenograft tumor growth by MEK inhibitors in combination with mTOR inhibitors (49, 57). In the Liu et al study, a better synergism between MEK and mTOR inhibitors was seen in cells with *BRAF* mutation than cells harboring the wild-type *BRAF* gene (49). A potential drawback of the current inhibitors targeting the MAPK pathway is that they only showed anti-proliferative effects on

thyroid cancer cells in all these studies and no pro-apoptotic effects were shown. The lack of pro-apoptotic effects of MEK inhibitors was seen even when they were combined with mTOR inhibitors (49, 57). Therefore, therapeutic targeting of MAPK and mTOR pathways using these particular inhibitors may not be able to eliminate thyroid cancers. Alternative combinations that can promote thyroid cancer cell apoptosis need to be identified for effective treatment of thyroid cancer. Genetic-based targeting of major signaling pathways, such as *BRAF* mutation-based targeting of the MAPK pathway, will likely be an important component of such combination therapy. Liu et al recently demonstrated a dependence of the PI3K/Akt pathway inhibitors on genetic alterations in this pathway in the inhibition of thyroid cancer cells and in the induction of cell apoptosis (58). These results support the concept of genetic-based targeting of thyroid cancer (59).

In summary, *BRAF* mutation is the most common and important oncogenic genetic alteration in PTC. Numerous studies from recent years have clearly demonstrated its clinical potential in the management of PTC. In particular, its value as a diagnostic and prognostic molecular marker and a therapeutic target has been widely recognized. A significant impact, as it has already started to occur, of this genetic marker on the practice of thyroid cancer medicine is expected.

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Management of Graves' orbitopathy on the basis of accurate diagnosis: the role of MRI.Yuji Hiromatsu¹, Junichi Tani¹, Yasuo Teshima², Yoichi Inoue³

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ABSTRACT

Magnetic resonance imaging (MRI) can visualize the inflamed lesions of Graves' orbitopathy (GO). Parasagittal, transverse and coronal sections of T1-weighted, T2-weighted and short inversion time inversion recovery (STIR) images can correlate clinical manifestations with the location of the inflamed lesions. In addition, the measurement of T2 relaxation time or signal intensity ratio of the enlarged muscles in T2-weighted fat suppression images or STIR images provide a precise quantitative evaluation of disease activity and may predict the outcome of immunosuppressive therapy for GO. MRI has potential in the evaluation of new drugs for GO. Therefore, we recommend MRI as a useful tool for the management of GO.

Key-words: Magnetic resonance imaging; Graves' orbitopathy; Glucocorticoids; Clinical activity score.

Introduction

Graves' orbitopathy (GO) is an autoimmune disorder frequently associated with autoimmune thyroid diseases. GO is clinically relevant in 25–50% of patients with Graves' disease and in 2% of patients with chronic thyroiditis (1–5). At the onset of ophthalmopathy, 90% of GO patients have hyperthyroidism and the rest have either euthyroidism or hypothyroidism. It often develops concomitantly with hyperthyroidism, but it may precede or follow hyperthyroidism (4). GO is usually bilateral, but it can be asymmetric or unilateral in 15% of patients (5). Sight is threatened in 3–5% of GO patients and, thus, requires urgent treatment (4).

Histological studies showed that extraocular muscles are widely separated by lymphocytic infiltrations and amorphous accumulation of glycosaminoglycans in the active stage. There are also lymphocytic infiltrations in the expanded fat tissue and lacrimal glands. In the inactive stage, atrophy and fibrosis of muscle bundles are evident. Although there is increasing evidence that the thyrotropin receptor may be the primary autoantigen in GO, and insulin-like growth factor-1 receptor and other antigens shared between the thyroid and orbit are hypothesized as important autoantigens in GO, the precise mechanisms of the development of GO are still unclear (3). The mechanism of the frequent association of GO with Graves' disease also remains unclear. The underlying genetic factors for susceptibility to GO have not been fully elucidated.

As various sites are involved in GO, various symptoms and signs are observed (6) (Fig. 1). In most cases, the diagnosis of GO is obvious: lid retraction combined with unilateral or bilateral proptosis in a person with a history of hyperthyroidism or hypothyroidism. However, in unilateral or unusual cases, orbital imaging is necessary for accurate diagnosis. Orbital imaging, such as magnetic resonance imaging (MRI), computed tomography (CT) and echography, show enlargement of the extraocular muscles, an increase in orbital fat tissue volume and enlargement of the lacrimal glands (7). In addition, orbital imaging reveals abnormalities in 90% of patients with Graves' disease.

Recently, the European Groups on Graves' Orbitopathy (EUGOGO) published recommendations for the assessment of GO using the clinical activity score (CAS) and ophthalmologic examination (8,9). In North America, the International Thyroid Eye Disease Study Group proposed the VISA (Vision-Inflammation-Soft tissue-Activity) scoring system for the assessment of GO (10). According to EUGOGO's recommendation, in their combined thyroid eye clinics, clinicians first assign their GO patients to "sight-threatening", "moderate-to-severe" or "mild" categories, and then determine whether or not the disease is active using CAS. If CAS is more than 3, immunosuppressive therapy is recommended for the treatment of GO (8).

In Japan, orbital MRI has been widely used to assess the activity and severity of GO, since we established the usefulness of MRI using short inversion time inversion recovery (STIR) images or T2 relaxation time (11–18). In this review, we examine the value of MRI for diagnosis and management of GO and for assessment of disease activity and severity, as well as allowing visualization of the involved lesions.

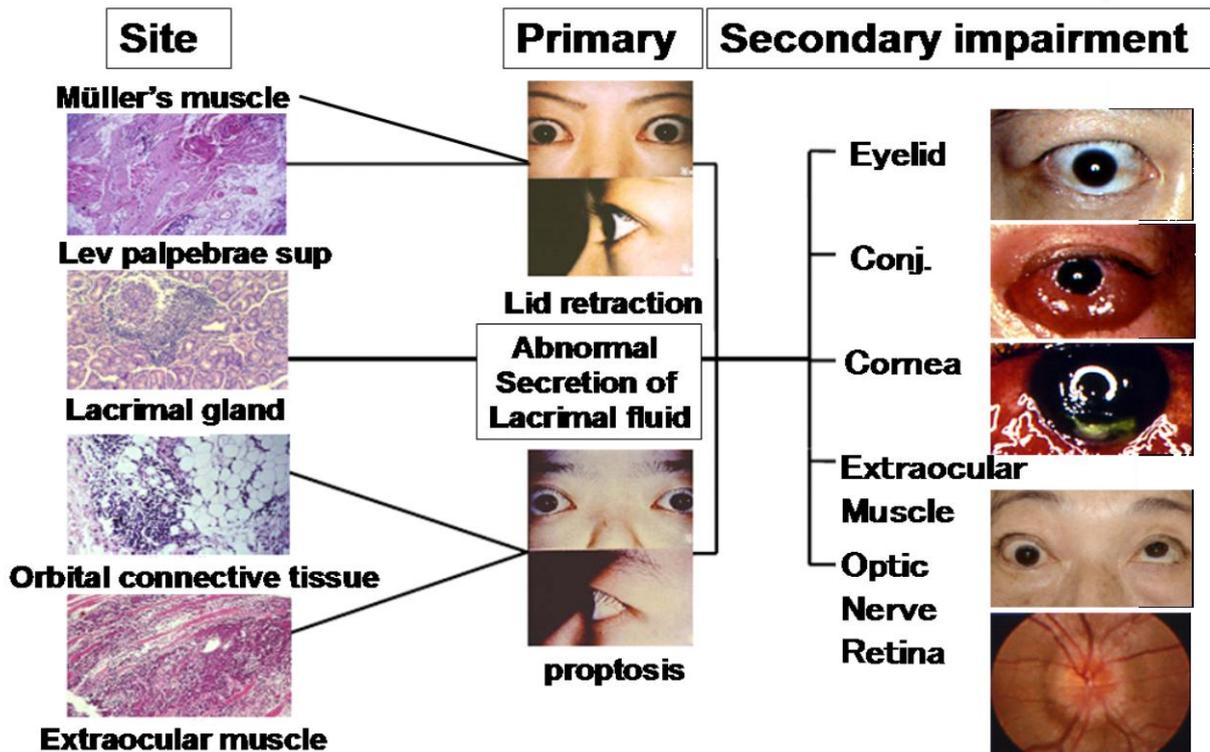


Fig. 1. Orbital lesions and clinical manifestations in Graves' orbitopathy. (modified from ref. # 6)

Magnetic resonance imaging

Our currently used MRI protocols for GO patients are: 1) T1-weighted image of the transverse, coronal and parasagittal sections; 2) T2-weighted fat suppression image or STIR image of the transverse, coronal and parasagittal sections; and 3) measurement of T2 relaxation time of the enlarged rectus muscles or signal intensity ratio in the coronal T2-weighted fat suppression image, or STIR image. The protocols should be adjusted for each machine.

Relationship between clinical manifestations and MRI

As mentioned above, patients with GO show a variety of symptoms and signs. The NOSPECS classification (19) has been used for long periods and is now outdated as a means of assessing patients with GO in clinical studies. However, it is still useful as a reminder of the clinical features that should be assessed.

Class 0: No physical signs or symptoms

More than a half of patients with Graves' disease do not have evident ophthalmopathy. Villadolid et al.(17) reported that 71% of patients with untreated Graves' disease without symptoms or signs of ophthalmopathy had enlargement of the extraocular muscles. Similar results were reported by Enzmann et al. (20) using CT. Therefore, subclinical involvement of the extraocular muscles is common in GO and is termed "occult thyroid eye disease" (21,22). Although we do not know who will

develop overt ophthalmopathy, we recommend MRI for patients with symptoms such as sensation of grit, light sensitivity, photophobia, excess eye-watering and blurring of vision, but no signs by ophthalmological examination. These symptoms may be initial symptoms during early GO. Future studies are indicated to clarify the factors associated with susceptibility to GO. It is important to control thyroid function in euthyroidism and abstain from tobacco smoking at this stage. Fig. 2A and 2B show T1-weighted images of the parasagittal section of orbital MRI in 2 patients with class 0.

Class I: Only signs, no symptoms (upper lid retraction, staring, and eyelid lag)

Lid retraction has been observed in 58–79% of GO patients (23,24). The sympathetic nerve stimulation associated with hyperthyroidism causes contraction of Müller's muscles, resulting in lid retraction. In this condition, it is important to achieve and maintain euthyroidism and guanethidine may alleviate lid retraction. However, two-thirds of patients may not respond to guanethidine. MRI reveals the enlargement of the "levator palpebrae superioris" (LPS) muscle and/ or "superior rectus"(SR) muscle (Fig. 2C, 2D). In patients with von Graefe's sign, MRI reveals the enlargement of the LPS muscle and/ or other extraocular muscles. Unilateral eye changes occur in 15% of patients with GO. In patients with unilateral lid retraction, enlargement of the LPS muscle and/or SR muscle are observed in the affected eye. Local injection of glucocorticoids (25,26), or botulinum toxin (27,28), or systemic administration of glucocorticoids indicated in those patients. MRI therefore aids treatment decision-making.

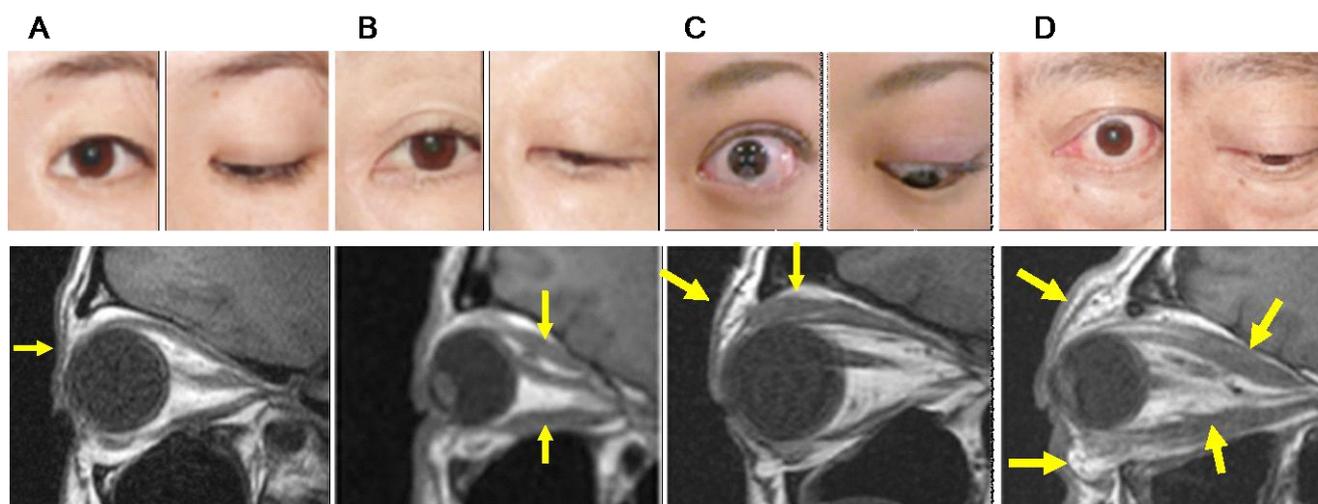


Fig. 2 Orbital MRI (T1-weighted parasagittal sections) in 4 patients. A: Patient without signs, or symptoms; MRI indicates no enlargement of muscles; B: Patient without signs, or symptoms; MRI reveals the enlargement of superior and inferior rectus muscles; C: Patient with Dalrymple sign, von Graefe's sign and proptosis; MRI reveals the enlargement of levator palpebrae superioris muscle; D: Patient with Dalrymple sign; MRI shows the enlargement of superior and inferior rectus muscles. Arrows indicate the lesions. (modified from Hiromatsu Y, *Nippon Naika Gakkai Zasshi*. 2010; 99:755-62, 2010).

Class II: Soft tissue involvement (symptoms and signs)

Lid swelling is frequently observed in 47%–68% of patients (23,24), and conjunctival injection and edema are observed in 32% of GO patients. MRI reveals the accumulation of fat tissue in the upper

and lower eyelids (Fig. 1C, 1D).

Class III: Proptosis

Proptosis is seen in 24% of untreated patients with Graves' disease and in 74%–85% of GO patients (23,24). Proptosis is usually accompanied by lid retraction (46%), lid swelling (19%), or both (16%). MRI reveals two types of exophthalmos. One is a result of the increase in fat tissue volume, and the other arises from enlargement of the extraocular muscles. The former type is observed in young female patients, the latter is more dominant in older male patients. In patients with the former type of exophthalmos, orbital fat decompression surgery is indicated in the inactive stage. The latter type of exophthalmos is classified as class IV. MRI can visualize the proptosis in transverse and parasagittal sections.

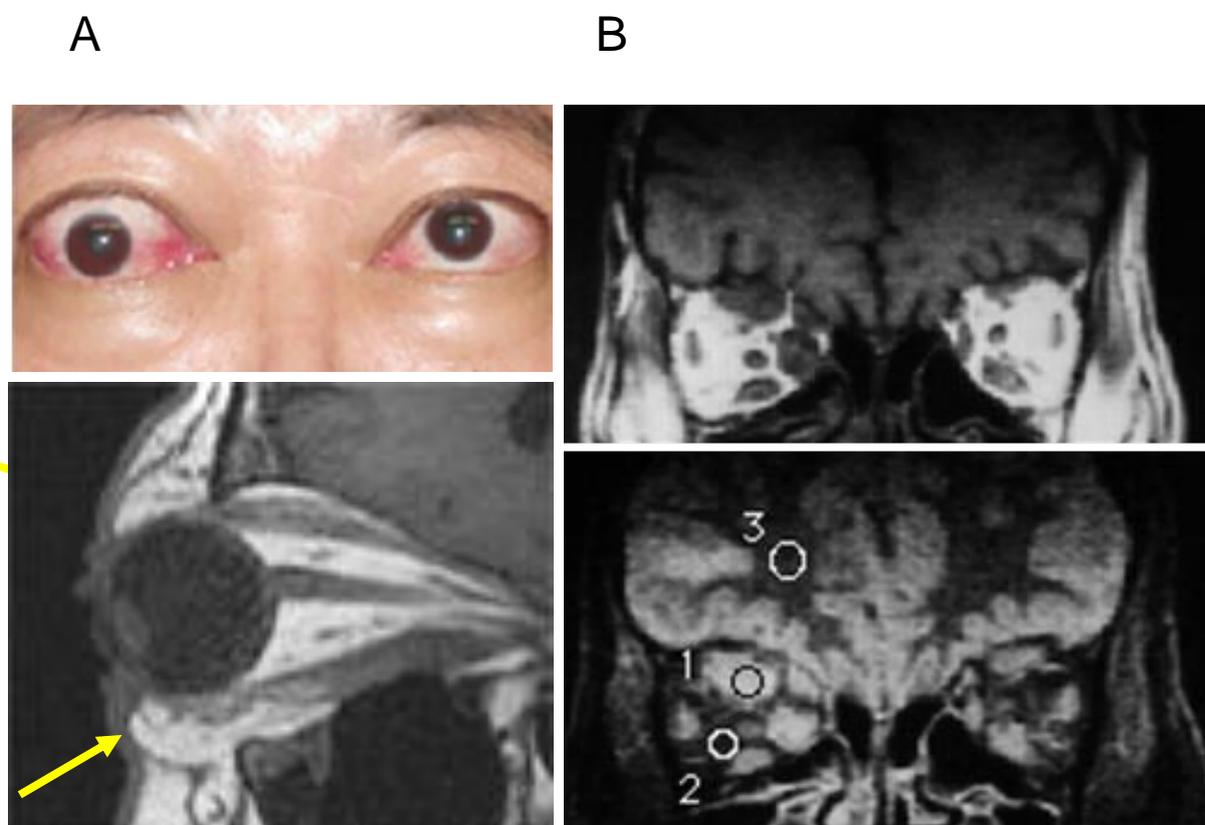


Fig. 3. Orbital MRI before steroid pulse therapy for patients with diplopia. A: Patient with diplopia; MRI reveals the enlargement of superior and inferior rectus muscles, proptosis and the accumulation of fat tissue in the eyelids B: Another patient with diplopia; upper snapshot, coronal section of T1-weighted image; lower snapshot, coronal section of STIR image Open circles shows the area where the signal intensity is measured. 1, orbital connective tissue; 2, eye muscle; 3, cerebral substantia alba.(modified from ref. # 11).

Class IV: Extraocular muscle involvement

About 22% of patients with GO suffer from diplopia (23). Enlargement of the inferior rectus muscle causes restriction of motion in upward gazing, and vertical deviation of the affected eye (Fig. 3A). The enlarged eye muscle is no longer able to lengthen. In most GO patients, several rectus muscles are affected to various degrees. Therefore, MRI is useful to evaluate the orbital lesions in

GO.

Class V: Corneal involvement

When the cornea cannot be protected by the closed eye, stippling of the cornea and ulceration may occur. Although punctuate staining is seen in 10%–17% of patients, the incidence of sight-threatening ulceration was <2% a century ago, and is probably lower now (5).

Class VI: Sight loss (optic nerve involvement)

Optic nerve involvement, so-called dysthyroid optic neuropathy, is observed in 3–7% of GO patients (8,24). Papilledema, papillitis and optic nerve atrophy are the main findings in this stage. MRI shows narrowing of apical crowding as a result of the enlargement of all rectus muscles, which compress the optic nerve. Intravenous injection of methylprednisolone is urgently indicated. If an improvement of sight is not obtained within 2 weeks, decompression surgery is warranted.

Differential diagnosis

In most cases, the diagnosis of GO is obvious: lid retraction combined with unilateral or bilateral proptosis in a person with a history of hyperthyroidism or hypothyroidism. Euthyroidism does not exclude GO. When the diagnosis is uncertain in atypical cases, orbital imaging is warranted. Myasthenia gravis, orbital myositis, carotid-cavernous fistula, orbital tumors and progressive external ophthalmoplegia should be ruled out.

Disease severity

The severity of disease should be assessed by measuring lid aperture, swelling and redness of the eyelids, redness and edema of the conjunctivae, inflammation of the caruncle or plica, exophthalmos, diplopia, eye muscle involvement, corneal involvement and optic nerve involvement, etc (8). Three classes of disease are described below (see also Fig. 4):

1) “Sight-threatening” GO

Dysthyroid optic neuropathy or corneal breakdown is sight-threatening and requires immediate treatment (8). Deterioration of vision, changes in intensity or quality of color vision, or disk swelling on fundoscopy, or if there is orbital subluxation, corneal opacity, or lagophthalmos with visible cornea, urgent referral is important.

2) “Moderate-to-severe” GO

For patients without sight-threatening GO, but whose eye disease has a marked impact on daily life, the risks of immunosuppression (if active) or surgical intervention (if inactive) are justified. Patients with moderate-to-severe GO usually have any one or more of the following: lid retraction ≥ 2 mm, moderate or severe soft tissue involvement, exophthalmos ≥ 3 mm above normal for race and gender, inconstant or constant diplopia, moderate corneal involvement.

3) "Mild" GO

In patients whose features of GO have only a minor impact on daily life, immunosuppressive or surgical treatment is not justified (8). Making treatment decisions requires detailed assessment of the eyes, understanding of the natural history of the disease, insight into the impact of GO on individual patients, and appreciation of the efficacy and side effects of therapies.

Disease activity

1) Clinical activity score

The CAS was proposed as a measure of disease activity by EUGOGO (8). CAS is calculated according to the presence or absence of the seven characteristic symptoms and signs (painful oppressive feeling in or behind the orbit, gaze-evoked pain, eyelid swelling, eyelid erythema, conjunctival redness, chemosis, caruncle or plica swelling). A CAS with 0 to 2 characteristics indicates inactive GO, and with 3 to 7 characteristics indicates active GO. The score has been shown to be predictive of a patient's response to immunosuppressive therapy. Mourits et al.(29) reported that GO patients whose CAS was over 4 of 10 points had a good response to glucocorticoid therapy. However, 10 of 28 patients whose CAS was 3 points or less also responded to immunosuppressive therapy (30). A recent study in Japan showed that two of eight GO patients whose CAS was 0 had active GO according to orbital MRI (31). Thus they hypothesized that MRI is more sensitive for detection of disease activity than CAS alone. Therefore, we recommend orbital MRI for assessment of GO.

2) T2 relaxation time and signal intensity ratio assessed by orbital MRI

Just et al.(32) reported that a long T2 time in the extraocular muscles before treatment was associated with a good response to orbital radiation. We reported that STIR imaging was useful for predicting the outcome of immunosuppressive therapy (11) (Fig. 3B). In 23 patients, we found a positive predictive value of 69% and a negative predictive value of 86% (11). Yokoyama et al. (18) reported that a homogeneously high signal intensity in T2 images was associated with a good response to methylprednisolone pulse therapy. Since then several reports have confirmed these findings. Thus, MRI seems a promising modality to detect disease activity. There are correlations between CAS and signal intensity ratio in STIR (33) and TIRM (turbo inversion recovery magnitude) sequences in T2-weighted and fat-suppressed images (34) and T2 relaxation time (31). Therefore, we recommend MRI as a useful tool for GO management.

There are some criticisms that MRI is not suitable for the assessment of GO because of the high cost, poor reproducibility of T2 relaxation times, and the dependence of T2 relaxation times on specific MRI equipment in different institutions. However, we recommend evaluating the disease activity by selecting either T2 relaxation time or signal intensity ratio in STIR in each institute. The good negative predictive value should be confirmed in a larger study.

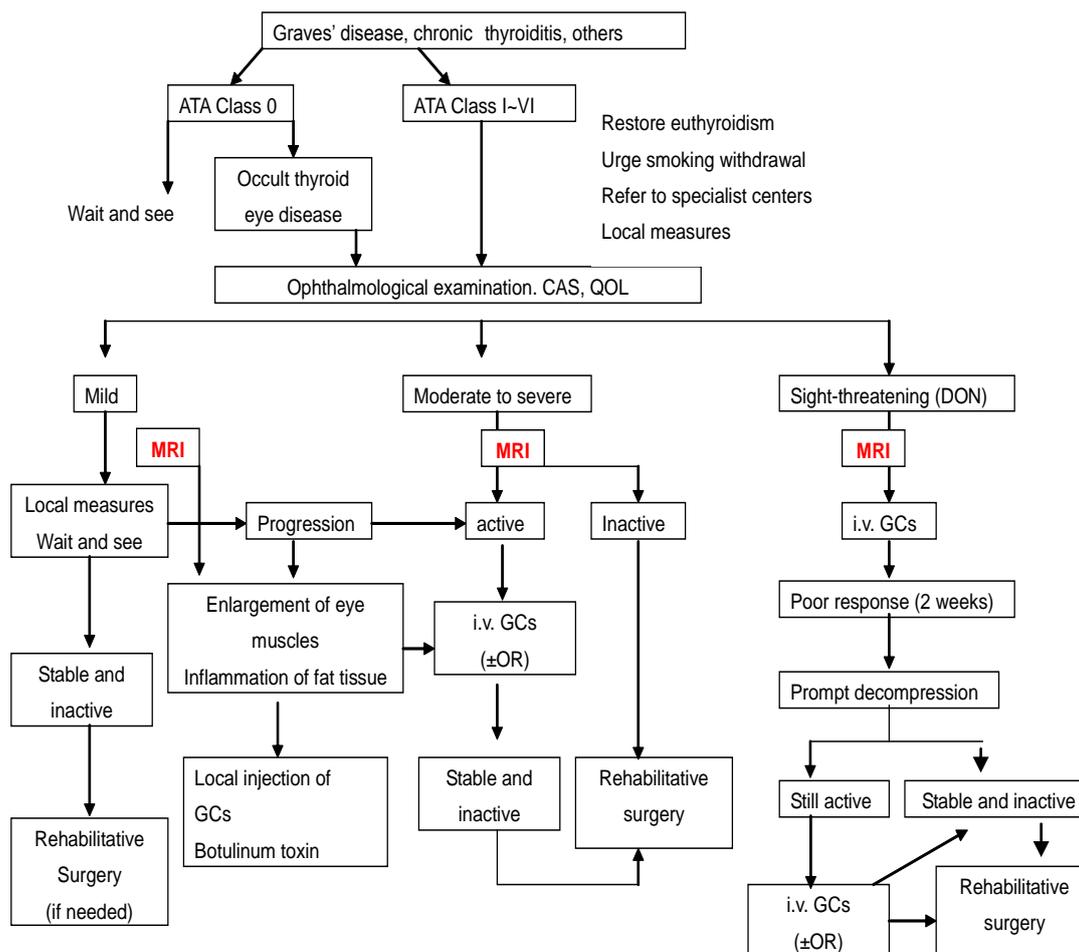


Fig. 4. Management of Graves' orbitopathy. We recommend that MRI should be considered to all the GO patients in the specialized center. It helps the diagnosis of GO (severity and activity) and the decision making of management of GO. Rehabilitative surgery includes orbital decompression, squint surgery, lid lengthening, and blepharoplasty/browplasty. i.v.GCs, intravenous glucocorticoid; OR, orbital radiotherapy; DON, dysthyroid optic neuropathy. (modified from ref. #8)

Treatment of hyperthyroidism

Because uncontrolled thyroid function is more likely to be associated with severe GO than patients with euthyroidism, all patients with GO should be treated promptly to restore and maintain euthyroidism (Fig. 4). Anti-thyroid drugs and thyroidectomy do not affect the course of GO, whereas radioiodine treatment is associated with a small risk of exacerbation of the disease (35,36). Development or exacerbation of GO after radioiodine therapy has been reported in randomized controlled trials (RCTs). In one RCT, radioiodine therapy caused progression of GO in about 15% of patients, whereas anti-thyroid drugs did not modify the natural course of GO (36). Risk factors for progression of GO after radioiodine therapy for hyperthyroidism include cigarette smoking (37), severe hyperthyroidism (free thyroxine >5 nmol/L), high levels of thyrotropin-receptor antibodies (38), and uncontrolled hypothyroidism. In two RCTs concomitant treatment of high risk patients with oral

prednisolone prevented progression and ameliorated the preexisting GO. The risk of exacerbation of pre-existing GO after radioiodine therapy is negligible as long as post-radioiodine hypothyroidism is avoided with levothyroxine (39).

Treatment of ophthalmopathy

1) Glucocorticoids

Patients with sight-threatening dysthyroid optic neuropathy require immediate treatment, usually with high-dose intravenous glucocorticoid agents (8). A common initial regimen is the administration of 1g of methylprednisolone intravenously for 3 consecutive days. If there is little or no improvement after 1 to 2 weeks, patients should promptly undergo surgical orbital decompression. There was no significant difference in outcome between decompression performed as first-line treatment, and initial treatment with intravenous glucocorticoids followed by oral prednisone (40).

Systemic administration of glucocorticoids is also indicated in moderate-to-severe and active GO (41). In a placebo-controlled, randomized trial, intravenous glucocorticoids (four cycles of methylprednisolone at a dose of 500 mg for 3 consecutive days at 4-week intervals) were effective in treating inflammatory changes and ocular movements in five of six patients as compared with one of nine patients who received placebo (42). In RCTs, intravenous therapy results in a higher rate of favorable responses than oral therapy and is better tolerated, with a reduced risk of the development of cushingoid features (43,44). However, severe and acute liver damage has been reported during intravenous glucocorticoid therapy (45–47). The risk of life-threatening liver failure has been reported with very high cumulative doses in 0.8% of patients. Therefore, EUGOGO recommends that the total cumulative dose of methylprednisolone should not exceed 8 g in one course of therapy (8,48). There is no consensus regarding the optimal dose and schedule. Thus, intravenous glucocorticoid therapy should be given only with close monitoring in specialized centers. All the patients should be closely followed for other potential adverse effects of glucocorticoid treatment (e.g., hypertension, hyperglycemia, electrolyte abnormalities, gastric ulcer and infections).

2) Orbital radiotherapy

Orbital radiotherapy is also useful for GO. In open trials about 60% of patients have had overall favorable responses to orbital irradiation (4). A common cumulative dose of radiation is 20 Gy per eye, given in 10 sessions over a 2-week period. An alternative regimen of 1 Gy per week over a 20-week period was equally effective and better tolerated (49). A lower dose (10 Gy) may be as effective as the standard 20 Gy regimen. Although one RCT has questioned the efficacy of orbital irradiation (50), a recent review of 18 studies (8 cohort and 10 RCT) showed that orbital radiotherapy is effective treatment for GO and the combination of orbital radiotherapy with intravenous methylprednisolone is more effective than either modality alone (51). Diabetes mellitus and severe hypertension are relative contraindications for orbital irradiation, because they increase the risk of retinopathy. Although the risk of tumors secondary to orbital irradiation is extremely small, orbital irradiation should be avoided in

patients younger than 35 years of age because of the potential long-term carcinogenic effects (8).

3) Surgery

Orbital decompression is required for sight-threatening dysthyroid optic neuropathy if high dose glucocorticoids do not ameliorate this condition within 1 to 2 weeks. Orbital decompression is also required for imminent corneal breakdown, if local measures and eyelid closure do not provide rapid, substantial improvement. Orbital CT is the modality of choice to plan orbital decompression surgery because CT can provide precise imaging of the orbital apex and especially of the osseous structures. Rehabilitative surgery is indicated for moderate-to-severe GO in the inactive stage. EUGOGO recommends that rehabilitative surgery should be performed in patients who have had inactive GO for at least 6 months, and surgical management should proceed in the following sequence: orbital decompression, then squint surgery, and then lid lengthening with or followed by blepharoplasty/browplasty, since the side effects of each step can interfere with the subsequent step (8).

Treatment of mild GO

EUGOGO recommends that simple measures and watchful waiting are appropriate for the majority of patients with mild GO, because glucocorticoids are rarely justified in mild GO as the risks outweigh the benefits (8). In a minority of patients with mild GO, whose quality of life is profoundly affected, glucocorticoid therapy is indicated. Although GO is a self-limiting disease, new efficacious drugs with minimal side effects are warranted. Antioxidants, such as selenium and pentoxifylline may be beneficial candidates for mild GO (52, 53). Parasagittal MRI provides information on eyelids and LPS and RS muscles, which is useful for planning therapy such as local injection of a glucocorticoid or botulinum toxin.

Other possible pharmacologic treatment

Randomized trials have not shown a benefit of somatostatin analogs (octreotide and lanreotide) for GO (54). The efficacy of the new somatostatin analog, SOM230, which has a relatively broad spectrum of activity, has not yet been evaluated. Cyclosporine was less effective than oral glucocorticoids in a randomized trial but may help to reduce the dose of glucocorticoids. Preliminary data suggest that immunomodulatory drugs such as rituximab (anti-CD20) (55–57) and etanercept (inhibitor of tumor necrosis factor- α)(58) may be beneficial for GO. In an open-label study, rituximab showed better outcomes and fewer adverse effects than intravenous steroid treatment. Randomized, controlled trials are required to evaluate the role of rituximab in the treatment of GO.

Conclusions

Patients with GO should be evaluated in a specialist center or by both an endocrinologist and ophthalmologist experienced in the management of this disorder. Orbital MRI provides useful

information regarding the inflamed lesions in the orbit, especially in extraocular muscle involvement and optic nerve involvement. Both T2 relaxation time and signal intensity ratio in STIR images indicate the activity of the lesion. Furthermore, MRI can visualize the inflamed lesion in the orbit. Thus, MRI is useful for decision-making regarding immunosuppressive therapy and prompt surgery for GO. It is also useful for predicting the outcome of immunosuppressive therapy. It may also provide a rationale to select new drugs for GO. Although there are some limitations in MRI, because of the reproducibility of the T2 relaxation time, cost and availability in some countries, we recommend that MRI should be considered for assessment of GO in specialized clinics.

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Chernobyl Thyroid Cancer 25 years after: in search of a molecular radiation signature

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ABSTRACT

Chernobyl accident, the worst technogenic catastrophe involving massive radiation release into the environment, will soon reach the 25th anniversary. Its major internationally recognized health consequence is thyroid cancer among the individuals affected by radioiodines at early ages. The largest in the world and unique series of radiation-induced thyroid malignancies has been a subject of investigations in many different aspects of sciences for decades. Here we review the results of investigations aimed at the elucidation of the “radiation signature”, a molecular classifier that could help discriminating between radiation-induced and sporadic tumors. The attempts to determine such employ a large variety of techniques, including measurements of DNA copy number variation on microarrays, differential gene expression profiling, proteomics, immunohistochemistry and genotyping of selected target genes or of the whole genome. From the point of view of study design and result interpretation, they could be broadly subdivided into those exploring molecular differences occurring after exposure to different etiological factors (i.e. radiation or other), thus looking for the damage pattern, and the ones seeking the markers of susceptibility to different etiological forms of thyroid cancer. There have been certain advances in both lines of investigations suggestive that establishment of the discriminative molecular signature is plausible. However, studies are far of being accomplished and require further efforts in following-up and investigating the Chernobyl cohort. Possible solutions to create comprehensive molecular concept will likely be integrative approaches combining clinico-pathological and extensive molecular data, and in-depth bioinformatic analyses.

Key-words: Chernobyl accident, thyroid cancer, molecular marker, genomics, gene expression, genetic association study

Introduction

Ionizing radiation is a well known genotoxic agent that induces a variety of DNA lesions including nucleotide base modifications, abasic sites, strand cross-linking, DNA adducts, and single- and double-strand DNA breaks (DSBs) (1-3). Although all these types of lesions may potentially result in gene mutations, DSBs are considered to be the most significant for chromosomal aberrations, mutagenesis, genetic instability and carcinogenesis (2, 4-7). The multiplicity of DNA damages produced by radiation is thought to be one of the reasons for the diversity in biological consequences of exposure.

Human thyroid is an organ particularly vulnerable to ionizing radiation as was initially seen in the series of patients subjected to external beam therapy of the head and neck area for medical indications who then developed thyroid cancer (8). The Chernobyl accident, which occurred nearly 25 years ago on April 26, 1986, provided evidence of carcinogenic effect of environmental exposure to radioiodine isotopes, especially to ^{131}I . A significant increase in thyroid cancer incidence was documented since early 1990-ies in Belarus, Ukraine and southwestern regions of Russia (9-12) (Fig. 1).

By 2002, the number of thyroid cancer cases registered in the individuals aged less than 18 years at the moment of exposure in the three most affected countries approached to 5,000 (13). Epidemiological studies have established qualitative and quantitative characteristics of causative association of thyroid cancer risk with internal exposure to radioiodine demonstrating that it is comparable to that after external irradiation (11, 14-20).

The outbreak of thyroid cancer in young patients suffered from the radioactive Chernobyl fallouts led to a great number of medical, epidemiological, dosimetric, sociological and laboratory investigations all aimed at evaluation of health impact, short and long-term consequences of the catastrophe for individuals, society and the environment as well as at elucidating the distinctive features of radiation-induced tumors. They resulted in important evidence-based conclusions which may be called lessons from Chernobyl; some of them could be drawn only after decades of observations. Applicably to the thyroid, the most important would be that ingestion of ^{131}I at childhood may later cause thyroid cancer, that period of latency after exposure may be as short as only 4 years, that the use of stable iodine as a dietary supplement or as a thyroid-blocking agent may have a protective effect against cancer. From the molecular and pathological point of view, it has been recognized that radiation excess in thyroid cancer incidence is due to the papillary thyroid carcinoma (PTC) whose morphology and molecular characteristics, such as histological architecture and mutational pattern, appear to be changing with increasing latency or correlate with patient's age (see ref. 20, 22, 23 for extensive reviews). The relative prevalence of *RET/PTC3*, *RET/PTC1* and *BRAF* mutations implicated in molecular carcinogenesis of PTC has been proposed to tentatively parallel the dynamics of thyroid cancer incidence in children, adolescents and adults, respectively, shown in Fig. 1 (20).

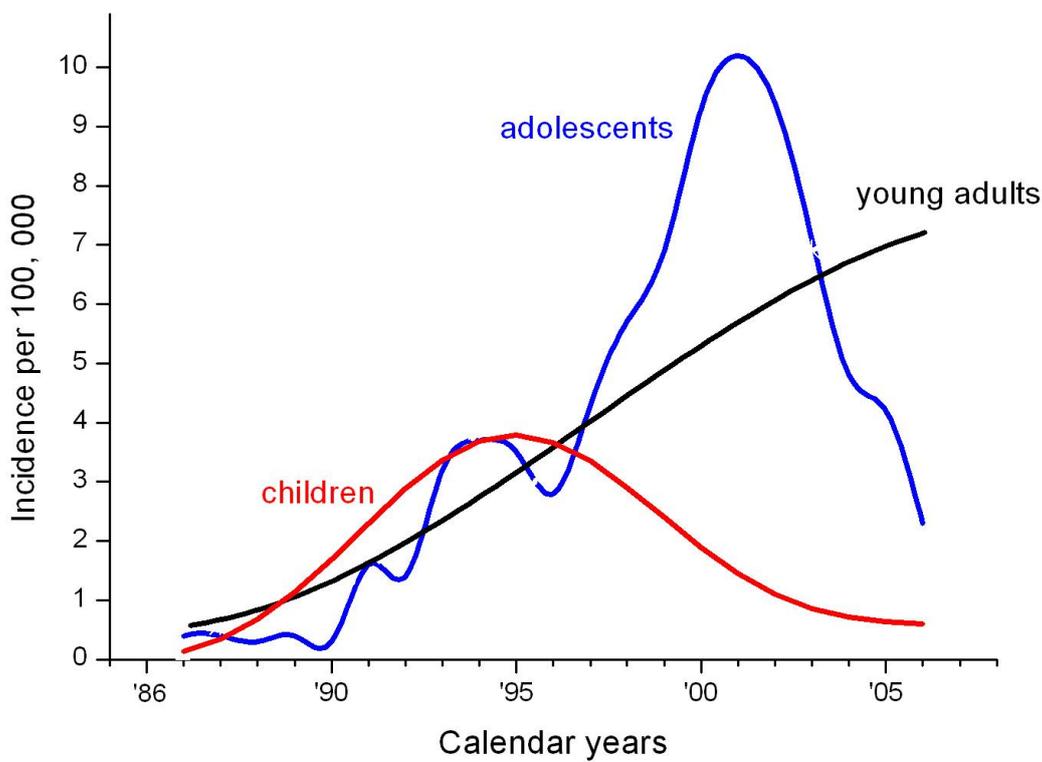


Fig. 1. Incidence of thyroid cancer in Belarus among the residents of radiocontaminated territories by age groups. This graph is inferred from the original one published earlier (21).

Molecular studies in Chernobyl thyroid cancer, depending on design, could be broadly classified into those attempting to determine a “damage signature” or “susceptibility signature” (24-26). The first type of investigations explores frequencies and distribution of various mutations, in a comparative manner, between radiation-induced and sporadic thyroid cancers. Initial works on Chernobyl series were mostly mutational studies. As a whole, they demonstrated that none of oncogenes such as gene rearrangements (*RET/PTC*, *NTRK*, *AKAP9-BRAF*) or point mutations (*BRAF*, *RAS* family genes) could have been identified as radiation-specific.

Studies of the second type investigate if gene expression patterns or genetic factors may modify or serve as markers of inherited predisposition for developing cancer after radiation exposure. They generally require more advanced techniques because of the need to cover a large number of targets, ideally the whole genome. So far, several factors have been established to affect risk for developing thyroid cancer following internal exposure: radiation dose for the thyroid, younger age at exposure and iodine deficiency. Whether or not the genetics particularities of the individuals who developed thyroid cancer after Chernobyl remains largely unknown, but some facts, such as inter-

patient variations in the clinical course and latency as well as development of cancer only in a small proportion of the exposed victims, may be indicative of such a possibility.

In this review we focus on the works performed to establish molecular classifiers capable of distinguishing radiation-induced Chernobyl cancers from sporadic PTCs. The importance and a need of a classifier is determined by the necessity to improve radiation risk assessment and risk communication, as well as to better manage and justify occupational and medical exposures tending to be expanding in the modern era of nuclear technologies.

Chromosomal imbalances

In an early study, chromosomal imbalances were examined using conventional comparative genomic hybridization (CGH) in a group of 60 Chernobyl childhood and adolescent PTCs (27). About 30% tumors were found to carry copy number variation (CNV). Both DNA gains (chromosomes 2, 7q11.2-21, 13q21-22, 21) and losses (16p/q, 20q, 22q) were found. Interestingly, deletions or loss of heterozygosity (LOH) on chromosomes 22q and 16p/q have been reported previously in PTC, FTC or ATC and associated with an aggressive tumor behavior (28-30). This study did not reveal correlations between the *RET/PTC* status of a tumor and specific DNA imbalance, yet the observation of a deletion at 22q in both *RET/PTC*-positive and *RET/PTC*-negative tumors was suggestive of the existence of alternate routes contributing to carcinogenesis, genetic heterogeneity or oligoclonal tumor development. The latter suggestion is supported by the observation of non-homogenous distribution of *RET/PTC*-harboring nuclei across tumor tissues (31). In a later work of the same group, employing an BAC-based array CGH, it was shown that *RET/PTC*-positive and *RET/PTC*-negative cases could be discriminated by the alteration pattern of chromosomes 1p, 3q, 4p, 7p, 9p/q, 10q, 12q, 13q and 21q (32). Furthermore, there was a significant difference between *RET/PTC*-positive childhood and adult PTCs: deletions on 1p35–36 were more frequent in adult cases. Regardless of *RET/PTC* rearrangement, chromosomal losses were more common than gains. In line with the previous study, the existence of additional, sometimes multiple, DNA alterations in both *RET/PTC*-positive and in *RET/PTC*-negative tumors could be interpreted as pointing at alternative paths of tumor development.

Another CGH study of 23 Chernobyl and 20 sporadic PTCs demonstrated that the overall prevalence of DNA gains was 2-4 higher in exposed patients as compared to non-exposed, and even more frequent (up to 10-fold) for recurrent gains (33). It was possible to determine the alteration pattern that discriminated radiation-related PTCs from sporadic (chromosomes 1p36.32-33, 2p23.2-.3, 3p21.1-.31, 6p22.1-.2, 7q36.1, 8q24.3, 9q34.11, 9q34.3, 11p15.5, 11q13.2-12.3, 14q32.33, 16p13.3, 16p11.2, 16q21-q12.2, 17q25.1, 19p13.31-qter, 22q11.21, 22q13.2) but because of limited sample size and non-uniform distribution of individual thyroid doses in the investigation the assessment of dose-response relationship has proved difficult. It was concluded that CNV, in addition

to carcinogenesis-related alterations, also depend on radiation exposure and patient's age at exposure.

Using a 50K Mapping array, 10 childhood Chernobyl PTCs were recently analyzed to demonstrate that DNA gains were more consistently observed at chromosome 1p, 5p, 9q, 12q, 13q, 16p, 21q, and 22q, while losses were found at 1q, 6q, 9q, 10q, 13q, 14q, 21q, and 22q (34). CNV amplifications were more frequent than deletions in line with the study by Kimmel et al. (33); no significant LOH was registered. This study is interesting because an overlay analysis was done to evaluate the concordance between CNV and gene expression. As a result, none of genes mapped to deleted regions was found to be downregulated. On the contrary, 87 genes that were amplified on CGH also displayed overexpression. After filtering gene expression profiles in Chernobyl PTCs against those reported previously for sporadic tumors and available from Gene Expression Omnibus, a radiation-related PTC identifier was established that included 113 messages among which 24 were downregulated and 41 were upregulated at least 3-fold. Six genes, *CAMK2N1*, *AK1*, *DHRS3*, *FBXO2*, *ECE1* and *PDE9A* were unique to childhood radiation-induced PTC.

As a whole, the results of CGH analyses performed to date are not yet comprehensive enough to derive a CNV-based radiation signature. Usually the studies deal with small sample size, do not report validation experiments on independent specimens and employ platforms that are quite different in their resolution cumulatively making cross-analysis difficult. They, however, provide insights into the genomic regions, candidate genes and functional pathways involved in radiation-related thyroid carcinogenesis.

Gene expression profiles

Several studies have been undertaken to elucidate characteristic expressionsome features of Chernobyl thyroid cancers. The earliest one analyzed 12 Ukrainian and 8 sporadic PTCs from French patients, and 13 thyroid adenomas using Micromax microarrays with a set of 2400 known human cDNA probes (24). Neither unsupervised nor supervised classification algorithms could distinguish radiation-related from sporadic PTCs, perhaps in part due to the relatively small number of tested genes. However, separation from benign thyroid neoplasia was effective: based on a 36-gene signature a 3% misclassification rate was achieved. The importance of this investigation was in obtaining molecular evidence of similarity between PTCs of different etiology which confirmed previous observations of their morphological resemblance once again proving that radiation-induced and sporadic PTCs are closely related diseases presumably having much in common pathogenetically.

The whole genome study used Human Genome Survey Microarray V2.0 platform that combines >29000 genes (35). Screening was done on pooled RNA samples from 11 Chernobyl patients aged 15-22 years at diagnosis and 41 patients from southeastern Germany aged 15-83 years and the results were confirmed on an RTQ-PCR low-density array for selected genes.

Microarray analysis detected 646 differentially upregulated and 677 downregulated genes (>5-fold difference) between the groups. Interestingly, the genes predominantly overexpressed in Chernobyl tumors included G-proteins (RAS family genes), growth factors and receptors (*VEGFA*, *EGFL9*, *PDGFC*, *PDGFRB*, *IGF1R*, *IGBP1*) and some of oxidoreductases (cyclooxygenase 2 (*PTGS2*), superoxide dismutase (*SOD1*)) which were associated with tumor aggressiveness and poorer prognosis in previous studies (36-42). Such overexpression was interpreted as supportive to the notion that Chernobyl PTC manifested particularly high aggressiveness with frequent lymph node metastases and extrathyroidal invasion. This work also identified a molecular classifier consisting of 7 genes (*SFRP1*, *MMP1*, *ESM1*, *KRTAP2-1*, *COL13A1*, *BAALC* and *PAGE1*) that enabled a confident classification into radiation-related and sporadic PTCs.

One more investigation explored transcriptomes in 12 Chernobyl and 14 French patients using Human 1 cDNA Microarray slides covering 8000 genes (43). Similarly to the previous report from this group (24), unsupervised classification did not provide distinction between the two groups of cancers on a global scale. A supervised analysis, however, using four different algorithms, succeeded to determine classifiers that included from one to several thousands genes (median 256) with overall error rates ranging 12-27%. This study is noteworthy because the effects of possible etiological agents, which are presumably gamma radiation in Chernobyl tumors and hydrogen peroxide in sporadic tumors, were taken into account. Hydrogen peroxide is produced during thyroid hormone synthesis (44) and may play a role in thyroid tumorigenesis (45). Furthermore, it is a potent DNA-damaging substance which produces not only single-strand DNA breaks and base modifications but also double-strand breaks and, as recently shown, is capable to generate *RET/PTC1* rearrangement in a human thyroid cell line (46). Using previously available data (47), the authors found that in a B-lymphocyte cell line treated with 10 different genotoxic agents, *in vitro* gene expression responses to 200 μ M of hydrogen peroxide and 2.5 Gy of gamma-rays were the most resembling. There were however 293 genes whose expression levels differed >1.5-fold between the two types of treatment of which, after removing genes related to immune reactions, 118 were present on the arrays used to profile PTCs. These genes were tested as a molecular classifier and, as a result, led to the separation of Chernobyl and sporadic PTC with the error rates 15-27%. In addition, whether the genes whose products are involved in five major DNA repair mechanisms, i.e. base-excision repair, mismatch-excision repair, nucleotide-excision repair, homologous recombination and nonhomologous end joining, may constitute a classifier was explored. Thirteen genes of homologous recombination pathway were found to make a classifier that distinguished radiation-induced and sporadic PTCs with error rates of 15-31%. It was proposed that, given DNA repair is largely accomplished within hours after damage while differential gene expression in the tumors persisted for many years, such profile may be a signature of susceptibility to different etiological forms of thyroid cancer. If these results find further support in independent PTC series, they may well be considered as a piece of evidence suggesting the existence of inherited predisposition to radiation-induced PTC.

Similarly to the results obtained in CGH studies, gene expression data provide valuable information for the attempts of elucidating molecular radiation signature, but they are not completed yet. So far reported works, being generally encouraging, have been done using relatively small series of cancers and produce the results that do not converge to yield a reliable set of markers. This points at the need to expand the number of analyzed cancers of both etiologies with better matching in terms of clinico-pathological and molecular characteristics to achieve the desired reproducibility and avoid biases.

Proteomic investigation

To date only one proteomic study involving Chernobyl thyroid cancers has been reported to the best of our knowledge. Boltze et al. analyzed protein extracts from 86 Chernobyl and 91 sporadic PTCs from patients of southeastern Germany (48). On 2-D electrophoresis, around 2000 spots were identified on the reference gels and among them 18 candidates upregulated in radiation-induced PTCs were determined. Immunohistochemistry was performed for all these candidates and in addition for two other proteins, potential markers for PTC. The results were evaluated semiquantitatively eventually leaving 6 proteins (NTRK1, MMP-1, MMP-13, MMP-9, Cathepsin W and Cathepsin X) that allowed most efficient separation between the groups. When adjusted for patients' age, NTRK1, MMP-1 and MMP-13 staining resulted in a complete separation of the two etiological groups. Without age adjustment, NTRK1 alone and a combination of either two MMPs or of two Cathepsins also worked well with no false positive and false negative test results. Note that *MMP1* gene upregulation in Chernobyl PTCs was reported previously (35). Interestingly, NTRK1 overexpression in radiation-induced PTCs may indicate structural mutation-independent role of this receptor tyrosine kinase as chromosomal rearrangements involving the *NTRK1* gene are observed in less than 10% of Chernobyl cancers (49).

Whether a relatively simple immunostaining approach can be universally used to discriminate radiation-induced from sporadic PTCs remains to be established. Concerns are related first of all to patients' age (and/or duration of latent period) and associated changes in tumor morphology as well as underlying mutational events all potentially leading to the shifts in the spectrum of expressed proteins. This direction certainly needs further investigation.

Genetic association studies

The purpose of this type of investigations is to determine genetic factors associated with disease thus addressing issue of inherited susceptibility. In general, there are two methodologies of selecting gene polymorphisms, usually SNPs, to be analyzed. The first one, termed candidate gene approach, is based on a hypothesis that genetic variations in one or in a limited number of genes may affect risk for or the phenotype of a given disease. A more comprehensive way is initially hypothesis-free and employs analysis throughout the genome; it is termed genome-wide association study

(GWAS). While a substantial number of studies has been done in sporadic thyroid cancers, only few explored radiation-induced thyroid malignancies.

Candidate gene approach

In a study by Stephens et al. (50) no evidence for LOH in the *RET* gene was found in 28 of 46 PTCs from Ukraine heterozygous for at least one of three SNPs of interest (G691S, S904S and L769L); this observation is in line with the later microarray findings (33). Investigation of the additional 68 cases demonstrated that the rare S allele of G691S was significantly overrepresented in patients aged more than 30 years (30-72 years old, range and exposed 10-14 years before operation) as compared to the younger ones. Since excess radiation risks for PTC in the individuals exposed at the age older than 20 years old is very low and further declines with age at exposure, it was proposed that *RET* polymorphisms may influence carcinogenesis in sporadic but not in radiation-induced PTCs.

The Arg72Pro polymorphism of the *TP53* gene (encodes tumor suppressor protein p53) was assessed in 48 pediatric/adolescent and 68 adult Ukrainian and Russian patients with PTC, residents of radiocontaminated territories in Chernobyl areas (51), and 53 adult patients with sporadic PTC and 313 healthy controls from Russia. The Arg/Arg homozygotes were found to be significantly underrepresented in adult patients, but not in children and adolescents. In tumor tissues, no LOH or imbalanced *TP53* allele expression in heterozygous individuals was found. These findings suggested that germline *TP53* allele combinations other than Arg/Arg may contribute to the risk of development of PTC in individuals exposed to radiation during their late childhood, adolescence or in young adulthood, particularly females aged between 18 and 30. Of note, elevated risk for thyroid cancer was reported in females exposed to Chernobyl radiation at the age below 30 years in an epidemiological investigation (52).

A recent study of 9 SNPs in 5 genes (*ATM*, *XRCC1*, *TP53*, *XRCC3* and *MTF1*) involved in DNA damage response in 255 PTC patients (123 from Chernobyl areas and 132 sporadic) and 596 healthy controls (198 residents of Chernobyl areas and 398 subjects without history of radiation exposure) showed that the *ATM* G5557A and *XRCC1* Arg399Gln polymorphisms, regardless of radiation exposure, were associated with a decreased risk of cancer (53). Interestingly, the *ATM* IVS22-77 T>C and *TP53* Arg72Pro SNPs interacted with radiation exposure: the *ATM* IVS22-77 associated with the increased risk of sporadic PTC whereas *TP53* Arg72Pro correlated with the higher risk of radiation-induced PTC in adult patients, in support to the previous report (51). A possibility of gene-gene and gene-environment interactions was demonstrated. Some particular *ATM/TP53* genotypes strongly associated with either sporadic or radiation-induced cancer indicating that variability of these genes may be potential risk modifiers for developing PTC of different etiology.

Molecular epidemiology based on whole genome association data

To date only one investigation of Chernobyl PTCs employing GWAS has been published (54). A total of 667 patients from Belarus diagnosed for PTC in 1989–2009 and 1275 controls from Belarus and Russia were studied, of which 408 cases and 627 controls were genotyped using Illumina Human610-Quad BeadChips (>500,000 SNPs) and the remaining samples were used for validation study. Statistical meta-analysis identified 4 SNPs at chromosome 9q22.33 showing significant association with disease. For one of them, rs965513, used for validation, a P -value of 4.8×10^{-12} was obtained which far surpasses the threshold of genome-wide significance of 5×10^{-8} (55). This SNP is located within a linkage disequilibrium (LD) block centromeric to the *FOXE1* gene which encodes a thyroid-specific transcription factor TTF2 playing pivotal roles in thyroid morphogenesis. In addition, two candidate SNPs on chromosomes 9p and 12p that strongly tended to associate with disease risk were identified but genotyping of additional samples would be necessary to validate the significance of those.

To better understand the importance of this finding, it is necessary to mention two studies of genetic predisposition to sporadic differentiated thyroid cancer published last year just before the study by Takahashi et al. The first one reported rs965513, the same polymorphism described in the Chernobyl series, as the strongest genetic marker associating with thyroid malignancy in individuals of European descent. This study also claimed another SNP, rs944289 on chromosome 14q13.3 in the proximity of the *NKX2-1* gene that encodes the TTF1 transcription factor, to be a marker for thyroid cancer (56) but it was not confirmed in the Chernobyl series. The second study, employing candidate gene approach, initially genotyped 768 SNPs in 97 genes in 615 cases and 525 controls from Spain and used 482 patients and 532 controls from Italy for validation (57). The target genes were selected based on their differential expression in primary thyroid tumours or the involvement in thyrocyte biology, metabolism and/or carcinogenesis such as the MAP kinase, JAK/STAT and TGF-beta pathways. An SNP, rs1867277, within the LD block spanning *FOXE1* and located at the 5'UTR of the gene was identified as associating with PTC. Functional study demonstrated that this SNP affects *FOXE1* expression by recruiting the USF1/USF2 transcription factors. Since forkhead transcription factors have been implicated in several human cancers (58-61) including epithelial-mesenchymal transition in colon cancer (62), it was proposed that *FOXE1* may influence thyroid tumor cell migration and invasion. While its precise role remains to be elucidated, this was an important clue to the understanding the molecular pathogenesis of PTC.

Thus, the three studies, two of sporadic thyroid cancers and one of radiation-induced tumors, have concordantly identified the *FOXE1* (*TTF2*) locus as a marker of inherited susceptibility for PTC of different etiology. This leads to an important corollary that among the genetic factors affecting risk for radiation-induced Chernobyl PTC the strongest one is the same that confers predisposition to the sporadic form of this type of malignancy. Therefore, it is likely that “radiation-sensitive genotype”, whose existence may be expected given the possible existence of putative radiation-associated

markers on chromosomes 9p and 12p and the absence of sporadic PTC marker on 14q13.3 (i.e. *NKX2-1* or *TTF1*), comes next to and after, in terms of the effect strength, the general susceptibility to thyroid cancer. As outlined in Fig. 2, the results of genetic association studies allow to add genetic predisposition to the list of risk factors for radiation-induced thyroid carcinogenesis known from the earlier experience. Further investigation of etiology-specific marker(s) will probably refine our understanding of radiation-induced carcinogenesis by addressing issues of gene-gene and gene-environment interactions.

Risk factors for papillary thyroid carcinoma

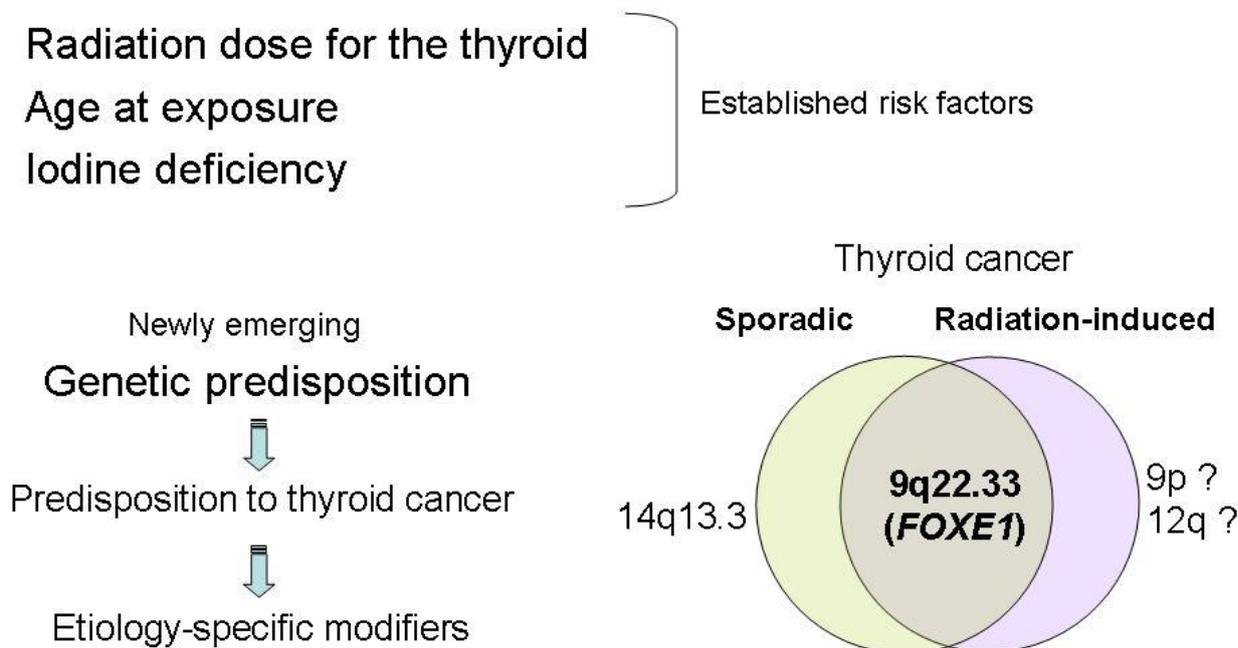


Fig. 2. Genetic predisposition as an emerging risk factor for both sporadic and radiation-induced papillary thyroid carcinoma. Sporadic and radiation-induced PTC share the major genetic determinant of inherited susceptibility to thyroid cancer, *FOX E1* at chromosome 9q22.33, which appears to be stronger than possible etiology-specific genetic markers: on chromosome 14q13.3 (*NKX2-1* or *TTF1*) for sporadic PTC and putative markers on chromosomes 9p and 12q for radiation-induced PTC.

Conclusion

A rapidly growing body of evidence suggests that the identification of molecular “radiation signature” in thyroid cancer is likely to become possible, with certain degree of certainty, in the coming years. The advances in exploring both the damage pattern by genomic microarrays, differential gene expression or immunohistochemically and inherited susceptibility by GWAS and

expression arrays keep on bringing encouraging results yet they are far of being finalized. At present they rather contribute to work out a proof of principle that radiation-induced and sporadic thyroid cancers could be distinguished using a definite set of validated markers. Perhaps this set will include not only the above-mentioned markers as well as essential clinico-pathological information but also other, such as e.g. miRNA and proteomics, whose integration into the spectrum of potential targets and in-depth analyses may enable better insights into the possible classifiers. Its availability will likely allow future personalized cancer risk prediction which is of a significant importance in view of the growing thyroid cancer incidence in the world and also because of the relevance to occupational and expanding medicinal exposures, and radiation emergency medicine issues.

Undoubtedly, Chernobyl cohort is an inestimable source of knowledge in the area. Continuous observation, follow-up and thorough studies are warranted to yield the higher level of understanding. In this regard, international initiatives, such as the Chernobyl Tissue Bank (<http://www.chernobyltissuebank.com/>) or EC-coordinated GENRISK-T consortium (<http://www.helmholtz-muenchen.de/isb/genrisk-t/index.html>), Nagasaki University GCOE Program Global Strategic Center for Radiation Health Risk Control (http://www-sdc.med.nagasaki-u.ac.jp/gcoe/projects/index_e.html) and other cooperative efforts would be the principal roadways to solving the problem.

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A putative role for deiodinases in muscle during illness.

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ABSTRACT

During illness changes in thyroid hormone metabolism occur, collectively known as the non-thyroidal illness syndrome (NTIS). NTIS is characterized by low serum thyroid hormone levels, while TSH and TRH expression do not increase, indicating a resetting of the central feedback regulation. NTIS may be a useful adaptation of the body to illness aimed at conserving energy. In this review we discuss changes in skeletal muscle deiodinase type 2 and type 3 expression during illness, including their possible physiological implications. Both in human and animal studies, differential regulation of muscle deiodinase expression during illness has been reported. On closer examination, both type and severity of illness as well as food-intake are determinants of muscle deiodinase expression. As deiodinase expression is a determinant of local T₃ or 3,3'-T₂ bioavailability, ultimately affecting metabolic state of the muscle tissue, the observed changes in deiodinase expression during illness are expected to be physiologically relevant.

Key-words: deiodinase, non thyroïdal illness, cytokines, muscle, cAMP, mitochondria

Introduction

The thyroid gland predominantly releases the prohormone thyroxine (T₄), which can be metabolized by deiodinating enzymes via inner- or outer ring deiodination. Deiodinase type 2 (D2) converts T₄ into the active hormone triiodothyronine (T₃) and it can also convert reverse T₃ (rT₃) into 3,3'-diiodothyronine (T₂) by outer ring deiodination (figure 1). D2 is present as an active dimer in the endoplasmic reticulum and is expressed in brain, pituitary, skeletal muscle, brown adipose tissue and placenta (1;2). Deiodinase type 3 (D3) is localized in the plasma membrane and is considered the main thyroid hormone inactivating enzyme as it converts T₄ into the inactive metabolite rT₃ and, in

addition, T_3 into T_2 by inner ring deiodination (Figure 1). $D3$ is mainly expressed in brain and placenta and it plays an important role during embryonic development. With the exception of the brain, $D3$ expression levels are very low in healthy mature tissues (3).

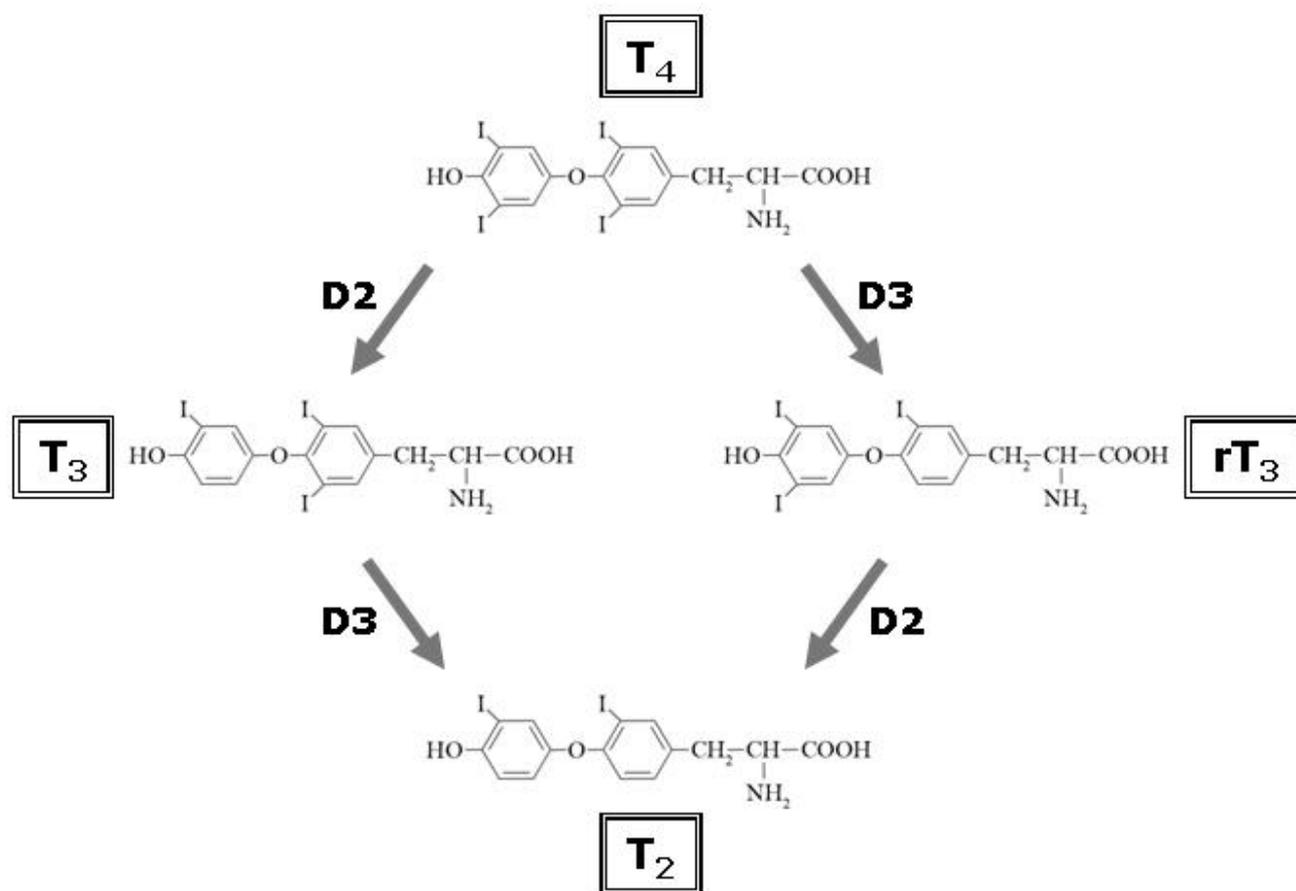


Figure 1 Overview of the deiodination of thyroxine (T_4) into tri-iodothyronine (T_3), reverse tri-iodothyronine (rT_3) and di-iodothyronine (T_2) by deiodinase type 2 ($D2$) and type 3 ($D3$).

Thyroid hormone metabolism in muscle has received much attention in recent years. Of note, muscle $D2$ was reported to contribute to serum T_3 levels in humans (4). Furthermore, it appeared that bile acids may activate $D2$ expression through the $TGR5$ receptor, which ultimately leads to increased oxygen consumption in human skeletal muscle cells (5). Finally, activation of the metabolic regulator peroxisome-proliferator activated receptor ($PPAR$) $_{\gamma}$ in primary murine myotubes increases $D2$ and decreases $D3$ activity, linking muscle $D2$ and $D3$ expression to metabolism (6).

Non thyroidal illness syndrome

During illness, thyroid hormone metabolism changes at various levels and this is collectively known as the non thyroidal illness syndrome (NTIS). The hallmarks of NTIS are decreased serum thyroid hormone levels, while thyroid stimulating hormone (TSH) and hypothalamic thyrotropin releasing

hormone (TRH) expression do not increase, indicating a resetting of the central negative feedback regulation. NTIS may reflect a useful adaptation of the body to counteract excessive catabolism during acute illness and it may, therefore, be viewed as a part of the acute phase response (7). However, during prolonged critical illness NTIS may turn maladaptative (8). To date, the pathogenesis of NTIS is incompletely understood, although hypothalamic, pituitary, and hepatic changes in thyroid hormone metabolism are known to be involved. In addition, alterations in muscle deiodinase expression have recently been postulated to contribute to decreased serum thyroid hormone levels observed during illness (9;4).

Muscle deiodinase expression during illness in humans

Both D2 mRNA and activity were reported to be upregulated in post-mortem skeletal muscle of intensive care unit (ICU) -patients compared to healthy controls. In addition, muscle D2 expression correlated negatively with serum thyroid hormone levels and –although less strictly- with free cortisol levels (10). In contrast, a study by Rodriguez-Perez *et al* reported decreased muscle D2 and increased muscle D3 expression in septic patients (11). Peeters *et al* also found substantial D3 activity levels in post-mortem skeletal muscle biopsies of ICU patients, which correlated with serum rT3/T4 ratio, but not with rT3/T3 ratio. The same authors suggested that muscle D3 expression might be associated with tissue hypoxia (9).

The different outcomes of D2 expression in these studies might be associated with differences in illness severity, illness duration, or with food intake. The latter possibility is supported by a recent study reporting decreased D2 mRNA expression in humans after 62h of fasting, suggesting that food intake regulates skeletal muscle D2 expression (12). Thus, differences in timing, composition and route of administration (parenteral or enteral) of food may be more important determinants of muscle deiodinase expression than previously thought.

Muscle deiodinase expression during illness in mice

Differential regulation of muscle D2 and D3 expression has been observed in different animal models of illness. During acute illness induced in mice by bacterial endotoxin (lipopolysaccharide, LPS) administration, muscle D2 mRNA increases, whereas muscle D3 mRNA decreases (13;14). Another pattern is present in muscles of mice with a local chronic inflammation induced by s.c. turpentine injection in the hindlimb (15), which increases D2 and D3 mRNA and activity simultaneously, in conjunction with an activation of the cAMP pathway (16). Finally, a severe bacterial infection with *S.pneumoniae* results in decreased D2 expression while D3 remains unchanged (16). These observations clearly show that changes in thyroid hormone metabolism in muscle during illness depend on the type of illness, and probably on its time course as well.

Possible mechanisms of illness-induced muscle deiodinase expression

Involvement of cellular signalling cascades

During inflammation, proinflammatory cytokines such as interleukin (IL)-1, IL-6 and tumor necrosis factor (TNF)- α are released. Proinflammatory cytokines exert their actions via nuclear factor (NF) κ B, extracellular-signal related kinase (ERK)1/2 and activator protein (AP)-1 signal transduction pathways. NF κ B and AP-1 response elements have been characterized in the D2 promoter (17;18) suggesting that activation of these pathways affects D2 expression. However, the involvement of NF κ B in hypothalamic D2 activation has been studied in sufficient detail to conclude that it is probably not the primary initiator of the LPS-induced D2 increase in tanycytes (19).

Not much is known about the mechanism of D3 induction during inflammation (15;20), although D3 can be stimulated via the ERK1/2 and p38 signalling pathway by 12-O-tetradecanoyl phorbol 13-acetate (TPA), basic fibroblast growth factor (bFGF) and transforming growth factor (TGF) β 1 (21; 22). Furthermore, hypoxia has been associated with D3 upregulation probably via the activation of hypoxia inducible factor (HIF)-1 α (23). During chronic inflammation, activation of the cAMP pathway is associated with an increase of muscle D2 mRNA and activity (16), suggesting increased D2 expression via the cAMP responsive element-binding (CREB) element present in the D2 promoter (24). In sum, it is presently far from established which signalling cascade is responsible for the changes in muscle D2 and D3 expression observed during illness.

Involvement of thyroid hormone, thyroid hormone receptors and PPAR γ

Both D2 and D3 have been reported to be regulated by thyroid hormones. The regulation of D3 expression by thyroid hormone has only been investigated in brain, where a thyroid hormone inactivating role of D3 was found (25). The regulation of D2 by thyroid hormones has been investigated more extensively. T₃ downregulates D2 mRNA expression (26), whereas both T₄ and rT₃ (the substrates of D2) increase D2 ubiquitination and, subsequently, proteasomal degradation, finally resulting in decreased D2 activity (27). This is in line with the study by Mebis *et al*, who observed a negative correlation of serum T₃ with muscle D2 mRNA in ICU patients. In addition, T₄ correlated with D2 activity (10). However, the animal studies showed no consistent correlation between serum thyroid hormone levels and muscle D2 and D3 expression during illness (14;16).

To investigate the possible involvement of thyroid hormone receptor (TR) α and TR β in the illness induced changes in muscle deiodinase expression, we studied muscle responses to inflammation in TR β ^{-/-} and TR α ^{0/0} mice. The LPS induced increase of muscle D2 was not different in TR α ^{0/0} mice, and less pronounced in TR β ^{-/-} mice compared to wildtype (WT), indicating predominant involvement of TR β (13;14). By contrast, The LPS-induced decrease of muscle D3 was less pronounced in TR α ^{0/0} mice, indicating differential involvement of TR isoforms in deiodinase changes in muscle (14).

A third route might be via PPAR γ . Stimulation of primary murine myotubes with an exogenous PPAR γ -ligand (pioglitazone) increases D2, while simultaneously decreasing D3 activity (6), similar to the effects of LPS administration on muscle D2 and D3 expression in mice. Among the endogenous ligands for the PPAR γ are nitrated-free fatty acids (FFA's) (28), which are synthesized by the addition of a nitric oxide (NO) group to FFA's. Nitric oxide is formed via the upregulation of inducible nitric oxide synthase (iNOS), which is induced in muscle after LPS injection (29). Although PPAR γ decreases in liver upon LPS (30), it remains unaltered in kidney (31) and it is currently unknown whether PPAR γ is affected in muscle tissue during inflammation. Thus, a role for PPAR γ in muscle D2 and D3 changes in acute inflammation cannot be confirmed or rejected at present.

Putative role for deiodinase changes in muscle during illness

During illness, muscle deiodinase expression changes profoundly, depending on type and severity of illness. A key question is what the role of these local deiodinase changes for the organism could be. Some investigators have postulated that decreased activation of thyroid hormone by muscle D2 induces the decreased serum thyroid hormone levels observed during illness (4). However, although muscle D2 expression decreases during sepsis both in humans and mice (11;16), the reverse pattern occurs in ICU patients and in LPS- and turpentine-treated mice (10;13;16). As in all these circumstances serum thyroid hormones (tend to) decrease, this hypothesis proves incorrect.

Another possibility is increased muscle D3 as a contributor to decreased serum thyroid hormone levels during illness (9). This was supported by the observation that D3 expressing infantile hemangiomas are associated with decreased thyroid hormone levels (32). Furthermore, increased D3 expression has been reported in ICU patients (9) and in septic shock patients (11). In addition, muscle D3 increases during sepsis and chronic inflammation in mice (16). However, severe bacterial sepsis in D3 knock-out (KO) mice resulted in a similar decrease of serum thyroid hormone levels in D3KO compared to WT mice (33). Moreover, abundant D3 expression in infiltrating granulocytes in a turpentine-induced sterile abscess does not result in decreased serum T₃ levels compared to pair-fed controls (15). Finally, muscle D3 mRNA decreases after LPS administration in mice, while serum thyroid hormone levels decrease as well (14). These observations exclude an important role for D3 in decreasing serum thyroid hormone levels during illness in mice, while more data are needed in the clinical setting before the putative role of D3 in lowering serum thyroid hormone levels is settled.

As muscle D2 and D3 do not appear to contribute in any major way to the illness induced decreased serum thyroid hormone levels and in view of the fact that the reported D2 and D3 activity levels in muscle are very low, a local effect in skeletal muscle seems more plausible. The differential regulation of muscle D2 and D3 expression theoretically will lead to a different outcome with regard to local T₃ and T₂ tissue concentrations in the different mouse models of illness as depicted schematically in figure 2. Both T₃ and T₂ are known to regulate metabolic state (34;35). It is tempting

to speculate that differential regulation of deiodinase expression contributes to alterations in muscle metabolic state during different stages of disease.

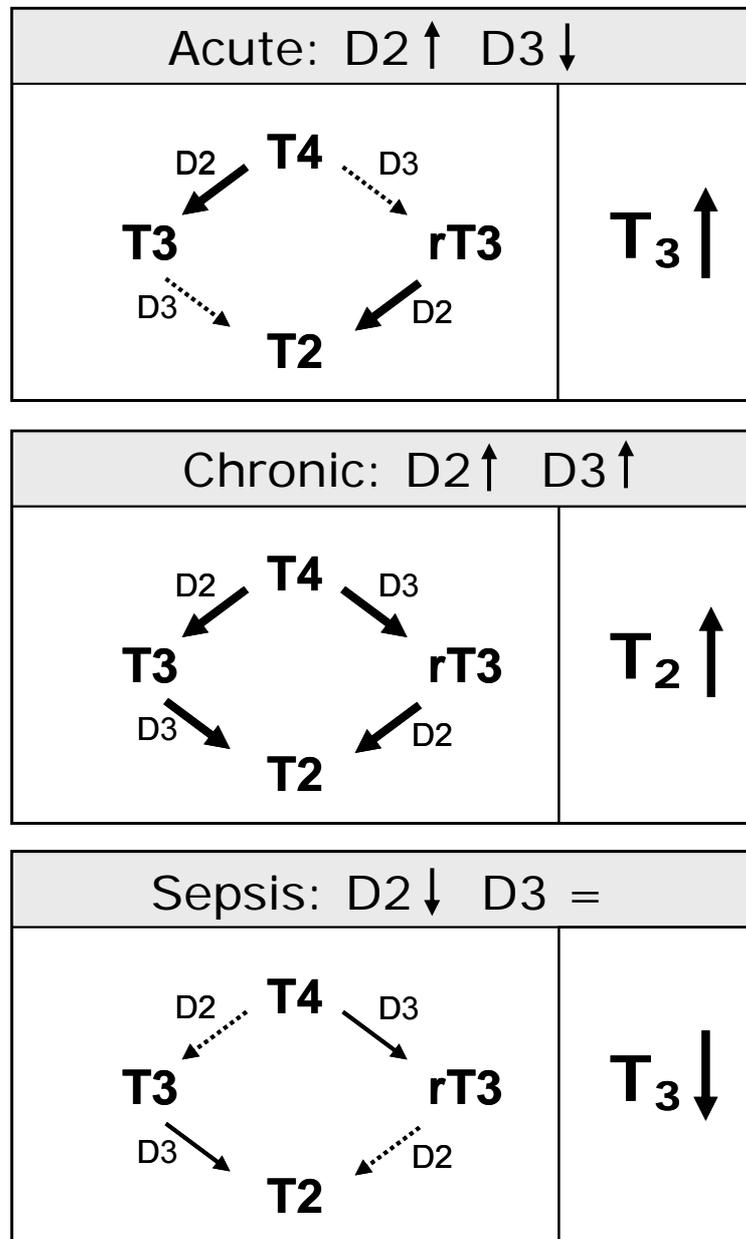


Figure 2. Schematic representation of the alterations in muscle deiodinase expression in mouse models of illness: acute inflammation (LPS administration, upper panel), chronic inflammation (turpentine induced abscess, middle panel) and severe bacterial infection and sepsis (*S.pneumoniae* infection, lower panel). On the left side the observed alterations in deiodinase expression, on the right side the theoretical net result on local T_3 and T_2 concentrations.

Both T_3 and T_2 affect mitochondrial activity. T_3 has profound effects on mitochondrial activity via mitochondrial $TR\alpha$ isoforms (36), by inducing changes in the mitochondrial inner membrane

protein and lipid compositions (34). Indeed, muscle D2 induction increases oxygen consumption (5). Furthermore, T_2 activates the mitochondrial enzyme cytochrome C oxidase (37;38), thereby increasing oxygen consumption. Finally, T_3 promotes the transition from type I (slow twitch) to type IIb (high twitch) muscle fibres, which have a lower mitochondrial density and are therefore less oxidative and more dependent on glycolysis (39). Of note, T_3 is involved in muscle proteolysis, as hyperthyroid rats show increased skeletal muscle protein degradation (40).

During illness, a selective loss of type I muscle fibre type has been proposed, leaving primarily type II muscle fibres (41), which are more prone to fatigue (39). This switch in fibre type might play a role in the muscle weakness frequently observed during ICU admission, which is known as critical illness myopathy (CIM). CIM increases morbidity in ICU patients as it leads to prolonged mechanical ventilation and delayed rehabilitation (42). CIM is supposed to be the result of a dysbalance in protein turnover resulting in muscle wasting, partly mediated via proinflammatory cytokines and NO-production by iNOS (42).

During sepsis mitochondrial dysfunction is frequently observed (43) and this is thought to contribute to CIM (42). As both T_3 and T_2 are important in mitochondrial biogenesis and activity, a shortage of T_3 and/or T_2 might contribute to sepsis-induced mitochondrial dysfunction.

Based on these observations we now hypothesize that during the acute stage of illness increased D2-mediated T_3 production plays a role in protein wasting and in the muscle fibre type switch into type II. Due to this switch fewer mitochondria are present in muscle tissue. The proposed increased T_2 production (due to simultaneously increased D2 and D3 observed during chronic inflammation) might be instrumental to activate cytochrome C oxidase which is severely affected in skeletal muscle during sepsis (43). Furthermore, it is known that adding exogenous cytochrome C oxidase improves cardiomyocyte mitochondrial function in an animal model of sepsis (44). Decreased D2 and increased D3 observed during end stage sepsis in both humans and mice might be a reflection of damaged muscle tissue with severe mitochondrial dysfunction.

To support the proposed role of differential expression of D2 and D3 in muscle during illness, the quantification of local thyroid hormone concentrations in muscle will be a prerequisite. In addition, functional studies will be needed to establish a causal role for T_3 and T_2 in mitochondrial dysfunction and in muscle metabolic state during illness.

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Obesity and Thyroid Function: Pathophysiological and Therapeutic

Implications

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ABSTRACT

The association between thyroid hormones and energy expenditure (EE) is well established. Furthermore, an inverse relationship between obesity and EE is also well-known. Therefore, the enhancement of EE emerges as a plausible treatment for obesity. At present, no clinically approved drugs for this aim are available. However, the new thyromimetic compounds may fill in this gap.

This review summarises the influence of thyroid hormones (TH) in obesity development, body composition changes and thermogenesis. The potential mechanisms involved in the variation of serum TH concentrations across the range of broad body mass index are briefly outlined. Finally, a synopsis of the thyroid mimetic compounds is presented.

Key-words: Obesity; Thyroid; Thyromimetic compounds; DITPA; GC-1; TRIAC

1.0. Introduction

1.1. Obesity and thyroid hormones

Over the last few years an unprecedented increase in obesity prevalence has settled worldwide, especially in industrialised countries (1). The reasons for this pandemic are still a matter of debate, but they are certainly related to the profound changes associated with modern lifestyle that implies a deep transformation in energy balance. Albeit well known for decades that thyroid hormones (TH) play a key role in regulating energy homeostasis (2), the obesity pandemic has driven new interest in the relationship between TH and weight status (3). Weight loss is a typical sign of thyroid hyperfunction, whereas hypothyroidism is generally associated with weight excess. The relationship between weight and TH has been broadly spread by the media, and not always with precise information. Therefore, practitioners commonly deal with overweight patients who believe that small changes in thyroid function have significant impact on body composition. This type of patient usually blames his or her thyroid as the cause of obesity. And they might be right. If not fully right, at least partially right (4). In this scenario it is plausible to speculate that TH analogues may be used in the treatment of overweight and obese people in the future.

1.2. The normal thyroid function

There is a universal agreement that thyroid function is initially determined by serum thyrotropin (TSH) concentration. Unfortunately, the agreement is lacking as regards the definition of normal thyroid function as such (5-8). Wide TSH level variations are common when serum samples from different healthy subjects are compared, even if the analysis is performed within the same age range. For instance, there is compelling evidence that normal TSH levels increase with age, although a recent study found that TSH secretion is gender invariant and depends on age in women only (9). Therefore, a specific TSH normal range for different situations is needed, including ethnic origin, age, sex, health status and, probably, body mass index (BMI) (10).

1.3. Thyroid function and body composition

More than a century has elapsed since the first clinical observation that hyperthyroid patients tend to lose weight and the reversibility of this tendency once treatment is established (11). However, unfortunately the explanation for this behaviour is still elusive in many aspects.

Epidemiological data, albeit limited, generally show a higher prevalence of overt and subclinical hypothyroidism (~20%) in morbid obese individuals (12). Although the range of TH values may vary in different populations (regarding, for instance, dietary iodine intake or other factors), the usual finding is that TSH levels correlate with body weight (10,13,14). Many groups have reported that baseline serum TSH levels are usually in the upper limit (or slightly over it) of the normal range in euthyroid obese individuals (12,14-23). Additionally, in these subjects (even in euthyroid individuals), the increase in TSH concentrations is associated with elevated waist circumference and BMI (24,25). However, this is a non-consistent finding as several clinical observations show conflicting results (12,26-28). For instance, a small French study of 20 hypothyroid women and 17 controls found no significant differences in body composition, heart rate, energy metabolism, or muscular function between the treated and untreated groups. The authors concluded that the increase in circulating thyroxine (T4) does not appear to modify the body composition or muscular function of women (29). A similar conclusion is provided by a larger British study on a cohort of 401 euthyroid nonobese and obese subjects (27). Nevertheless, these are not unexpected findings because there are consistent observations showing that a reciprocal relation between weight and serum T4 levels is lacking. Furthermore, it has been found that those morbidly obese subjects with higher TSH concentrations exhibit higher levels of triiodothyronine (T3) (which is quite a constant observation) and, only in some studies, also high T4 (30). Additionally, in an Italian cohort of women an increase in free T3 and TSH levels was associated not only with BMI, but waist circumference and fat accumulation as well (31). This TSH profile was also observed in a sample of obese children (32). As expected, the decrease in TSH and free T3 associated with weight loss in obese women indicate a reduction in energy expenditure (EE) in response to caloric restriction (30). In line with these observations, another Italian group reported that weight loss after laparoscopic gastric banding induces a decrease in free T3, while free T4 increased and TSH remained steady despite the fact that all values were within the normal range both before and after surgery (33). The interpretation of the former results suggests that progressive central fat accumulation is associated with a parallel increase in free T3 levels (FIGURE 1), probably as an adaptive thermogenic phenomenon, and the regulation of TSH secretion by free TH is possibly impaired in obesity (31).

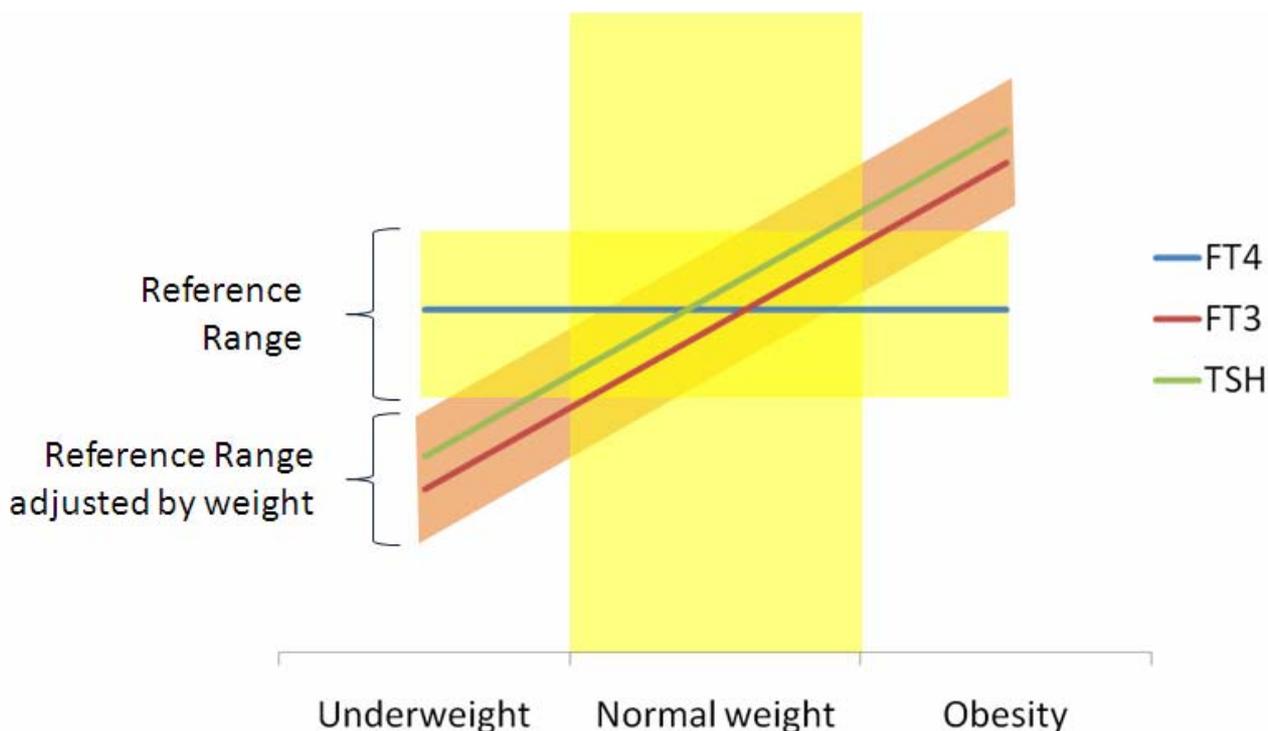


Fig. 1. The reference range for serum concentration of thyroid hormones does not take into account special circumstances like weight, age, sex, iodine intake, etc. All these situations affect the reference range limits. There is a good amount of evidence showing that weight is directly correlated with serum Thyrotropin (TSH) and free triiodothyronine (FT3) levels, whereas free thyroxine (FT4) remains unchanged. The illustration summarises this idea. The means for normal serum FT4, FT3 and TSH levels are depicted by a blue, red and green line, respectively. The "normal" reference range remains useful for all three thyroid hormones in normal weight subjects (yellow shaded areas), while TSH and FT3 are usually below the "normal" reference range in underweight patients, as is the opposed to the obese. Thus a "new normal" reference range should be considered and adjusted for the subject's weight (red shaded area).

Some authors have studied the contribution of thyroid autoimmunity to the elevation in serum TSH levels of morbid obese subjects (34). They found that autoimmunity is not a major cause sustaining the high rate of subclinical hypothyroidism in these patients. Thus, in this population, the diagnosis of subclinical hypothyroidism, as assessed by an isolated high serum TSH level, remains questionable (3,34). Additional information has been offered by a study on patients with differentiated thyroid cancer. Resting EE (REE) and body composition were evaluated during the short-term of hypothyroidism previous to the whole body scan, and on TSH-suppressive LT4 treatment. The results were compared with healthy controls. REE was significantly lower, whereas the percentage of body fat was significantly higher in patients than in controls. It was concluded that LT4 treatment enhances REE, but the increase was not significantly different from controls. Short-term deprivation of TH has an impact on body composition and influences EE (35).

1.4. Thyroid function and energy consumption

EE is determined mainly by REE and physical activity (FIGURE 2).

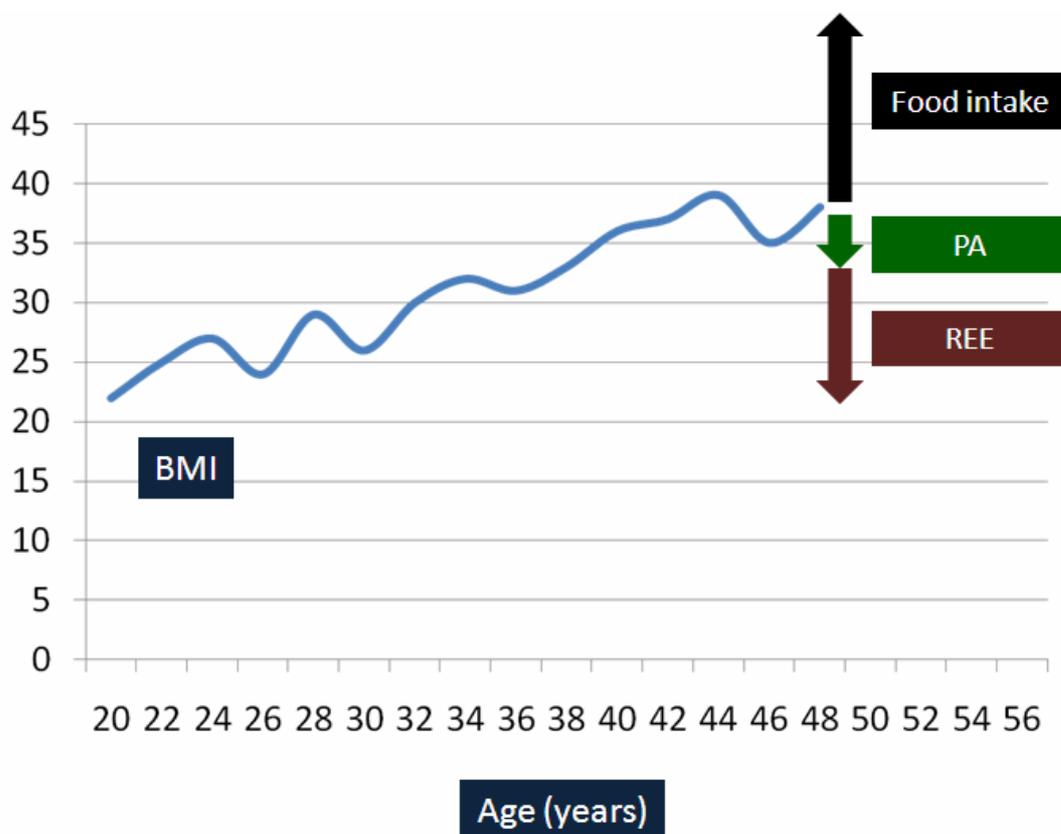


Fig. 2. Body Mass Index (BMI) depends on the balance between Energy intake (ie Food intake) and Energy consumption. Energy consumption is determined mainly by Physical Activity (PA) and Resting Energy Expenditure (REE). REE depends on adaptative and obligatory thermogenesis, which is in part mediated by thyroid hormones. The evolution of BMI over the life span depends on the equilibrium of these factors.

REE depends on obligatory and adaptative thermogenesis. Controlling thermogenesis is one of the major tasks of T3 (36). TH are key regulators of metabolism, although it is uncertain which T3-responsive-energetic processes are most important for the determination of the basal metabolic rate (37). Around 30% of obligatory thermogenesis depends on TH, and this fraction is essential for the temperature homeostasis. This action (the rise in basal thermogenesis and therefore the obligatory thermogenesis) is driven through speeding up ATP turnover. T3 raises basal metabolic rate and promotes thermogenesis by inducing an increase in the mitochondrial respiratory chain activity.

TH are also important for adaptative thermogenesis (38). Moreover, the most specific example of TH-dependent EE is not related to the basal metabolic rate, but rather to the adaptative thermogenesis (38). Adaptative thermogenesis is characterized by an uncoupling of oxidative

phosphorylation in cold-exposed brown adipose tissue (BAT), which is dependent on locally generated TH (3). A decisive component in this process is the type 2 thyroxine 5'-deiodinase (D2), which converts T4 to the active metabolite T3. Particularly, D2 can increase local, intracellular T3 production from T4 without affecting serum T3 levels (39). It has traditionally been thought that the role of BAT in humans was limited. However, a number of recent studies have changed this view, opening new fascinating perspectives. By the use of PET scans, several authors have shown the presence of 18-FDG uptake in BAT areas as well as regions of hypermetabolism in relation with cold exposure (40-42).

TH induce changes in behaviour and physical activity. Overactive thyroid patients display more physical activity than normal subjects (43). This association is most likely related to the recognized relationship between TH action and body mass. In situations with a lack of physical activity there is an increase in serum reverse T3 (rT3) levels that denotes an increase in thyroxine 3 5-deiodinase enzyme (D3) activity, which converts T3 to the inactive metabolite rT3 (43).

T3-driven mechanisms in tissues other than skeletal muscle may eventually prove to be important as well, such as the work performed by the heart, which may be responsible for up to 15% of the overall EE at rest (3,44).

1.5. Thyroid hormones and age

Extreme longevity has been associated with an increase in serum TSH concentrations (45) with thyroid diseases being more prevalent in older people (46). However, it is not known whether the rise in serum TSH levels represents a physiological trend related to aging or the consequence of illness (47). Therefore, an elevation in serum TSH level may be the translation of two situations with opposite influence on longevity (48). A low metabolic rate (which is associated with high TSH level) is a longevity marker. Furthermore, caloric restriction slows down the aging process. On the other hand, illness is a known cause of elevation in TSH – there are a good number of pathological conditions associated with mild or subclinical hypothyroidism (43,49,50). Thus, mild hypothyroidism seems to be detrimental for young or middle aged subjects, whereas it may be harmless or perhaps beneficial for advanced aged individuals (48). Therefore, it looks as if some of the metabolic alterations related with obesity may regulate thyroid homeostasis; and, this situation seems to be mitigated with age (15).

2. Pathophysiology

Many teams of investigators have been speculating on possible mechanisms that could explain the relation between obesity and thyroid gland activity. However, as recently published, fat and energy economy in hypothyroidism and hyperthyroidism are not the mirror image of one another (11). The observed positive association between TSH and BMI could be due to alterations in TH activity or as a result of an alteration in the regulation of the hypothalamic-pituitary-thyroid (HPT) axis. The hypothesis that involves a direct effect of TSH is also plausible as the TSH receptor is expressed in adipose tissue (51). It has been published that circulating cytokines related with metabolic syndrome can suppress thyroid function either at hypothalamic or pituitary or thyroid levels (52). The contribution of autoimmunity or iodine deficiency to the rise in serum TSH levels in the obese population has been ruled out (53).

The more suitable contributing factor is the deregulation of the HPT axis in the obese population, since a direct relationship between TSH and BMI has been consistently observed (16). However, as aforementioned, there are conflicting data in the literature regarding the relationship between obesity and TH. Some studies, but not all, demonstrated low T3 and low T4 at higher body weight and BMI levels, whereas other authors found a direct relationship between free T3 and BMI. Therefore, there are a number of factors that contribute to free T3 levels in obese subjects. These factors could vary among different subjects with same BMI, like body composition, underlying thyroid diseases, iodine intake, etc.

In this scenario, it is then plausible that a neuroendocrine dysfunction resulting in an abnormal secretion rate of TSH could be the cause of elevated TSH concentrations in obese subjects. It has been observed that D2, which is the main pituitary deiodinase isoenzyme, and its activity, is the key point to release TSH under T3 control, but does not work appropriately in these individuals. This mechanism may be damaged according to the observation that pituitary D2 expression does not reach the normal range in obese subjects. In addition, an Ala92 D2 variant in humans induces obesity and an insulin resistant state in comparison with wild type 92Thr D2 (54). Consequently, a resetting of the HPT axis, and not merely insufficient TH levels, seems to be a key factor that shifts the EE

equation in obese subjects. Fasting, for instance, induces profound changes in the HPT axis as well. Studies in rodents have shown a dramatic down-regulation of TRH gene expression in the paraventricular nucleus (PVN) during fasting. Direct and indirect effects of decreased serum leptin, in addition to effects on increased local T3 concentrations in the hypothalamus during food deprivation, contribute to a decreased activity of TRH neurons in the PVN. Pituitary TSH β mRNA expression also decreases during fasting, and this may be relatively independent of leptin and/or TRH, since leptin administration in this setting does not fully restore pituitary TSH expression, while it does restore TRH expression in the PVN. The observed decrease in serum TH concentrations is the result to some extent of a diminished thyroidal secretion of TH. The overall result of these complex HPT axis changes in various tissues during fasting is down-regulation of the HPT axis, which is assumed to represent an energy-saving mechanism, instrumental in times of food shortage (55).

Thus, it seems that the adipocyte-derived hormone, leptin, may be at the origin of this dysfunction (56) (see below), although other possibilities may also exist. Some investigators have suggested the existence of partially bioinactive TSH in obese subjects, although this hypothesis is very speculative (3). Other authors suggest that there may be certain TH resistance, as well as decreased T3 receptors in obese subjects (57).

3.0. Associations related to thyroid hormones and obesity

3.1. Insulin resistance

The association between thyroid disease and glucose metabolism is well documented (58). Insulin resistance with hyperinsulinemia are important features of the metabolic syndrome and generally accompanies obesity (59). Insulin resistance has been related with both ends of the thyroid dysfunction spectrum, although the poorly understood mechanisms might be different in each case (60). Hyperthyroidism is a well-known cause of hyperglycaemia. The explanation for this relationship could be an unopposed activation of gluconeogenesis (61). Moreover, hyperthyroidism, even in subclinical forms, is associated with a reduced insulin half-life, most likely because of accelerated insulin degradation (58,60,62). On the other hand, hypothyroidism-associated insulin resistance could

be the result of diminished tissue sensitivity to insulin. Accordingly, glucose disposal is then reduced in this situation (58). In hypothyroidism, insulin resistance is counterbalanced by a parallel reduction in gluconeogenesis, making it habitually irrelevant without clinical consequences (58).

3.2. Thyroid and adipokines

Several groups have studied the relationship between leptin and the pituitary-thyroid axis (15,22,62-67). Interestingly, TSH and leptin display similar serum concentration profiles in their circadian rhythm, which may indicate a leptin regulatory effect on TSH secretion. Considering that leptin is an indicator of fat mass, the observed association between serum leptin levels and thyroid function is interesting. Unfortunately, once more the results of these studies fall under discrepancy (56,63,65,68,69), making difficult the elucidation of the reasons for this relationship. Different investigators have found almost all possible combinations between thyroid function and leptin. Some of them have associated hypothyroidism with serum leptin levels below (69-71), above (72) or in the normal (56,73) range. Something similar has been shown in hyperthyroid subjects where high serum TH concentrations have been linked with low (71), high (70,74) or normal (69) serum leptin levels.

As aforementioned, some authors have hypothesised on the role of leptin in the modulation of the pituitary-thyroid axis. This has been demonstrated in premenopausal obese women (67), despite conflicting findings. A recent study was also completed in a sample of premenopausal women with hyperthyroidism or hypothyroidism. Treatment of thyroid dysfunction – not associated with changes in BMI or % of body fat – did not influence serum leptin but did affect serum ghrelin. The authors concluded that thyroid status itself, in the absence of alterations in BMI and % body fat, exerts an important influence on circulating ghrelin but not leptin (75). In our experience (15), in agreement with former studies (76,77), there is a significant positive correlation between circulating leptin and TSH levels in a sample of obese men. On the other hand, we also observed that the correlation between leptin and age was negative (15).

Nowadays, there is no clear explanation to disclose the reasons for these disagreements between different studies. We can speculate that the catabolic condition of thyrotoxicosis is very similar to the fasting state, and both situations seem to lead to a reduction in both serum leptin and TSH secretion. On the other hand leptin itself directly stimulates TRH (38) secretion, and

subsequently TSH and TH. A rise in serum TSH levels is usually interpreted as a hypothyroid status, but may also be the result of an effort to stimulate the thyroid and, therefore, the induction of gland overactivity. In addition, leptin has been shown to have a direct inhibitory effect on several components involved in TH production from thyrocytes (62); and, leptin may directly affect the sensitivity of the thyrotroph or the thyrocyte (4).

Data regarding the relationship between ghrelin levels and thyroid function exist, but are scarce. Serum ghrelin levels are increased by 32% in the hypothyroid state and became normalized after L-thyroxine replacement. Therefore, serum ghrelin levels are reversibly increased in hypothyroid patients (78). In agreement with this information, it has been found that hyperthyroid subjects present low serum ghrelin levels (79). In addition, circulating ghrelin has been significantly correlated with age, fasting, glucose and TSH, but not with BMI (79). In any case, it seems that there are complex mechanisms involved in these observations that are worth of clarification.

4.0 Thyroid hormones and mimetic compounds in the treatment of obesity

Obesity is defined as an excessive accumulation of body fat. Therefore, the ideal treatment for patients with obesity aims to achieve a negative caloric balance, not only to reduce weight, but also to improve body composition by decreasing as selectively as possible the percentage of body fat. However, caloric deprivation usually also leads to reduction of both fat tissue and fat-free mass. According to this, a desirable therapeutic response consists in not allowing a fat-free mass wasting superior to 25% of total body weight. Energetic balance, macronutrient proportion and physical activity are significant factors involved in the control of body composition in obesity treated subjects. Loss of fat-free mass due to a reduction in muscle tissue is at least in part, responsible for a fall in resting EE, which contributes to the frequent phenomenon of weight regain. Intense caloric deprivation is associated with a decrease in plasma leptin, T3 and sometimes T4 concentrations and a rise in rT3 levels in what has been considered as an adaptation process to reduce metabolic needs (80). This T3 decrease has also been involved in the weight loss-associated REE fall (81).

In an attempt to maintain or promote further weight loss and avoid weight regain, different trials with TH supplementation have been carried out (82). The purpose of this treatment would be to

increase fat loss through enhancing oxygen consumption and fatty acid oxidation without having either TSH suppression or side effects on muscle, central nervous system, bone or cardiac function.

4.1. Thyroid hormones

Recently, a systematic review has been published on the results obtained with TH treatment in obese patients submitted to caloric deprivation (82). The paper includes 14 studies designed to test the efficacy of T3 administration in obese treated patients. However, the heterogeneity in the quality and design of these trials prevented drawing any firm conclusions. T3 doses ranged from 18 to 117 mcg/70 kg of body weight. Significant weight loss was only achieved in five out of 26 comparisons and TSH and T4 values, when assessed, were systematically reduced. No firm data on protein breakdown due to catabolic effects of TH were obtained. No studies performed body composition evaluations to investigate if there were changes in the fat or fat-free mass compartments. Only few studies measured REE, nitrogen balance and 3-methylhistidine urinary excretion, showing no consistent results. No clear variations in heart rate were seen. Therefore, those studies did not demonstrate any sustained benefit on weight loss, whereas TSH suppression and a decrease in T4 values are compatible with inhibition of the HPT axis due to T3-induced subclinical hyperthyroidism. T3 administration increases leptin gene expression following caloric restriction in obese rats. Nevertheless, the contribution of this mechanism to weight loss or maintenance is unlikely since no increase in circulating leptin levels or significant weight reduction were observed in that experimental model (83).

4.2. Dextrothyroxine and TRIAC

Use of TH analogues have been tried in the past with the aim of taking advantage of beneficial actions such as fat mass reduction or control of hyperlipidemia while avoiding side effects on bone, brain and heart. Clinical experiences using dextrothyroxine for hyperlipidemia therapy have been unsuccessful (84). However, a natural TH metabolite, triiodo-thyroacetic acid, has shown thermogenic capacity in brown adipocytes in culture (85,86), though no clinical studies have confirmed its efficacy in the treatment of obesity.

4.3. Selective thyroid hormone receptor activation

The progressive knowledge in the mechanisms of TH action at the cellular level has opened new possibilities on the therapeutic application of selective TH receptor (THR) activation. THR encoding genes are differentially expressed in various tissues. Different studies carried out in mice with inactivation of different THR isoforms (87,88) and data from patients with resistance to TH (89) have demonstrated that different THR forms are responsible for tissue-specific responses to TH.

THR alpha (THR α) is mainly present in the brain and heart where it regulates cardiac function, whereas THR β is preferentially detected in liver, where it is responsible for the effects of TH on lipid metabolism (90). The THR β 1 is the predominant systemic form, while the pituitary form, which controls TSH secretion, is THR β 2. These investigations have opened new perspectives to look for selective stimulation of particular TH actions for treatment of some diseases such as dyslipidemia and obesity. A rise in REE as well as a reduction in fat mass and an improvement in insulin sensitivity and lipid profile represent interesting objectives to achieve in obesity treatment following selective thyroid receptor activation. At the same time, classical side effects due to TH overexposure such as muscle wasting, bone loss, nervousness, hypertension and cardiac dysfunction (arrhythmias, heart failure) should be avoided.

Changes in the molecular structure leading to selective receptor binding with a particular THR isoform as well as the capacity of different compounds to be taken up by specific tissues explain the specificity of actions (91). Special efforts have been made to develop THR β selective modulators due to their preferential effect on liver metabolism.

A selective thyromimetic compound, GC-1 (3,5-dimethyl-4-(4-hydroxy-3-isopropylbenzyl)phenoxy acetic acid -sobetirome-) with 10-fold preferential action on THR β 1 over THR α 1, was able to induce an increase of EE of 5-10% while provoking only mild tachycardia in mice (92). The drug also caused a 4% body weight reduction in cynomolgous monkeys treated for 7 days with no evidence of muscle wasting (93). Affinity of GC-1 for THR α 1 is 10 times lower than T3 (94), accounting for the attenuation in heart rate stimulation. Accordingly, the increase in heart rate related

to the rise in EE was less than that seen following treatment with T3. In addition, GC-1 administration to primates has been followed by an increase in oxygen consumption and body weight reduction (93).

More recently these results have been confirmed in female rats treated for 6 weeks with either T3 or CC-1. The animals showed a rise in oxygen consumption similar to that found after T3 treatment, whereas GC-1 induced a 20% reduction in fat mass without increasing food intake. In contrast with T3 administration, no changes were seen in heart mass; and skeletal muscle mass was minimally affected by GC-1. These results suggest that GC-1 may have a promising role as an anti-obesity agent, since it reduces fat mass without increasing food intake and facilitates the control of dyslipidemia without having deleterious effects on heart or bone mass (95).

Another $\text{THR}\beta$ agonist, KB-141, is 10-fold more selective for stimulating metabolic rate and 30-fold more selective for cholesterol lowering than for positive chronotropic effects. However, accumulation in the liver is much less than that seen with CC-1. To maintain or broaden this therapeutic window, reassuring the lack of cardiac side effects is essential before considering its use in humans. KB-141 has shown to induce weight loss, cholesterol and Lp(a) reduction. However, this compound decreases TSH values leading to secondary hypothyroidism in territories not accessible to selective $\text{THR}\beta$ agonists such as the brain (96).

GC-24 is another $\text{THR}\beta$ agonist that has demonstrated ability to reduce body fat accumulation and to prevent liver steatosis in rats with diet-induced obesity and increasing EE (97). These effects were seen without any change in food intake or cardiac weight, suggesting that this compound does not act significantly on myocardium. GC-24 also reduced the glucose response to a glucose load, improved insulin sensitivity and normalized the previous hypertriglyceridemia. However, GC-24 reduced only marginally total cholesterol levels and did not have any effect on free fatty acids or IL-6 levels. GC-24 increased the expression of Cpt 1, Sd and Acc 1 in BAT, suggesting that the drug has significant thermogenic effects as well as effects on EE. GC-24 displays a 40-100 fold preference for $\text{THR}\beta$ over $\text{THR}\alpha$ (98).

KB 2115 is the only compound that has been administered to human subjects. The drug was effective in achieving a 40% reduction in total and LDL-cholesterol plasma concentrations after 14

days of treatment (TABLE 1). Mechanisms seem related to an increase in bile acid synthesis. Interestingly, KB 2115 therapy was associated with a dose-dependent reduction in total and free T4 levels without inducing any variations in TSH concentrations (99). No changes in metabolic rate or body weight were observed.

Table 1. Effects and characteristics of different selective thyroid hormone receptor agonists.

	GC-1	KB-141	GC-24	KB-2115	DITPA
Effects on EE, Body Weight and Body composition	Increase in EE Body weight reduction Body fat reduction No food intake stimulation Increase in fatty acid oxidation Decrease in inflammatory markers Reduction of liver steatosis	Increase in EE Reduction in body weight Reduction in fat mass Glucose reduction in Zucker rats and ob/ob mice	Increase in EE Prevents increase in fat mass Upregulates BAT gene expression Increase in insulin sensitivity	No changes in EE No changes in body weight	Reduction in body weight
Effects on Serum Lipids	Reduction in Triglycerides Reduction in LDL cholesterol Reduction in Lp(a) Increase in HDL receptors Stimulates reverse cholesterol transport	Reduction in cholesterol Increase LDL receptor expression Reduction in Lp(a) Increase in CYP7A1 expression	Reduction in cholesterol Reduction in triglycerides	Reduction in total cholesterol Reduction in LDL cholesterol Reduction in bile acid synthesis	Reduction in total cholesterol Reduction in LDL cholesterol Reduction in triglycerides
Effects on circulating thyroid hormone levels	Reduction in TSH, T3, T4		Reduction in TSH, T3, T4	Reduction in T4 No changes in T3 or TSH	Reduction in TSH, T3, T4
Effects on heart and bone	No effect on muscle, bone or heart	Minimal heart effect	No effect on heart	No effect on heart	Increase in heart rate Increase in bone turnover Increase in SHBG
Thyroid Hormone Receptor affinity	10 times more selective for THR β than THR α	15 times more selective for THR β than THR α	40 times more selective for THR β than THR α		Low THR selectivity

EE: Energy expenditure. BAT: Brown adipose tissue. THR: Thyroid Hormone Receptor. Asterisks indicate the drugs that have been tried in human subjects.

Recently, a less selective thyromimetic, 3,5-diiodothyropropionic acid (DITPA), has been assessed in patients with stable congestive heart failure. DITPA was able to reduce body weight, total and LDL-cholesterol values and triglycerides to a lower extent. However, the drug induced suppression of the hypothalamic-pituitary thyroid axis and increased bone turnover, whereas it was ineffective in improving cardiac symptoms (100,101).

Experimental studies suggest that several obesity complications such as liver steatosis and type 2 diabetes can be improved by TH mimetics (96). Whether this is due to a direct effect or to an indirect action derived from fat mass loss remains unclear.

4.4. Bile acids

Dietary supplementation with bile acids can regulate EE and TH activation via changes in D2 expression, an enzyme involved in BAT thermogenic pathways that contribute to EE (102,103). Kaempferol, a polyphenolic molecule of dietary sources has been shown to increase skeletal myocyte oxygen consumption by rising cAMP generation and inducing protein kinase A activation. Also, this compound is able to influence the expression of genes involved in thermogenesis, such as UCP-3, and to up-regulate D2 gene expression, prolonging its half-life (104). These pathways may also represent a target with clinical application in the treatment of obesity and other metabolic disorders.

Selective manipulation of TH actions represents a promising therapeutic tool for the treatment of obesity and some of its complications. Future investigations to design new compounds with selective effects on different metabolic pathways may lead to the generation of valuable drugs to be used as monotherapy or in combination with already established modes of therapy for obesity, fatty liver, type 2 diabetes or dyslipidemia. Nevertheless, more studies are needed to gain more knowledge about adequate dosage, time and mode of administration, and especially on safety matters regarding integrity of other organs sensible to TH administration, such as the heart, bone, HPT axis and the central nervous system.

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Iodine status, thyroid and pregnancy

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Abstract

Iodine is an essential trace element for life. Its biological effects is due to the fact that iodine is an integral part of the thyroid hormones, and thus plays a crucial role in fetal organogenesis, and in particular in brain development. This takes place during early gestation and involves delicate targeting throughout the central nervous system. Iodine deficiency in pregnant women - defined as a median of urinary iodine excretion of less than 150 µg/L in pregnant and lactating women - is the leading cause of preventable mental retardation, affecting as many as 2 billion people –35.2%- at the beginning of the new century. Europe has a very high prevalence of iodine deficiency in the general population, with nearly 50% of the 600 million people in Western and Central Europe having insufficient iodine intake. Recent Studies involving pregnant women indicate that in Belgium, Poland, France, Italy, Denmark, Turkey, Portugal, and some regions of Spain, iodine deficiency has been detected. Iodine deficiency is often associated with a deficiency of other nutrients such as Selenium, Iron or Vitamin A, contributing to a worsening of the biological effects of iodine deficiency. Prevention of fetal iodine deficiency is feasible, provided that iodine supplements of 200-300 µg/day to the mother are given both, before and throughout gestation and continued through lactation. The presence of other micronutrients deficiencies cannot be forgotten, and a combined multi-supplement approach covering all these nutritional needs, seems the best practice in pregnancy.

Introduction

In this manuscript we will review some epidemiological data on the status of iodine deficiency in pregnant women in Europe, the current recommendations regarding when and how to supplement with iodine in pregnant women, and finally, what are the reported data related to the consequences for the progeny when iodine supplements are given to the mother during pregnancy. The consequences of iodine deficiency are due to the fact that iodine is an integral part of the thyroid hormones. Since the first reports by Pharoah in the 70s^{1,2} and Thilly³, the last three decades have brought to the fore both epidemiological and experimental data confirming that defective maternal iodine input, leading to insufficient maternal production of thyroxine during pregnancy is associated to a variety of tissue damage in the progeny. The spectrum of severity ranges from relatively mild neurocognitive defects to severe alterations of mental function in those lesions best documented relating to iodine related maternal hypothyroxinemia. We now know from experimental studies that these functional defects have demonstrable underlying structural lesions of an irreversible nature and originate in early pregnancy at the time of fetal organogenesis⁴⁻⁶. Most of the clinical data found in the literature are derived from studies performed in areas of extreme iodine deficiency or in cases of untreated maternal hypothyroidism; however, it has been demonstrated that even a moderate lack of iodine can have detrimental and irreversible consequences for the fetus, and therefore should be understood as a continuum in which the milder forms could be defined as fetal iodine deficiency disorder, and are currently neglected or ignored in the general medical practice⁷.

Iodine deficiency - defined as a median of urinary iodine excretion of less than 100 µg/L for general population and 150 µg/L in pregnant and lactating women^{8,9} - is the leading cause of preventable mental retardation, affecting as many as 1.6 billion people -29% of world population- according to WHO data in 1990¹⁰, increasing to approximately 2 billion people -35.2%- at the beginning of the new century. These data include 285 million school-age children in 2006¹¹ and indicate that the WHO's goal of avoiding new cases of iodine deficiency disorders by the year 2000 has not been accomplished; and the sad story is that even if this problem can be prevented with very cost-effective and efficient programs ensuring a sufficient iodine supply provided to the mother before and during pregnancy, the current achievements are far from what has been an international commitment by many governments¹², including those of the European Union.

For a long time, the idea that the principal factor accounting for impaired fetal neurologic development was maternal hypothyroidism -defined as a state of increased serum TSH- has prevailed^{13,14}. In the last decade, the progress in thyroid research has allowed a more in-depth knowledge of maternal, placental and fetal thyroid hormones interrelationships¹⁵, the trafficking of thyroxine through the placenta, as well as the full characterization of the ontogeny of thyroid hormone receptors in the placenta and embryonic tissues¹⁶. All this information taken together, has given the basis for a better physiopathological understanding of the diversity of clinical expression of endemic cretinism, or in modern terminology, fetal iodine deficiency disorder. This has at times led to confusion, being both,

the neurologic cretinic and the myxedematous forms – with considerable overlap in between – explained and driven by the timing and severity of pre and postnatal iodine deficiency. This confirms the crucial role of maternal euthyroxinemia in early pregnancy as a prerequisite for a normal embryonic development ¹⁷.

Epidemiology of iodine deficiency in pregnant women in Europe

In 2003 about 2000 million people were currently estimated to be iodine deficient worldwide (35.2% of the entire world population) ¹¹. Paradoxically, Europe, which has been thought to be free of iodine deficiency disorders and has been leading the research in the field, has in fact the highest prevalence of iodine deficiency, with nearly 50% of the 600 million people in Western and Central Europe having an insufficient iodine intake ¹⁸, defined as urinary iodine <100 µg/L ¹⁹. Northern and Southern America are at the other end of the spectrum, with a prevalence of iodine deficiency of less than 10%. According to very recent studies which have examined the European countries where iodine status is threatening pregnancy outcome, we realize that the list is long and includes (Table 1): Belgium, Poland, France, Italy, Denmark, Turkey, Portugal, and some regions of Spain.

Table 1. Urinary iodine in pregnant women in Europe (1990-2009)

^a Iodine Nutrition	publication year	N	S/C ^b	Trimester ^c	Median of urinary iodine
Iodine-Sufficient countries					
Germany ²⁰	1996	89	S	1	53 µg/g cr
		89	11 d P	-	50 µg/g cr
Germany ²¹	2003	109	C	3	181 µg/g cr ^d
Hungary ²²	1997	119	C	1,2,3	57 µg/g cr
Sweden ²³	1998	32	S	1	180 µg/day
		32	S	2	170 µg/day
		32	S	3	145 µg/day
Switzerland ²⁴	2001	511	C	2,3	138 µg/L
Switzerland ²⁵	2004	279	C	1	249 µg/L
Slovenia ²⁶	2009	118	C	3	176 µg/g cr
Spain (Pontevedra) ²⁷ (León) ²⁸	2002	81	C	3	111 µg/L
		161	S	1	71 µg/L ^d
		161	S	2	91 µg/L ^d
(Málaga) ²⁹	2004	161	S	3	120 µg/L ^d
		520	S	1	70 µg/L ^d
		520	S	2	77 µg/L ^d
		520	S	3	84 µg/L ^d
(Madrid) ³⁰	2006	112	C	2-3	101 µg/L
(Alicante) ³¹	2007	104	S	1	69 µg/L
		104	S	2	140 µg/L
(Bizkaia) ³²	2007	2191	S	1	88 µg/L
		1401	S	2	140 µg/L
(Extremadura) ³³	2008	761	S	1	123 µg/L
		362	S	2	125 µg/L
(Val Aran) ³⁴	2008	35	S	1	88 µg/L
		32	S	3	140 µg/L
(Valencia) ³⁵	2010	530	C	1	131 µg/L
		100	C	2	144 µg/L
(Guipuzcua) ³⁵	2010	126	C	1	171 µg/L
		475	C	2	168 µg/L
(Sabadell) ³⁵	2010	122	C	1	97 µg/L

<i>Table 1 (continued)</i>					
(Valencia) ³⁶	2009	161	C	2	95 µg/L
(Toledo) ³⁷	2009	232	C	1	100 µg/L
(Catalonia) ³⁸	2010	157	C	1	135 µg/L
	2010	220	S	1	163 µg/L
		121	S	3	172 µg/L
Iodine-deficient countries					
Belgium ³⁹	1990	230	C	1	58 µg/L
		265	C	2	58 µg/L
		370	C	3	53 µg/L
Poland ⁴⁰	1993	46	C	1-3	35 µg/L
Poland ⁴¹	2003		C	1-3	70 µg/L
Denmark ⁴²	1993	26	S	2	51 µg/L
		26	S	3	40 µg/L
Denmark ⁴³	1993	98	C	5 d PP	35 µg/L
France ⁴⁴	1997	306	S	1	50 µg/L
France ⁴⁵	2009	330	C	3	64 µg/L
Ireland ⁴⁶	2009	38	S	1	135 µg/L
		38	S	2	124 µg/L
		38	S	3	122 µg/L
		84	C	40 d PP	74 µg/L
Italy ⁴⁷	2002	67	C	1,2	74 µg/g cr
Italy ⁴⁸	2008	51	C	1	74 µg/L
Italy ⁴⁹	2009	220	C	1	96 µg/L
Turkey ⁵⁰	1995	90	C	1,2,3	91 µg/day
Turkey ⁵¹	2005	18	C	1	143 µg/L ^d
		28	C	2	137 µg/L ^d
		77	C	3	161 µg/L ^d
Turkey ⁵²	2004	70	C	5 d PP	30 µg/L
Turkey ⁵³	2009	824	C	1	77 µg/L
Portugal ⁵⁴	2009	136	S	1	65 µg/L
		128	S	2	57 µg/L
		119	S	3	70 µg/L

^aCurrent classification of countries based in iodine nutrition¹⁹

^bS/C, sequential (S) or cross-sectional study (C)

^cTiming of urinary iodine determination. PP, postpartum. Adapted from Gliozzi (2003)⁵⁵

^dMean of urinary iodine

In different Spanish studies performed in Catalonia, while most of schoolchildren⁵⁸ and general population⁵⁹ had acceptable iodine nutrition, about half pregnant women of this region had an insufficient iodine supply^{60,61}, and in some particular places of Catalonia, the proportion of women showing less than 150 µg/L of urinary iodine has shown to be up to 74%⁶². In these Catalan studies, the prevalence of maternal hypothyroxinemia ranged between 1.4 and 4.2%⁶³. Similar data have been found in other different regions of Spain^{27,29}. These and other studies indicate that in countries with an apparently acceptable iodine status, in pregnancy, the dietary iodine supply is insufficient for covering gestational requirements, and therefore, other strategies as, i.e., preconceptional oral supplementation with potassium iodide or others, are needed for solving the problem, although from epidemiological surveys, this practice less 15-50% of pregnant women in Europe⁶⁴.

When and how for a safe iodine supplementation in pregnancy

Without doubt, the best practice would be to ensure a sufficient replenishment of thyroid iodine stores before pregnancy, but as mentioned, this ideal situation is far from being a reality, and mostly in Europe. Up until now an early (or not so early) supplementation during pregnancy has usually been the case. The addition of iodine during gestation has not always been accepted and is not general practice by the obstetric community; moreover, it has even been questioned if not suspected of being

harmful for the progeny. Eight different trials aiming to study the effects of iodine supplementation early during pregnancy have been performed in Europe in the last two decades, including about 700 women in which iodine was given at a dose ranging 50-300 µg/day^{20;42;47;65-69}. In all these studies, median urinary iodine increased about two to three-fold and thyroid volume virtually did not change in treated women, while in 20-30% of the control population an increase in thyroid volume was detected by ultrasonography. Overall, treatment had no effect on maternal TSH and thyroglobulin, and cord thyroglobulin levels were significantly lower in the treated groups. No significant differences were found between groups comparing maternal or cord T4, T3, and FT4. However, in the studies in which the amount of iodine given was the highest, FT4 was lower when compared to the control groups in the third trimester, as in the study by Velasco⁶⁹, but also, FT4 decrease in comparison to first trimester values was less. Fetal TSH in this last study was higher in the babies in which the mothers were supplemented in comparison to those who were not, but their neuropsychological development was better according to the Bayley scales scores when compared to the non-supplemented control group. In general, for the newborn, most data suggest that supplementation is safe, although three of the mentioned studies showed higher newborn TSH levels –albeit within normal values– when supplementation was reported. Until now, the interpretation of this event has usually been seen as potentially harmful for the baby, but it may not be so deleterious if we consider that in two of the studies neurodevelopmental scores were better in these children and particularly in those where their mothers were supplemented earlier in their pregnancies^{68;69}. Considering this point, the time when iodine is introduced during pregnancy seems to be very important; in the study also performed in Spain by Berbel, neurocognitive development assessed by the Brunet-Lézine was better in the kids of mothers in which supplementation was started at 4-6 weeks of gestational age in comparison to those where supplements started at 12-14 weeks of gestation or no supplement was given. This implies that the therapeutic window is restricted to the very early pregnancy, and therefore if a pregnancy is planned, the time of trying to become pregnant is the ideal moment in which to replenish the maternal iodine thyroid stores. Also an analysis should be performed as soon as the woman is aware that she is pregnant.

The usual form of iodine supplements are potassium iodide tablets or iodine-containing prenatal multivitamin preparations⁷⁰⁻⁷⁴. Another option is i.m. iodized oil that has been used in South America; it is safe and its single dose is easy to administer, and may provide constant blood iodine levels⁷⁵⁻⁷⁷. According to the surprisingly low compliance of some very well established preventive programmes, such as oral folic acid supplementation, which is followed just by 7-42% pregnant women in some European studies^{78;79}, this latter possibility of using a single and simple depot administration of i.m. iodized oil injection prior to conception, seems to be a reasonable solution in a potentially non-compliant population.

Questions remaining to be answered in iodine supplementation for pregnant women

Despite all data previously commented, a substantial debate is still going on the dose to be given, and if an undetected thyroid disease in the mother maybe influenced by iodine supplementation. In relation to the dose to be used, no adverse effects of 50–300 µg daily iodine supplement have been documented in moderately iodine-deficient pregnant women. Theoretically, it is possible that doses higher than 500 µg/day of supplemental iodine could result in fetal hypothyroidism. The ability to escape from the acute Wolff-Chaikoff effect, and therefore to avoid iodine-induced hypothyroidism, seems not fully active until around 36 wk of gestational age ^{80;81}, and therefore the World Health Organization has stated that daily iodine intake greater than 500 µg/day may be excessive in pregnancy ⁸²; the European Food Agency has defined a similar limit of 600 µg/day; finally, the U.S. Institute of Medicine considers the safe upper limit for daily iodine intake as 1100 µg/day in pregnant women ⁸³, indicating that the safe upper limit seems to be quite high. Probably more studies are needed to better define this issue, but what is certain is that most women supplemented with 200-300 µg/day will reach a sufficient replenishment of their thyroid stores safely, and if this is done before conception the potential maternal and fetal Wolff-Chaikoff effects maybe avoided.

In the case that a pregnant woman is discovered to have an inhibited TSH in the first trimester, iodine supplementation should be maintained, although it is recommended to perform a clinical follow-up with measurement of total and free T4. It has to be remembered that TSH may not be the best parameter of thyroid function to be used during the first trimester as it decreases physiologically under the effect of increasing hCG concentrations. The same applies for those women having Graves disease in remission, and it is unlikely that unfavourable effects of 200-300 µg/day of iodine may happen. This should be clearly differentiated from a real pharmacological dose of iodine, i.e, the one given in the treatment with amiodarone. Furthermore, if a woman having an active Graves disease or a toxic multinodular goiter becomes pregnant, iodine supplementation should not necessarily be stopped if the case is treated with the usual antithyroidal drugs, and the follow-up should not be different to usual practice ⁸⁴. In fact Graves disease tends to remit during the second trimester, regardless if iodine is or is not included in the pregnancy supplementation protocol. It has been argued that patients under replacement thyroxine treatment because of previous hypothyroidism, might not require iodine supplements; if we take into account that levothyroxine supplies 65.3 µg of iodine/100µg of thyroxine, it is clear that the iodine released during thyroxine metabolism is insufficient for the fetus, which then needs iodine during the second half of the pregnancy for synthesising its own thyroxine ¹⁷.

In all the published data there was no increase in maternal thyroid autoimmunity, or in the prevalence or severity of post-partum thyroiditis⁸⁵, therefore even if a theoretical risk existed, the iodine requirements of the fetus and its benefits, justify the administration of potassium iodine to the mother. Finally, most of the studies seem to agree that iodine supplementation should be continued until the end of lactation, as the iodine content of maternal milk is enriched ^{64;86}

Iodine and other Nutrients

Iodine deficiency is often associated with deficits of other nutrients such as Selenium (Se), Iron (Fe) or Vitamin A (Vit A) ⁸⁷⁻⁸⁹. Se is a major constituent of enzymes such as glutathione peroxidase (GPx), thioredoxin reductase (TxnRd) and the iodothyronine deiodinases (D1O1- O3) which are essential for thyroid hormonogenesis and metabolism ⁹⁰. Concurrent deficiencies of one or more of these substances can intensify the effects of iodine deficiency. For example, in combined iodine and Se deficiency there is a failure to utilise H₂O₂ for thyroid hormonogenesis with build up of cytotoxic products leading to myxedematous cretinism ⁹¹. Giving Se alone can exacerbate the hypothyroidism due to increased deiodination of stored T₄. In this situation it is important to resolve the iodine deficiency before administering Se ^{87;91}. Deficiencies of Fe and Vit A are common in pregnancy. A combination of Fe and I deficiency can result in decreased thyroid hormone production as Fe is an important component of TPO ⁹². Fe deficiency may block a child's ability to use iodide. Iodide prophylaxis may be of no use if Fe is not given simultaneously ⁹³. Another aspect of the Fe/I story is the increased Fe requirement in pregnancy. Fe deficiency may contribute to the increased TSH seen in late pregnancy ⁹⁴. Vit A deficiency has been shown to be associated with increased TSH while high doses of Vit A can decrease production of the promoter on the TSH β subunit gene ⁸⁸. These findings strongly support the need for combined approaches for correcting nutritional deficiencies ⁹².

In summary, iodine status of pregnant women is clearly suboptimal in many regions of Europe, and iodine-containing supplements have a beneficial impact or are at least safe for the iodine and thyroid status of both the mother and the newborn. Pregnant women in these regions, if not adequately covered by iodized salt or even using iodized salt, should be supplemented with iodine in most cases, ideally during the preconceptional situation, or during early (as soon as possible) pregnancy and lactation in order to ensure a maximal normal neurodevelopment of their children. The presence of the others nutritional deficiencies cannot be forgotten.

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AMERICAN ASSOCIATION OF CLINICAL ENDOCRINOLOGISTS (AACE), ASSOCIAZIONE MEDICI ENDOCRINOLOGI (AME), AND EUROPEAN THYROID ASSOCIATION (ETA) MEDICAL GUIDELINES FOR CLINICAL PRACTICE FOR THE DIAGNOSIS AND MANAGEMENT OF THYROID NODULES ^a

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Note of the Authors

Medical Guidelines for Clinical Practice have been developed by AACE to assist health care professionals in medical decision making for specific clinical conditions. Most of the content herein is based on literature reviews. In areas of uncertainty, professional judgment was applied. These guidelines are a working document that reflects the state of the field at the time of publication. Because rapid changes in this area are expected, periodic revisions are inevitable. We encourage medical professionals to use this information in conjunction with their best clinical judgment. Any decision by practitioners to apply these guidelines must be made in light of local resources and individual patient circumstances.

^a *These guidelines are based on Endocr Pract. 2006 Jan-Feb;12(1):63-102. Used with permission.*

ABSTRACT

This document was prepared as a collaborative effort between the American Association of Clinical Endocrinologists (AACE), the Italian Association of Clinical Endocrinologists (AME), and the European Thyroid Association (ETA). This guideline covers diagnostic and therapeutic aspects of thyroid nodular disease but not thyroid cancer management.

The AACE protocol for standardized production of clinical practice guidelines was followed to rate the evidence level of each reference (on a scale of 1 to 4) and to link the guidelines to the strength of recommendations on the basis of grade designations A (action based on strong evidence) through D (action not based on any evidence or not recommended). The best evidence level (BEL), corresponding to the best conclusive evidence found, accompanies the recommendation grade. All

recommendations resulted from a consensus among the AACE, AME, and ETA primary writers and were influenced by input from the Task Force members and reviewers. Some recommendations were upgraded or downgraded on the basis of expert opinion. In these cases, subjective factors such as clinical experience, cost, risks, and regional availability of specific technologies and expertise took priority over the reported BEL.

The use of high-resolution ultrasonography (US), sensitive thyrotropin (TSH) assay, and fine-needle aspiration (FNA) biopsy is the basis for management of thyroid nodules. Thyroid scintigraphy is not necessary for diagnosis in most cases. However, it may be warranted in patients with a low serum TSH value or a multinodular gland, to detect functional autonomy, most common in iodine-deficient areas. Measurement of serum TSH is the best initial laboratory test of thyroid function and should be followed by measurement of free thyroxine and triiodothyronine if the TSH value is decreased, and of anti-thyroid peroxidase antibodies if the TSH value is above the normal range. A single, nonstimulated calcitonin measurement can be used in the initial work-up of thyroid nodules and is recommended before thyroid nodule surgery.

Although thyroid nodules are a common incidental finding, US should not be performed as a screening test. Most patients with thyroid nodules are asymptomatic, but the absence of symptoms does not rule out malignancy; thus, clinical and US risk factors for malignant disease should always be reviewed. All patients with a palpable thyroid nodule or with clinical risk factors should undergo US examination.

Thyroid FNA biopsy is best performed under US guidance because of the increase in diagnostic accuracy of the procedure. US-guided FNA (UGFNA) biopsy is recommended for nodules <10 mm if clinical information or US features are suspicious. Cytologic smears or liquid-based cytology should be interpreted by a pathologist with specific experience. A classification scheme in 5 cytologic diagnostic categories is recommended for the cytologic report: nondiagnostic, benign, follicular lesion, suspicious, or malignant. Currently, no single cytochemical or genetic marker is specific and sensitive enough to replace the morphologic diagnosis of follicular lesion or suspicious for neoplasm. However, use of these markers may be considered in selected cases. Hormone determination on washout from FNA biopsy may increase the diagnostic accuracy of FNA biopsy in suspicious node metastasis or hyperplastic parathyroid glands. US-guided core-needle biopsy should be reserved for patients with neck masses and uncertain FNA biopsy diagnosis.

Patients with benign thyroid nodules should undergo clinical and US follow-up. Symptomatic goiters, whether euthyroid or hyperthyroid, may be treated surgically or with radioiodine. Although we do not recommend routine levothyroxine suppressive therapy, it may be considered for small nodular goiters in young patients living in iodine-deficient regions. Percutaneous ethanol injection is useful in the treatment of benign cystic thyroid lesions. Symptomatic patients with benign nodules who decline surgery or who are at surgical risk may benefit from US-guided thermal ablation.

Malignant or suspicious nodules should be treated surgically. Preoperative evaluation with US and UGFNA biopsy is recommended for appropriate surgical planning.

Suggestions for thyroid nodule management during pregnancy and childhood are also presented.

Key-words and abbreviations: AFTN = autonomously functioning thyroid nodule; BEL = best evidence level; CNB = core-needle biopsy; CT = computed tomography; EL = evidence level; FNA = fine-needle aspiration; LNB = large-needle biopsy; MEN2 = multiple endocrine neoplasia type 2; MeSH = Medical Subject Headings; MNG = multinodular goiter; MRI = magnetic resonance imaging; MTC = medullary thyroid carcinoma; PEI = percutaneous ethanol injection; PLA = percutaneous laser ablation; PTC = papillary thyroid carcinoma; RFA = radiofrequency ablation; rhTSH = recombinant human TSH; TPOAb = anti-thyroid peroxidase antibody; TRAb = anti-TSH-receptor antibody; TSH = thyrotropin (thyroid-stimulating hormone); UGFNA = US-guided FNA; US = ultrasonography, ultrasonographic

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1. Thyroid Nodules: The Scope of the Problem

Thyroid nodules are a common clinical finding, with an estimated prevalence on the basis of palpation that ranges from 3% to 7% (1,2). The prevalence of clinically inapparent thyroid nodules is estimated with ultrasonography (US) at 20% to 76% in the general population, with a prevalence similar to that reported from autopsy data (3-5). Moreover, 20% to 48% of patients with 1 palpable thyroid nodule are found to have additional nodules on US investigation (5,6). Thyroid nodules are more common in elderly persons, in women, in those with iodine deficiency, and in those with a history of radiation exposure. The estimated annual incidence rate of 0.1% in the United States suggests that 300,000 new nodules are detected in this country every year (7,8).

This guideline covers diagnostic and therapeutic aspects of thyroid nodular disease but does not cover thyroid cancer management.

2. Clinical Evaluation and Diagnosis

2.1. History and Physical Examination

Both benign and malignant disorders can cause thyroid nodules (Box 1) (9). Hence, the clinical importance of newly diagnosed thyroid nodules is primarily the exclusion of malignant thyroid lesions (6,10) (Box 2). In iodine-deficient areas, however, local symptoms, functional autonomy, and hyperthyroidism are common clinical problems (11).

Benign nodular goiter
Chronic lymphocytic thyroiditis
Simple or hemorrhagic cysts
Follicular adenomas
Subacute thyroiditis
Papillary carcinoma
Follicular carcinoma
Hürthle cell carcinoma
Poorly differentiated carcinoma
Medullary carcinoma
Anaplastic carcinoma
Primary thyroid lymphoma
Sarcoma, teratoma, and miscellaneous tumors
Metastatic tumors

Box 1. Causes of Thyroid Nodules

History of head and neck irradiation
Family history of medullary thyroid carcinoma, multiple endocrine neoplasia type 2, or papillary thyroid carcinoma
Age <14 or >70 years
Male sex
Growing nodule
Firm or hard consistency
Cervical adenopathy
Fixed nodule
Persistent dysphonia, dysphagia, or dyspnea

Box 2. Factors Suggesting Increased Risk of Malignant Potential

2.1.1. History

During examination, patients should be asked about a family history of benign or malignant thyroid disease. Familial medullary thyroid carcinoma (MTC), multiple endocrine neoplasia type 2 (MEN2), familial papillary thyroid tumors, familial polyposis coli, Cowden disease, and Gardner syndrome should be considered (12-14).

Previous disease or treatments involving the neck (head and neck irradiation during childhood), recent pregnancy, and rapidity of onset and rate of growth of the neck swelling should be documented. Presence of thyroid nodules during childhood and adolescence should induce caution because the malignancy rate is 3- to 4-fold higher than in adult patients (15). The risk of thyroid cancer is also higher in older persons and in men (3,9).

2.1.2. Symptoms and signs

Most patients with thyroid nodules have few or no symptoms, and usually no clear relationship exists between nodule histologic features and the reported symptoms. Thyroid nodules are often discovered incidentally on physical examination, color Doppler evaluation of the carotid artery, or imaging studies performed for unrelated reasons (16).

In symptomatic patients, a detailed history and a complete physical examination may guide the selection of appropriate clinical and laboratory investigations. Slow but progressive growth of the nodule (during weeks or months) is suggestive of malignant involvement.

Sudden pain is commonly due to hemorrhage in a cystic nodule. In patients with progressive and painful enlargement of a thyroid nodule, however, anaplastic carcinoma or primary lymphoma of the thyroid should be considered (17). Symptoms such as a choking sensation, cervical tenderness or pain, dysphagia, or hoarseness may be perceived as attributable to thyroid disease, but in most patients, these symptoms are caused by nonthyroid disorders. Slow-onset cervical symptoms and

signs caused by the compression of vital structures of the neck or upper thoracic cavity usually occur if thyroid nodules are embedded within large goiters. When observed in the absence of a multinodular goiter (MNG), the symptoms of tracheal compression (cough and dysphonia) suggest an underlying malignant lesion. Surgical treatment should be considered in patients with growth of a thyroid mass and vocal cord paresis even if cytologic results are negative for malignancy (18,19). Differentiated thyroid carcinomas rarely cause airway obstruction, vocal cord paralysis, or esophageal symptoms at their clinical presentation. Hence, the absence of local symptoms does not rule out a malignant tumor (20).

Small differentiated thyroid cancers are frequently devoid of alarming characteristics on physical evaluation (21-23). However, a firm or hard, solitary or dominant thyroid nodule that clearly differs from the rest of the gland suggests an increased risk of malignant involvement (17). Therefore, despite the low predictive value of palpation (23,24), a careful inspection and palpation of the thyroid gland and the anterior and lateral nodal compartments of the neck should always be done (Box 2).

Suppressed or low levels of thyrotropin (thyroid-stimulating hormone; TSH) are associated with a decreased probability of malignancy (25), and autonomously functioning thyroid nodules (AFTNs) in adults need no further cytologic evaluation because the incidence of malignancy is exceedingly low (26). Hyperfunctioning MNGs, however, may harbor both hyperfunctioning areas and cold (potentially malignant) lesions (22). Nodules appearing in patients with Graves disease or Hashimoto thyroiditis should be managed in the same way as in any other patients (27).

2.2. Thyroid Incidentaloma

Thyroid lesions discovered on computed tomography (CT) or magnetic resonance imaging (MRI) performed for other reasons have an uncertain risk of malignancy and should undergo US evaluation before considering evaluation with fine-needle aspiration (FNA) biopsy (28,29). Nodules are detected infrequently by ^{18}F -fluorodeoxyglucose positron emission tomography, but when found have a high risk of malignancy (30,31). Such lesions should undergo focused US evaluation followed by FNA biopsy.

Focal lesions detected by technetium Tc99m sestamibi scans have a high risk of malignancy (32) and should be evaluated by US.

2.3. Key Recommendations

2.3.1. History

- Record the following information (grade B; best evidence level [BEL] 2):
 - Age
 - Family history of thyroid disease or cancer
 - Previous head or neck irradiation
 - Rate of growth of the neck mass

- Dysphonia, dysphagia, or dyspnea
- Symptoms of hyperthyroidism or hypothyroidism
- Use of iodine-containing drugs or supplements
- The vast majority of nodules are asymptomatic, and absence of symptoms does not rule out malignancy (grade C; BEL 3)

2.3.2. Physical examination

- A careful physical examination of the thyroid gland and cervical lymph nodes is mandatory (grade A; BEL 3)
- Record (grade C; BEL 3):
 - Location, consistency, and size of the nodule(s)
 - Neck tenderness or pain
 - Cervical adenopathy
- The risk of cancer is similar in patients with a solitary nodule or with MNG (grade B; BEL 2)

3. US and Other Diagnostic Imaging Studies

3.1. When to Perform Thyroid US

High-resolution US is the most sensitive test available to detect thyroid lesions, measure their dimensions, identify their structure, and evaluate diffuse changes in the thyroid gland (33,34).

If results of palpation are normal, US should be performed when a thyroid disorder is suspected on clinical grounds or if risk factors have been recognized (Box 2). The physical finding of suspicious neck adenopathy warrants US examination of both lymph nodes and thyroid gland because of the risk of a metastatic lesion from an otherwise unrecognized papillary microcarcinoma (35).

In all patients with palpable thyroid nodules or MNGs, US should be performed to accomplish the following:

- Help with the diagnosis in difficult cases (as in chronic lymphocytic thyroiditis)
- Look for coincidental thyroid nodules or diffuse thyroid gland changes
- Detect US features suggestive of malignant growth and select the lesions to be recommended for FNA biopsy
- Choose the gauge and length of the biopsy needle
- Obtain an objective measure of the baseline volume of the thyroid gland and of lesions that will be assigned to follow-up or medical therapy

Standardized US reporting criteria should be followed, indicating position, shape, size, margins, content, and echogenic and vascular pattern of the nodule. Nodules with malignant potential should be carefully described.

3.2. US Criteria for FNA Biopsy of Palpable Nodules

The risk of cancer is not significantly higher for palpable solitary thyroid nodules than for multinodular glands or nodules embedded in diffuse goiters (22,23). Moreover, in 50% of thyroid glands with a “solitary” nodule on the basis of palpation, other small nodules are discovered by US (24). For MNGs, the cytologic sampling should be focused on lesions with suspicious US features rather than on larger or clinically dominant nodules (34,36).

US and color Doppler features have varying abilities to predict the risk of malignancy. The reported specificities for predicting malignancy are 41.4% to 92.2% for marked hypoechogenicity, 44.2% to 95.0% for microcalcifications (small, intranodular, punctate, hyperechoic spots with scanty or no posterior acoustic shadowing), 48.3% to 91.8% for irregular or microlobulated margins, and about 80% for chaotic arrangement or intranodular vascular images (37,38). The value of these features for predicting cancer is partially blunted by the low sensitivities, however, and no US sign independently is fully predictive of a malignant lesion (21). A rounded appearance or a “more tall (anteroposterior) than wide (transverse)” shape of the nodule is an additional US pattern suggestive of malignant potential (39,40). The coexistence of 2 or more suspicious US criteria greatly increases the risk of thyroid cancer (21,39-41).

Large neoplastic lesions may be characterized by degenerative changes and multiple fluid-filled areas, findings rarely noted in microcarcinomas. Although most complex thyroid nodules with a dominant fluid component are benign, US-guided FNA (UGFNA) biopsy should always be performed because papillary thyroid carcinoma (PTC) can be partially cystic (42). Extension of irregular hypoechoic lesions beyond the thyroid capsule, invasion of prethyroid muscles, and infiltration of the recurrent laryngeal nerve are infrequent but threatening US findings that demand immediate cytologic assessment (34).

The presence of enlarged lymph nodes with no hilum, cystic changes, and microcalcifications is highly suspicious (43,44). Rounded appearance and chaotic hypervascularity are more common but less specific findings (44). Such nodes and any coexistent thyroid nodules, whatever their size, always warrant UGFNA biopsy.

3.3. US Criteria for FNA Biopsy of Impalpable Nodules and Nodular Goiters

Clinically unapparent thyroid lesions were detected by US in about half of the women in several studies (2,8). The prevalence of cancer reported for non palpable thyroid lesions ranges from 5.4% to 7.7% (21,37,45) and appears to be similar to that reported for palpable lesions (5.0%-6.5%) (22,37,44-46). Clinical criteria for a malignant nodule are lacking for most nonpalpable lesions (20). Hence, it is essential to determine which thyroid lesions have a high malignant potential on the basis of their US features.

The US characteristics suggestive of malignant involvement in impalpable thyroid nodules are the same as in palpable nodules (21,38,40). The combination of nodule isoechogenicity with a spongiform appearance, however, has a high predictive value for a benign lesion (38).

Malignant involvement is not less frequent in nodules smaller than 10 mm in diameter; thus, an arbitrary diameter cutoff for cancer risk is not justified (21) and suspicious lesions smaller than 10 mm should be assessed with FNA biopsy. Furthermore, early diagnosis and treatment of small tumors may be clinically important, but an aggressive disease course is rare in incidentally discovered microcarcinomas (47-49). Hence, incidental thyroid lesions with a diameter of about 5 mm should usually be followed up with US (48). A possible diagnostic algorithm for work-up of thyroid nodules and the strength of indication for FNA biopsy are shown in Figures 1 and 2.

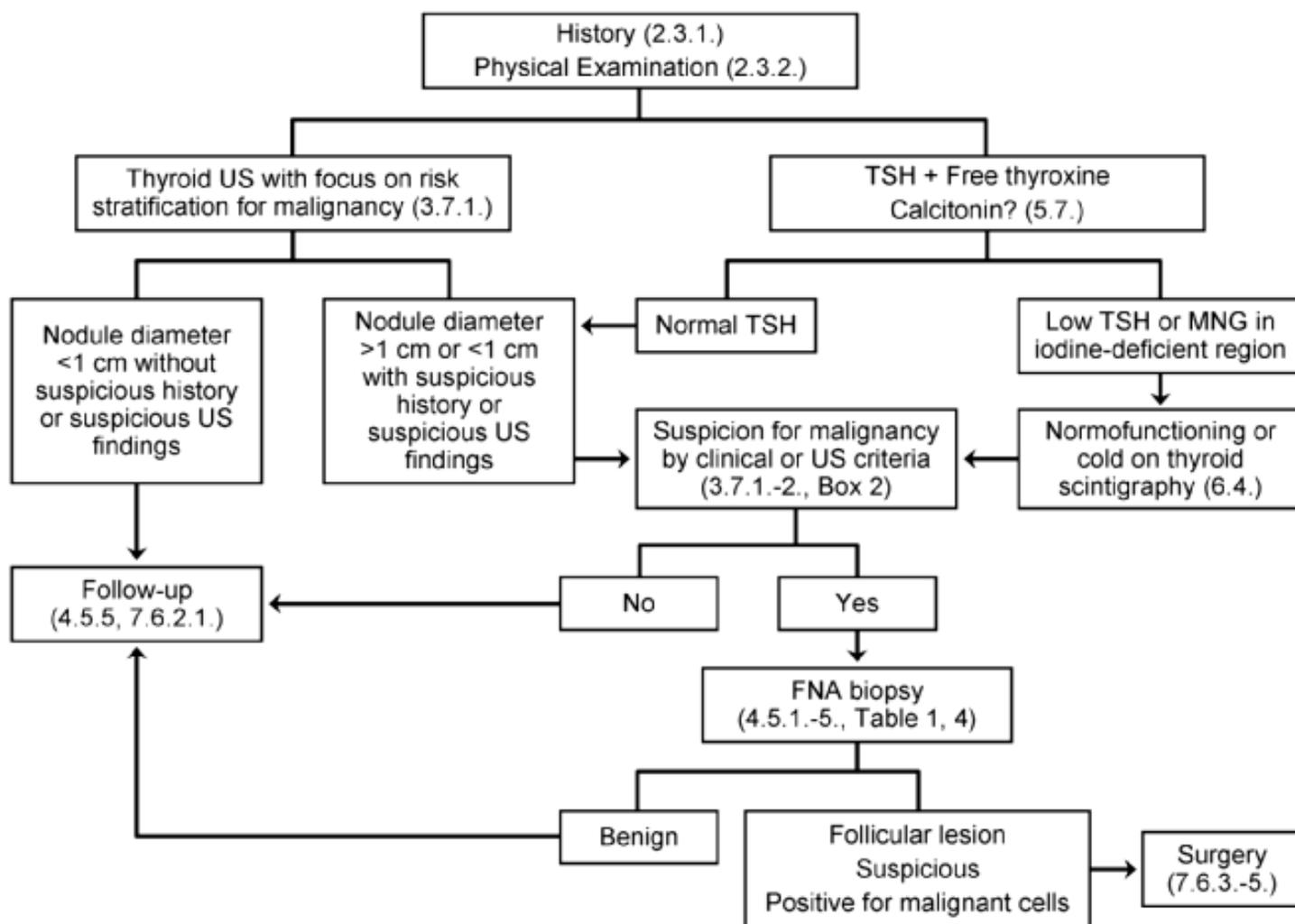


Figure 1. Flowchart indicating a scheme for the diagnosis and management of palpable thyroid nodules. Associated Key Recommendations shown in parentheses. FNA, fine-needle aspiration; MNG, multinodular goiter; TSH, thyrotropin; US, ultrasonography.

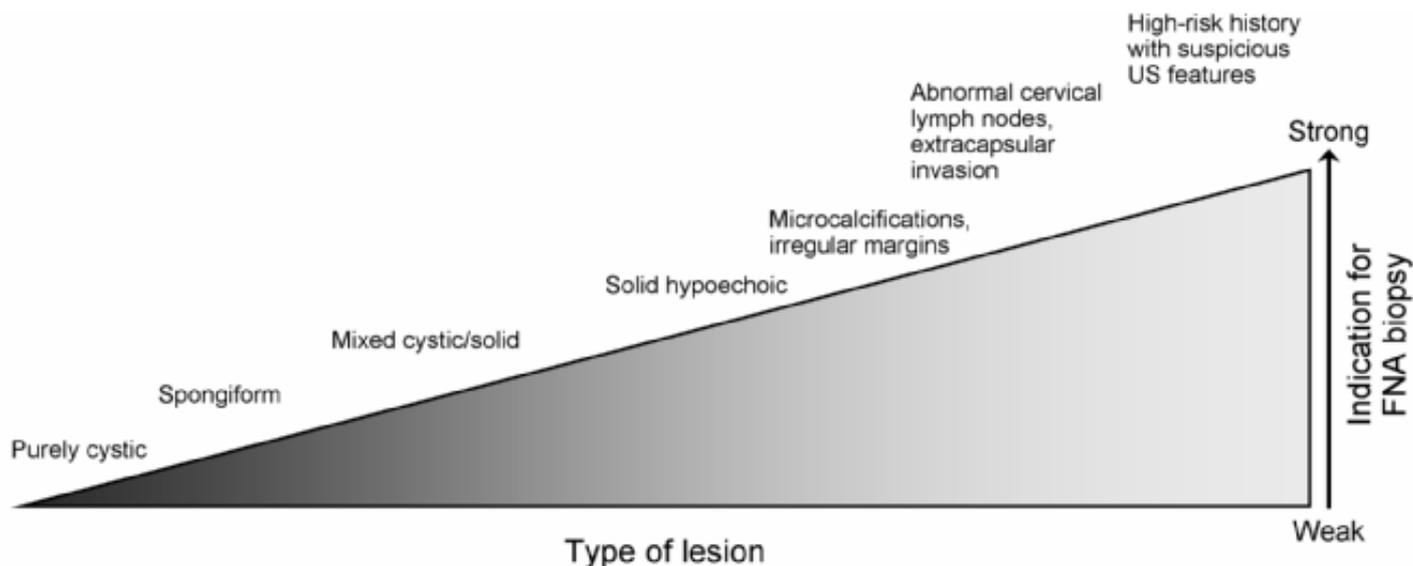


Figure 2. Strength of indication for fine-needle aspiration (FNA) biopsy of thyroid nodules on the basis of ultrasonography (US) findings.

3.4. US-Elastography

A thyroid nodule with firm or hard consistency is associated with an increased risk of malignancy. Elastography has recently been applied in the diagnostic approach to nodular thyroid disease and has shown a high sensitivity and specificity in selected patients. The predictive value of US-elastographic measurement seems to be independent of nodule size (50,51) and is maintained for lesions that are indeterminate on FNA biopsy (52).

Cystic nodules and nodules shown to have a calcified shell by US are not suitable for US-elastographic evaluation. Because the nodule to be examined must be clearly distinguishable from other nodules, MNGs with coalescent nodules are not suitable for this analysis (51). Larger prospective studies are needed to establish the diagnostic accuracy of this technique.

3.5. US Contrast Media

First- and second-generation contrast agents provide only ancillary data for the diagnosis of malignant thyroid nodules and offer a modest improvement over the information obtainable with traditional color Doppler or power Doppler examinations (53,54). Currently, use of US contrast agents should be restricted to definition of the size and limits of necrotic zones after US-guided ablation procedures (54).

3.6. Other Imaging Techniques

MRI and CT should not be used routinely in nodular thyroid disease because they are rarely diagnostic for malignant lesions except in very advanced cases (28,29). MRI and CT may be of value, however, if assessment of size or substernal extension of a nodular goiter is desired for clinical

management. CT contrast medium usually contains iodine (55), decreases subsequent uptake of radioiodine, and may also induce hyperthyroidism, especially in iodine-deficient geographic areas.

3.7. Key Recommendations

3.7.1. *Ultrasonography*

3.7.1.1. When to perform thyroid US

- US evaluation is not recommended as a screening test in the general population nor in patients with a normal thyroid on palpation and a low clinical risk of thyroid cancer (grade C; BEL 3)
- US evaluation is recommended for (grade B; BEL 3):
 - Patients at risk for thyroid malignancy
 - Patients with palpable thyroid nodules or MNGs
 - Those with lymphadenopathy suggestive of a malignant lesion

3.7.1.2. How to describe US findings

- Report should focus on risk stratification for malignancy (grade C; BEL 4)
- Describe position, shape, size, margins, content, echogenic pattern, and vascular features of the nodule(s) (grade C; BEL 3)
- For multiple nodules, detail the nodule(s) bearing the US characteristics associated with malignancy (hypoechoic pattern and/or irregular margins, a more-tall-than-wide shape, microcalcifications, or chaotic intranodular vascular spots) rather than describing the largest (“dominant”) nodule (grade C; BEL 3)

3.7.2. *Indications for FNA biopsy*

3.7.2.1. How to select nodule(s) for FNA biopsy (grade B; BEL 3):

- FNA biopsy is recommended for nodule(s):
 - Of diameter >10 mm, and solid, hypoechoic on US
 - Of any size with US findings suggestive of extracapsular growth or metastatic cervical lymph nodes
 - Of any size with: patient history of neck irradiation in childhood or adolescence; PTC, MTC, or MEN2 in first-degree relatives; previous thyroid surgery for cancer; increased calcitonin levels in the absence of interfering factors
 - Of diameter <10 mm along with US findings associated with malignancy (see section 3.7.1.2.); the coexistence of 2 or more suspicious US criteria greatly increases the risk of thyroid cancer

- Nodules that are hot on scintigraphy should be excluded from FNA biopsy (see difference in recommendations for children; section 8.4.)

3.7.2.2. FNA biopsy of multinodular glands

- It is rarely necessary to biopsy more than 2 nodules when they are selected on the basis of previously described criteria (grade D)
- If a radioisotope scan is available, do not biopsy hot areas (grade B; BEL 4)
- In the presence of suspicious cervical lymphadenopathy, FNA biopsy of both the lymph node and suspicious nodule(s) is essential (grade B; BEL 4)

3.7.2.3. FNA biopsy of complex (solid-cystic) thyroid nodule(s)

- Always sample the solid component of the lesion by UGFNA biopsy (grade B; BEL 4)
- Submit both the FNA biopsy specimen and the drained fluid for cytologic examination (grade B; BEL 4)

3.7.2.4. FNA biopsy of thyroid incidentalomas

- Thyroid incidentalomas should be managed according to previously described criteria for nodule diagnosis (grade C; BEL 3)
- Incidentalomas detected by CT or MRI should undergo US evaluation before consideration for UGFNA biopsy (grade C; BEL 3)
- Incidentalomas detected by positron emission tomography with ^{18}F -fluorodeoxyglucose should undergo US evaluation plus UGFNA biopsy because of the high risk of malignancy (grade C; BEL 3)

3.7.3. *Other diagnostic imaging techniques*

- MRI and CT are not indicated for routine thyroid nodule evaluation (grade D)
- MRI and CT are of value for assessment of size, airway compression, or substernal extension of a nodular goiter (grade C; BEL 3)

3.7.4. *Novel US techniques*

- Elastography and US contrast media currently are not used routinely in the evaluation of thyroid nodules (grade C; BEL 3)

4. Thyroid Biopsy

4.1. Thyroid FNA Biopsy

Clinical management of thyroid nodules should be guided by the combination of US evaluation and FNA biopsy (Figures 1 and 2) (8). FNA biopsy is currently the best triage test for the preoperative evaluation of thyroid nodules (56-58).

Because the most common cause of a false-negative cytologic diagnosis is sampling error (56), cytologic diagnosis is more reliable and the nondiagnostic rate is lower when FNA biopsy is performed with US guidance (UGFNA) (59-61). UGFNA biopsy is strongly recommended in impalpable nodules, MNGs, and generally in obese patients and in men with well-developed cervical muscles. Hence, UGFNA biopsy is currently the single most important procedure for the management of thyroid nodules.

4.2. Cytologic Diagnosis

Thyroid smears or liquid-based cytology should be reviewed by a cytopathologist with a special interest in thyroid disease (62). The request form accompanying the cytologic specimen should include all the relevant clinical and US information (63,64). The cytologic report should be descriptive and, whenever possible, a diagnosis should be made (58,63).

The FNA biopsy sample must be adequate enough for an interpretation that yields a low false-negative rate (65). FNA biopsy results should be classified as diagnostic (satisfactory) or nondiagnostic (unsatisfactory). Even if the evaluation of adequacy is difficult to standardize (65,66), the specimen is labeled "diagnostic" if it contains a minimum of 6 groupings of well-preserved thyroid epithelial cells, consisting of at least 10 cells per group (67).

Cytologic diagnoses should be organized into 5 classes: nondiagnostic, benign, follicular lesion, suspicious, and malignant (62,63):

Class 1. Specimens may be labeled as "nondiagnostic" because of an insufficient number of cells, which can be attributable to cystic fluid or bloody smears, or because of poor technique in preparing slides, leading to compromised preservation of the diagnostic material (66,67).

Class 2. A benign (or negative for malignancy) cytodiagnosis is the most common finding (66,67). Benign cytologic findings include colloid nodule, hyperplastic nodule, lymphocytic or granulomatous thyroiditis, and benign cyst.

Class 3. Follicular lesions include all follicular-patterned specimens for which a definite cytologic diagnosis of benign or malignant cannot be established on the basis of cytomorphology (62,63). These include adenomatoid hyperplasia, follicular adenoma and carcinoma, Hürthle cell neoplasms, and the follicular variant of PTC. Follicular lesions appear as hypercellular specimens with a monotony of cells, microfollicular arrangement, and decreased or absent colloid. Hürthle cell neoplasm is diagnosed in an aspirate that consists of almost entirely Hürthle cells, usually with absent

or scanty colloid, and that lacks an associated lymphoid cell population, as found in Hashimoto thyroiditis. In centers with specific experience in thyroid cytology, follicular cytology may be further subdivided into “follicular lesion/atypia of undetermined significance” and “follicular neoplasm” (64). This distinction may separate 2 cytologic groups at different risk for thyroid malignancy (56,64) but with the same operative indications.

Class 4. Suspicious results include either samples with adequate cellularity characterized by cytologic features suggesting but not fulfilling the criteria for a definite diagnosis of malignancy or samples with poor cellularity and/or poor fixation and preservation but clear features indicating malignancy (63,64,66,68).

Class 5. Malignant (or positive) results include samples characterized by malignant cytologic features that are reliably identified by the cytopathologist (63,64,69). The most frequent malignant lesion encountered is PTC. Other malignant lesions include MTC, anaplastic carcinoma, lymphoma, miscellaneous thyroid tumors, and metastatic cancers (66,69).

4.3. FNA Biopsy Results

Most (60%-80%) results of FNA biopsy are classified as benign; for the rest, 10% to 20% are follicular lesion/neoplasm, 3.5% to 10% are malignant, 2.5% to 10% are suspicious, and 10% to 15% are nondiagnostic (56,63,65,69,70). The results of FNA biopsy are critical in deciding whether to manage the nodule medically or surgically. Selection of patients for surgical treatment on the basis of FNA biopsy results has decreased the number of thyroid operations by about half and has increased the yield of cancer from 15% to 50% (57,71).

The sensitivity and specificity of FNA biopsy performed by experienced personnel are excellent, as shown in Table 1. The false-negative rate—a missed diagnosis of malignant disease—for palpation-guided FNA biopsy has been reported as 1% to 11% (mean, 5%) (56,70,72). The true incidence of malignancy in the benign class can only be determined with difficulty because a benign diagnosis is usually managed conservatively. However, with the use of UGFNA, the rate of false-negative FNA biopsy results, established on clinical grounds, is about 1% to 2% (61) and decreases further with repeated UGFNA biopsy (62). Methods for minimizing false-negative results are described in Box 3.

A false-positive diagnosis implies that no malignancy was detected in a surgically removed thyroid that had a class 5 FNA biopsy diagnosis. The reported incidence of false-positive results ranges from less than 1% to 7.7% (56,70). Most errors are interpretative, resulting from overlapping features, degenerative changes, an inadequate specimen, or cytopathologist inexperience (66,70). PTC is the most common false-positive diagnosis (66).

Table 1. Summary Characteristics for Thyroid Fine-Needle Aspiration: Results of Literature Survey

Feature	Mean %	Range %	Definition
Sensitivity	83	65-98	Likelihood that patient with disease has positive test results
Specificity	92	72-100	Likelihood that patient without disease has negative test results
Positive predictive value	75	50-96	Fraction of patients with positive test results who have disease
False-negative rate	5	1-11	Fine-needle aspiration negative; histology positive for cancer
False-positive rate	5	0-7	Fine-needle aspiration positive; histology negative for cancer

Adapted from Gharib H, Papini E, Valcavi R, et al; AACE/AME Task Force on Thyroid Nodules. American Association of Clinical Endocrinologists and Associazione Medici Endocrinologi medical guidelines for clinical practice for the diagnosis and management of thyroid nodules. *Endocr Pract.* 2006 Jan-Feb;12(1):63-102. Used with permission.

Use ultrasound-guided fine-needle aspiration (UGFNA) biopsy
Perform multiple punctures of the nodule so that several areas are sampled
Consider repeat UGFNA biopsy for follow-up of benign nodules
For multiple nodules, prioritize the nodule to biopsy according to ultrasonographic findings
For cystic lesions, sample solid areas with UGFNA biopsy and submit cyst fluid for examination
Obtain at least 6 properly prepared thin cell smears
Use immediate wet fixation for Papanicolaou staining technique
Review slides with an experienced cytopathologist

Box 3. Ways to Minimize False-Negative Results

4.4. Large-Needle and Core-Needle Biopsy

Large-needle biopsy (LNB), performed without US guidance with a large-bore needle, is not recommended for thyroid nodules because of local pain and risk of cervical bleeding. It also does not add any further diagnostic information to FNA biopsy in nodules with follicular cytology (73).

Core-needle biopsy (CNB), performed under US guidance with a 20- to 21-gauge cutting needle by experienced operators, may offer additional information to FNA biopsy in selected cases of thyroid or neck masses with repeated inadequate FNA biopsy cytology (74). In patients with suspicious

anaplastic tumor, thyroid lymphoma, pathologic lymph nodes, or other malignant neck disease, CNB frequently provides critical information for nodule management (64,75). However, CNB offers no additional diagnostic value in distinguishing a cellular hyperplastic nodule from a follicular adenoma or carcinoma (76). Hence, US-guided CNB should not be seen as an alternative to FNA biopsy but as a complementary investigational tool (64,75).

4.5. Key Recommendations

4.5.1. *Thyroid FNA biopsy*

- Clinical management of thyroid nodules should be guided by the combination of US evaluation and FNA biopsy (grade A; BEL 3)
- Cytologic diagnosis is more reliable and the nondiagnostic rate is lower when FNA biopsy is performed with US guidance (grade B; BEL 3)

4.5.2. *Cytologic reporting*

- Thyroid smears or liquid-based cytology should be reviewed by a cytopathologist with a special interest in thyroid disease (grade C; BEL 3)
- The request form accompanying the cytologic specimen should include all the relevant clinical and US information (grade D)
- The cytologic report should be descriptive, and, whenever possible, a diagnosis should be made (grade B; BEL 4)

4.5.3. *Cytologic diagnosis*

FNA biopsy results may be diagnostic (satisfactory) or nondiagnostic (unsatisfactory). Even if the evaluation of adequacy is difficult to standardize, the specimen is labeled “diagnostic” if it contains a minimum of 6 groupings of well-preserved thyroid epithelial cells, consisting of at least 10 cells per group (grade D; BEL 4).

Cytologic diagnoses should be organized into 5 classes (grade B; BEL 3):

- **Class 1. Nondiagnostic** (inadequate or insufficient): samples with processing errors or an insufficient number of follicular cells
- **Class 2. Benign** (or negative for malignancy): includes colloid or hyperplastic nodules, Hashimoto or granulomatous thyroiditis, and cysts
- **Class 3. Follicular lesions**: all follicular-patterned lesions, including follicular neoplasms, Hürthle cell lesions, and the follicular variant of PTC. In centers with specific experience in thyroid cytology, follicular cytology may be further subdivided into “follicular lesion/atypia of undetermined significance” and “follicular neoplasm.” This distinction separates 2 cytologic groups at different risk for thyroid malignancy but with the same operative indications

- **Class 4. Suspicious:** samples that suggest a malignant lesion but do not completely fulfill the criteria for a definite diagnosis
- **Class 5. Malignant** (or positive): samples characterized by malignant cytologic features that are reliably identified by the cytopathologist and are diagnostic of primary or metastatic tumors

4.5.4. *Pitfalls in FNA biopsy*

- False-negative results are usually due to inadequate sampling or inappropriate target selection (grade D)
- False-positive results are usually due to specimens with suspicious findings (grade D)
- Gray zones in cytologic reports are follicular lesions and cytologic findings suggestive of but not diagnostic for PTC (grade D)
- In follicular lesions, consider performing thyroid scintigraphy to exclude a hot nodule at very low risk for malignancy (grade B; BEL 3)

4.5.5. *Ways to minimize false-negative results*

- Use UGFNA biopsy (grade C; BEL 3)
- Aspirate multiple nodule sites (grade C; BEL 4)
- For multiple nodules, prioritize the nodules to biopsy according to US findings (grade B; BEL 3)
- For cystic lesions, sample solid areas with UGFNA biopsy and submit cyst fluid for examination (grade C; BEL 4)
- Review slides with an experienced cytopathologist (grade D)
- Follow up cytologically benign nodule(s) (grade D)
- Consider repeat UGFNA biopsy for follow-up of benign nodules (grade C; BEL 3)

4.5.6. *Core-needle biopsy*

- CNB performed under US guidance may offer additional information in selected cases with thyroid or neck masses and inadequate FNA biopsy cytologic results (grade C; BEL 3)

5. Laboratory Evaluation

5.1. Assessment of Thyroid Function

The high sensitivity of the TSH assay for detecting even subtle thyroid dysfunction makes it the most useful laboratory test in the initial evaluation of thyroid nodules (77). Measuring serum levels of free thyroid hormones and anti-thyroid peroxidase antibody (TPOAb) or anti-TSH-receptor antibody

(TRAb) should be the second diagnostic step, which is necessary for confirmation and the subsequent definition of thyroid dysfunction if TSH concentration is outside the normal range (78).

5.2. TSH Assay

Third-generation TSH chemiluminometric assays, with detection limits of about 0.01 microunits/mL, should be used in current clinical practice. They can detect decreased TSH levels even in mild cases of hyperthyroidism and allow a reliable diagnosis of mild (subclinical) thyroid hyperfunction (77,78).

5.3. Serum Free Thyroxine and Free Triiodothyronine

If the serum TSH level is within the normal range, the measurement of free thyroid hormones adds no further relevant information. If TSH levels are low, however, measurement of free thyroxine and free triiodothyronine levels is necessary to confirm the presence of hyperthyroidism or consider central hypothyroidism, in which TSH can be normal or low and free thyroxine levels may be low (79).

To limit unnecessary laboratory testing, the following strategy should be followed for most patients with thyroid nodules (78,79):

- Serum TSH level within normal limits: no further testing (unless suspicion of central hypothyroidism)
- Increased serum TSH: test free thyroxine and TPOAb to evaluate for hypothyroidism
- Decreased serum TSH: test free thyroxine and triiodothyronine to evaluate for hyperthyroidism

5.4. Antibody Assays

TPOAb should be measured in patients with high levels of serum TSH (79,80). High serum TPOAb values and a firm, diffusely enlarged, or small thyroid are very suggestive of autoimmune or Hashimoto thyroiditis (11,80,81). Occasionally, a nodular goiter may represent Hashimoto thyroiditis (80).

Antithyroglobulin antibody testing should be reserved for patients with US and clinical findings suggestive of chronic lymphocytic thyroiditis in conjunction with normal serum TPOAb levels (79).

TRAb determination should be performed in patients with hyperthyroidism for more complete etiologic clarification (82), since 17% of patients in iodine-deficient areas with scintigraphic criteria for toxic MNG are positive for TRAb (83).

5.5. Thyroglobulin Assay

Assessment of serum thyroglobulin is not recommended in the diagnosis of thyroid nodules (84). In patients undergoing surgery for malignancy, testing of serum thyroglobulin may be considered so as not to overlook a false-negative serum thyroglobulin value due to decreased thyroglobulin immunoreactivity or heterophilic antibodies (85).

5.6. Calcitonin Assay

Calcitonin is a serum marker for MTC and correlates with tumor burden (86). Calcitonin testing is imperative in patients with a history or a clinical suspicion of familial MTC or MEN2 (87). Calcitonin measurement is recommended if FNA biopsy results are suspicious for MTC and in patients with nodular goiters undergoing thyroid surgery to avoid the risk of inadequate surgical treatment (88,89). Routine testing of serum calcitonin for MTC in all patients with unselected thyroid nodules is still debated (90). Studies of nodular thyroid disease have reported a prevalence of MTC ranging from 0.4% to 1.4% of all patients (80,88,89,91,92). Calcitonin levels can be increased in patients with pulmonary or pancreatic endocrine tumors, kidney failure, autoimmune thyroid disease, or hypergastrinemia (resulting from proton-pump inhibitor therapy); other factors that increase calcitonin are alcohol consumption, smoking, sepsis, and heterophilic anticalcitonin antibodies (93-95). In addition, sex, age, weight, increased calcium levels, and the assay itself also affect the calcitonin level (93-95). Cutoff values, such as 10 or 20 pg/mL, have been effectively used for the screening of unselected nodules (92,96). The false-positive rate decreases with increasing cutoff levels. Therefore, a single nonstimulated calcitonin measurement can be used in the routine work-up of thyroid nodules. If the calcitonin value is increased, the test should be repeated and, if confirmed in the absence of the above modifiers, pentagastrin stimulation testing will increase the diagnostic accuracy (92,96). The availability of pentagastrin is limited outside Europe; in the United States, calcitonin stimulation may be performed with calcium. The diagnostic value of calcium-stimulation test results has not been completely assessed (96), but a cutoff for the response in normal subjects is under investigation (97). Screening of at-risk family members should be done by testing for germline mutations of the *RET* protooncogene (87,98). Screening for *RET* protooncogene germline mutations in apparently sporadic MTC may detect MEN2 in about 5% of cases (99).

5.7. Key Recommendations

5.7.1. Laboratory evaluation in patients with thyroid nodules

- Always measure serum TSH (grade A; BEL 3)
- If TSH level is decreased, measure free thyroxine and total or free triiodothyronine; if TSH level is increased, measure free thyroxine and TPOAb (grade B; BEL 3)
- Testing for antithyroglobulin antibodies should be restricted to patients with US and clinical findings suggestive of chronic lymphocytic thyroiditis when serum levels of TPOAb are normal (grade C; BEL 3)
- Assessment of serum thyroglobulin is not recommended in the diagnosis of thyroid nodules. In patients undergoing surgery for malignancy, serum thyroglobulin measurement may be useful to detect potential false-negative results (grade C; BEL 3)

- TRAb measurement should be performed in patients with TSH levels below normal (grade D)

5.7.2. Calcitonin

- Measurement of basal serum calcitonin level may be a useful test in the initial evaluation of thyroid nodules (grade B; BEL 3)
- Measurement of nonstimulated serum calcitonin level may be considered before thyroid surgery for nodular goiter (grade B; BEL 3)
- Measurement is mandatory in patients with a family history or clinical suspicion of MTC or MEN2 (grade A; BEL 2)
- If calcitonin level is increased, the test should be repeated and, if confirmed in the absence of modifiers, a pentagastrin or calcium stimulation test will increase the diagnostic accuracy (grade B; BEL 3)

5.7.3. Other tests

- Measure serum calcium, parathyroid hormone, or both if a nodular lesion is suspicious for intrathyroidal parathyroid adenoma on US examination (grade D)

6. Radionuclide Scanning

6.1. Thyroid Scintigraphy

Thyroid scintigraphy is the only technique that allows for assessment of thyroid regional function and detection of areas of AFTN (100).

6.2. Diagnostic Accuracy

On the basis of the pattern of radionuclide uptake, nodules may be classified as hyperfunctioning (“hot”), hypofunctioning (“cold”), or indeterminate (100). Hot nodules almost never represent clinically significant malignant lesions, whereas cold or indeterminate nodules have a reported malignancy risk of 3% to 15% (42,101-103).

Because the vast majority of thyroid lesions are cold or indeterminate and only a small minority of them are malignant (104,105), the predictive value of hypofunctioning or indeterminate nodules for the presence of malignant involvement is low. The diagnostic specificity is further decreased in small lesions (<1 cm), which are below the resolution threshold of scintigraphy (100,106,107).

The role of scintigraphy in the diagnostic work-up of thyroid nodules is limited in countries with iodine-rich diets, in which serum TSH measurement and thyroid US can correctly diagnose autonomous nodules in most patients (106,107), and FNA biopsy facilitates accurate diagnosis of a malignant lesion (71). Moreover, because the resolution of US is considerably greater than that of scintigraphy,

radionuclide scanning has little place in the topographic assessment of nodular goiter and no place in the measurement of thyroid nodules.

However, in geographic regions with iodine deficiency, thyroid scintigraphy is used as part of the evaluation of patients with MNG (100,108) because it provides useful information on the functional characterization of thyroid nodules. It allows early diagnosis of thyroid autonomy and prioritization of cold and indeterminate nodules in MNGs for FNA biopsy (100). In patients in these regions, the serum TSH may remain unsuppressed even if autonomy is present because of the low proliferation rate of thyroid epithelial cells and the low synthesis rate of thyroid hormones by iodine-depleted thyroid glands (109). Moreover, in the early phases of autonomy, the bulk of autonomous tissue may be insufficient to suppress the TSH level (100,106,109,110). The early recognition of autonomous nodules, before they induce the suppression of TSH, enables early treatment to avoid thyroid growth and progression toward manifest hyperthyroidism (111). Furthermore, in iodine-deficient euthyroid goiters, microscopic areas of hot thyroid tissue contain constitutively activating TSH receptor mutations, which increases the risk of iodine-induced hyperthyroidism (111).

Quantitative pertechnetate scintigraphy (calculation of technetium thyroid uptake under suppression) is a sensitive and specific technique for the diagnosis and quantitation of thyroid autonomy and is a reliable predictor of hyperthyroidism in the setting of euthyroid autonomy (100).

Thyroid scintigraphy can be performed with ^{123}I or $^{99\text{m}}\text{TcO}_4^-$ (sodium pertechnetate). Each of these imaging agents has advantages and disadvantages.

$^{99\text{m}}\text{TcO}_4^-$

- Advantages: Less expensive; more readily available; more rapid examination
- Disadvantages: Technetium is trapped but not organified (risk of false-positive images); activity in esophagus or vascular structures can be misleading; poor image quality when uptake is low

^{123}I

- Advantages: better visualization of retrosternal thyroid tissue; better images when thyroid uptake is low; real iodine clearance of the thyroid may be measured instead of Tc uptake as a surrogate parameter
- Disadvantages: higher cost; less comfortable for the patient (delayed imaging at 24 hours is often used); less readily available; imaging times usually longer

6.3. Indications for Thyroid Scintigraphy

Thyroid scintigraphy is indicated in the following settings (108-111):

- With a single thyroid nodule and suppressed TSH level; FNA biopsy is not necessary for hot nodules

- For MNGs, even without suppressed TSH, to identify cold or indeterminate areas for FNA biopsy and hot areas that do not need cytologic evaluation
- For large MNGs, especially with substernal extension
- In the diagnosis of ectopic thyroid tissue
- In subclinical hyperthyroidism to identify occult hyperfunctioning tissue
- In follicular lesions to identify a functioning cellular adenoma that may be benign; however, most such nodules are cold on scintigraphy
- To determine eligibility for radioiodine therapy
- To distinguish low-uptake from high-uptake thyrotoxicosis.

6.4. Key Recommendations

6.4.1. *When to perform thyroid scintigraphy*

- Perform scintigraphy for a thyroid nodule or MNG if the TSH level is below the lower limit of the normal range or if ectopic thyroid tissue or a retrosternal goiter is suspected (grade B; BEL 3)
- In iodine-deficient regions, consider performing scintigraphy for a thyroid nodule or MNG even if TSH is normal to exclude autonomy (grade C; BEL 3)

6.4.2. *How to perform thyroid scintigraphy*

- Either ^{123}I or $^{99\text{m}}\text{TcO}_4^-$ (sodium pertechnetate) can be used for thyroid scintigraphy (grade B; BEL 3)
- ^{131}I thyroid uptake is not recommended for routine diagnostic use unless low-uptake thyrotoxicosis is suspected (grade A; BEL 3)

7. Management and Therapy

Clinical management of thyroid nodules should be guided by the results of US evaluation and FNA biopsy (8,112) (Figures 1 and 2).

7.1. Nondiagnostic Nodules by FNA Biopsy (Class 1)

Nondiagnostic FNA biopsy specimens usually result from cystic nodules that yield few or no follicular cells; benign or malignant sclerotic lesions; nodules with a thick or calcified capsule; abscesses; and hypervascular or necrotic lesions (113-115). Additional causes of nondiagnostic results may be sampling errors or faulty biopsy techniques. Reaspiration yields satisfactory results in 50% to 62% of cases (66,69). The timing of repeat needle aspiration has not been established, but a waiting period of at least 3 months should elapse before reaspiration, unless the clinical suspicion for malignancy is high (64,66).

Despite good initial technique and repeated biopsy, 5% to 30% of nodules remain nondiagnostic because of factors inherent to the lesion (66). In these cases, the use of US guidance (59) and a stylet (116) or thin-core needle may further decrease the risk of a nondiagnostic sample (75).

In nondiagnostic specimens, the reported malignancy rate is from 2% to 12% (66). Nondiagnostic aspirates composed of pure colloid and obtained from a nodule that is completely cystic on US require clinical and US follow-up. Aspirates of complex lesions containing blood and histiocytes need careful correlation with family history and with clinical and US findings, and, in the case of repeat nondiagnostic UGFNA, should be considered for surgical resection (57,66). Most nondiagnostic solid nodules should be surgically excised, but some of them, on the basis of clearly favorable clinical and US findings, may be followed up with close clinical and US surveillance (8,112).

7.2. Benign Nodules by FNA Biopsy (Class 2)

7.2.1. *Follow-up or levothyroxine suppressive therapy*

Most thyroid nodules with benign cytologic results and no clinical and US risk factors should be followed up clinically (2,3,8). The timing of clinical and US follow-up and the role of routine rebiopsy of benign nodules are still unclear (117,118). In most cases, clinical and US examination and TSH measurement are appropriate in 6 to 18 months. A routine repeat FNA biopsy may be considered in patients with initially benign cytologic results because of the low, but not negligible, possibility of false-negative results (119). Reaspiration under US guidance is recommended if a nodule significantly enlarges, if a cyst reappears, or in case of suspicious clinical or US changes (8,112).

A clinically significant (>50%) decrease in nodule volume is obtained with levothyroxine therapy in a minority of patients with palpable thyroid nodules (120). Reduction of nodule volume with levothyroxine seems to be more effective in small thyroid nodules with colloid features at FNA biopsy and in geographic regions with iodine deficiency (121). Long-term TSH suppression may prevent an increase in size of a thyroid nodule and of the thyroid gland itself (122,123), but nodule regrowth occurs after cessation of therapy; thus, commitment to long-term therapy seems inevitable. Levothyroxine suppressive therapy is not useful for prevention of goiter recurrence after lobectomy in patients with normal levels of TSH (124,125). Moreover, sustained subclinical hyperthyroidism is associated with a decrease in bone density in postmenopausal women (126,127). In elderly patients with suppressed levels of serum TSH, a 3-fold increase in atrial fibrillation has been reported (128,129).

Routine levothyroxine treatment in patients with nodular thyroid disease is not recommended. Levothyroxine therapy or iodine supplementation (130) may be considered in young patients who live in iodine-deficient geographic areas and have small thyroid nodules and in those who have nodular goiters and no evidence of functional autonomy (122,123). The use of levothyroxine should be avoided in patients with large thyroid nodules or long-standing goiters, in patients with low-normal

TSH levels, in postmenopausal women, in men older than 60 years, and in patients with osteoporosis, cardiovascular disease, or systemic illnesses (127,129).

7.2.2. *Surgical indications*

The following situations are indications for surgical treatment in a patient with a benign thyroid nodule: neck pressure, dysphagia, a choking sensation, shortness of breath (especially when supine), dyspnea on exertion, hoarseness, or pain (2). It is important to verify that the symptoms are associated with the nodule or goiter and not with other disease processes, such as pulmonary or cardiac disease, esophageal disorders, or other head, neck, or lung tumors (8). If a thyroid nodule shows a significant increase in volume or a change in its US features, despite benign FNA biopsy results, surgical resection should be considered (112).

A symptomatic uninodular goiter or MNG, whether euthyroid or hyperthyroid, may be treated surgically or with radioiodine. The preferred extent of resection is lobectomy plus isthmectomy for benign uninodular goiter and (near) total thyroidectomy for MNG (62,125).

7.2.3. *US-guided minimally invasive procedures*

Minimally invasive thyroid surgery may be performed with minimum surgical risk in patients with small nodules (131,132). In recent years, percutaneous, image-guided, minimally invasive therapeutic procedures have been proposed for the nonsurgical management of thyroid nodules in selected cases (133,134).

7.2.3.1. *Percutaneous ethanol injection*

Percutaneous fluid drainage may cure thyroid cysts; however, recurrences are common and surgery is often the final treatment of large relapsing lesions (135). Prospective randomized trials and long-term studies have shown that percutaneous ethanol injection (PEI) is significantly superior to aspiration alone for inducing volume reduction in cysts and complex nodules with a dominant fluid component (136-139). Volume reduction is followed by disappearance of local pressure symptoms (140). The recurrence rate of cystic lesions successfully treated with PEI is low, but in large or multilocular thyroid cysts several injections may be necessary (136).

For hyperfunctioning thyroid nodules, short-term volume reduction is satisfactory (141,142), but 5 years after PEI, serum TSH is suppressed in most cases (136). Hence, PEI is not indicated for hyperfunctioning nodules or nodular goiters because of a high recurrence rate and the availability of effective alternative treatment options.

Clinically significant decreases in nodule size after PEI are reported in solid thyroid nodules that are cold on scintigraphy (143,144). The response, however, is less impressive than in cysts, more treatments are needed, and adverse effects are more frequent (136).

7.2.3.2. Thermal ablation

Thermal ablation with radiofrequency has been proposed for the debulking of large benign thyroid nodules (145,146). Radiofrequency ablation (RFA) is based on the percutaneous insertion of large needle electrodes (14- to 18-gauge) or hook needles and is usually performed under conscious sedation. Because of some disadvantages and the absence of prospective randomized trials, RFA is currently not recommended in the routine management of benign thyroid nodules.

US-guided thermal ablation with laser (percutaneous laser ablation [PLA]) allows the use of small (21-gauge) and multiple (up to 4) needles with a minimally invasive procedure (134). In most patients with thyroid nodules, 1 to 3 sessions of PLA or a single treatment with multiple fibers induces a clinically significant decrease in nodule volume and the amelioration of local symptoms (147). PLA is performed with local anesthesia. Two randomized trials have confirmed its safety and clinical efficacy (148,149).

Because of the novelty of the PLA technique, long-term follow-up studies are lacking (150). Therefore, PLA should be restricted to patients with pressure symptoms or cosmetic concerns who decline surgery or are at surgical risk. Because of potential complications, thermal ablation procedures should be performed only by experienced operators.

7.2.4. Radioiodine treatment for hyperfunctioning nodules

Radioiodine is indicated for the treatment of hyperthyroidism attributable to a hyperfunctioning nodule or a toxic MNG (151). The aims of radioiodine treatment are the ablation of the autonomously functioning areas, the achievement of euthyroidism, and the reduction of goiter size (80,151-153). AFTNs are usually more radioresistant than are toxic diffuse goiters, and higher radiation doses may be needed for successful treatment, especially in countries with iodization programs leading to decreased uptake of radioactive iodine (80,154).

Radioiodine therapy normalizes thyroid function in 85% to 100% of patients with hyperfunctioning thyroid nodules or toxic MNGs (154). After treatment, the thyroid volume generally decreases substantially (median decrease, 35% at 3 months and 45% at 24 months) (152). Radioiodine treatment is generally thought to be effective and safe. Although some investigators have indicated that radioiodine treatment may be associated with increased cardiovascular and cancer mortality (155), other large-scale epidemiologic studies have demonstrated discordant results (156).

After ablation of the autonomous tissue, most patients become euthyroid because of residual normal thyroid tissue, which is no longer suppressed. Nevertheless, depending on the dose of radioiodine used, follow-up of thyroid function, and the possible presence of autoimmune thyroiditis, postradioiodine hypothyroidism may develop in up to 60% after 20 years (157,158). In up to 5% of patients, immunogenic hyperthyroidism may result from radioiodine treatment of toxic or nontoxic

nodular goiter (158,159) because of induction of TRAbs (160). This typically occurs 3 to 6 months after radioiodine treatment and could be due to initially undetectable TRAbs in Graves disease (161). Ingestion of drugs with a high iodine content (such as amiodarone or a saturated solution of potassium iodide) should be avoided before administration of radioiodine, so as not to impair radioiodine uptake by the thyroid. If possible, antithyroid drugs (especially propylthiouracil) (162) should be withdrawn at least 1 week before treatment to prevent radioiodine uptake by normal thyroid tissue and to increase the uptake in the hot thyroid tissue. Use of antithyroid drugs during the first week after radioiodine therapy also decreases the efficacy of the radioiodine treatment. However, it also decreases biochemical and clinical hyperthyroidism and complications such as atrial fibrillation (163).

Radioiodine treatment is best suited for small to medium-sized benign goiters, for patients previously treated surgically, for those with serious comorbid conditions, or for those who decline surgery (164). However, radioiodine is not suited for large nodules that require high doses of radioiodine and may be unresponsive to treatment, or if an immediate resolution of hyperthyroidism is desired (164). The only absolute contraindications to radioiodine treatment are breastfeeding and pregnancy, which should be excluded by a pregnancy test (80,151,153). There is no consensus on a lowest age limit.

7.2.5. Radioiodine treatment for nodular goiter

The use of radioiodine for the treatment of nontoxic nodular goiter has been reported in numerous studies from geographic areas with relatively low dietary intake of iodine (80,165-169). In these reports, patients with MNG had increased or high-normal 24-hour radioiodine uptake compared with that in similar patients with MNG in the United States. There are no studies comparing radioiodine therapy with and without dietary iodine restriction.

In general, a 40% to 50% decrease in thyroid size after 1 year (80,165,166,170) and a 50% to 60% decrease after 3 to 5 years can be achieved with radioiodine therapy (79,164), half of which is seen within 3 months (164). The degree to which goiter volume decreases varies greatly, and 20% do not seem to respond at all. In a randomized study (170), levothyroxine had no effect, whereas radioiodine decreased goiter size by 50% after 1 to 2 years. In very large goiters (>100 mL), goiter volume decreased by only 30% to 40% after 1 year, and the amount of decrease correlated inversely with initial goiter size (167,171). Theoretically, the effect of radioiodine depends on the retained dose in the thyroid. Generally, radioiodine activities have been adjusted according to radioiodine uptake, aiming at an absorbed dose of 100 Gy (156,171), but it has been questioned whether this adjustment is worthwhile (172). Because of regulations regarding allowed radiation doses, which vary considerably between countries, many physicians use fixed doses limited to the maximum outpatient activity in order not to hospitalize the patients. Use of radioiodine usually improves symptoms and respiratory function (80,171).

Early adverse effects of radioiodine are generally mild and transient (164). They include radiation thyroiditis in approximately 3%, transient thyrotoxicosis in 5%, and occasionally an up to 25% increase in thyroid size. Late adverse effects are currently limited to hypothyroidism in 22% to 58% within 5 to 8 years after therapy. Although the risk of malignancy generally is not thought to be increased, no large-scale studies have been conducted in patients with nontoxic goiter, as opposed to toxic goiter. There are no studies comparing radioiodine therapy with surgery and no quality-of-life data using a validated thyroid-specific quality-of-life questionnaire.

7.2.6. Recombinant human TSH–stimulated radioiodine for nontoxic goiter

Currently, the use of recombinant human TSH (rhTSH) for nontoxic goiter is off label; however, studies are under way to obtain United States Food and Drug Administration and European Medicines Agency approval. The main reason for using rhTSH is based on a desire to increase radioiodine uptake in the vast number of patients with low uptake and to decrease extrathyroidal radioiodine uptake, thereby decreasing the risk of malignancy and facilitating the decrease in goiter size (169,173).

The optimal dose of rhTSH and its timing in relation to subsequent radioactive iodine therapy are not clear. Recent data, however, suggest that radioiodine uptake is doubled with use of rhTSH doses as small as 0.03 to 0.1 mg without an evident dose-response relationship (169). Knowing that it takes time to activate the thyroid sodium-iodine symporter, an interval of 24 to 48 hours between rhTSH stimulation and radioiodine administration seems optimal (169).

When used in combination with radioiodine therapy, rhTSH decreases goiter volume 35% to 56% more than nonstimulated radioiodine therapy (171,174,175). It also improves respiratory function (171). However, it is unclear whether its use increases patient satisfaction (174). The goiter-decreasing effect increases with increasing thyroid size, in contrast to the effect without rhTSH prestimulation. An alternative is to reduce radioiodine activity corresponding to the increase in radioiodine uptake obtained with rhTSH stimulation, while obtaining the same decrease in goiter size (176). This decreases radioiodine activity and, thereby, the theoretical risk of extrathyroidal malignancy. The induction of transient dose-dependent hyperthyroidism is the main adverse effect, starting 4 to 8 hours after rhTSH injection and peaking after 24 to 48 hours, with normalization within 3 weeks. With rhTSH doses of 0.1 mg or less, thyroid hormone levels are maintained within the normal range for most patients (176), with no alterations in structural or functional parameters of the heart (177). Acute (within 24-48 h) dose-related swelling of the normal thyroid has been demonstrated, with an increase in mean thyroid volume of 35% for 0.9 mg rhTSH, 24% for 0.3 mg rhTSH, and 10% for 0.1 mg rhTSH (173,174). Therefore, the optimal rhTSH dose seems to be 0.1 mg or less (178,179).

The major long-term complication of rhTSH use is an increase in the rate of hypothyroidism: 3 randomized studies (171,174,175) showed up to 5-fold increases in the rate of hypothyroidism in the

rhTSH groups (21%, 61%, and 65%) as compared to the corresponding control groups (7%, 11%, and 21%, respectively). As seen with conventional radioiodine therapy, the incidence of hypothyroidism is positively related to goiter volume reduction. It is unclear whether rhTSH-stimulated radioiodine therapy increases the risk of thyroidal and extrathyroidal malignancy.

7.3. Follicular Lesions by FNA Biopsy (Class 3)

The follicular category is used when cytologic features indicate a follicular-patterned lesion for which a definite cytologic diagnosis of malignancy cannot be made. Currently, no clear-cut morphologic criteria are available to distinguish benign from malignant lesions (57,64,66). Repeat biopsy of nodules classified as follicular neoplasm is not recommended because it creates confusion and does not provide additional useful information for management (8). However, FNA biopsy may be repeated in cases diagnosed as “atypical cells” to exclude a follicular neoplasm (64). At surgical intervention, about 20% of such specimens are found to be malignant lesions (57,64,180). CNB is not recommended in the management of follicular nodules because it does not provide additional information (64).

Clinical criteria (Box 2) may be associated with increased risk of malignancy (181), but their predictive value is low (180). US features and US-elastography may provide adjunctive information for assessing the risk of malignancy in cases with follicular cytology. However, the specificity and reproducibility of these tools are limited (52,112).

Molecular and immunohistochemical markers may improve the accuracy of cytologic diagnosis, but they do not have consistent predictive value for malignancy and their use is still expensive and restricted to specialized centers (182,183). On the basis of current limited evidence, routine use of molecular and immunohistochemical markers in clinical practice is not recommended and should be reserved for selected cases (14,63).

Surgical excision of the lesion and histologic examination should be performed in most cases. Patients with follicular thyroid lesions can be treated with thyroid lobectomy and isthmectomy or total thyroidectomy, depending on the clinical situation and patient preference. Frozen section is usually not recommended (63,64) but may be useful in nodules with an ill-defined capsule, or in case of nontotal thyroidectomy to decrease the risk of completion thyroidectomy in case of diagnosis of cancer.

In cases with favorable clinical, cytologic, and US features, a multidisciplinary team may consider clinical follow-up without immediate diagnostic surgery (62,63,184).

7.4. Suspicious Nodules by FNA Biopsy (Class 4)

This category includes samples characterized by cytologic features that suggest malignancy but do not fulfill the criteria for a definite diagnosis; it also includes samples with inadequate cellularity but with cellular features strongly suggestive of malignancy (62,63). The rate of histologically confirmed

malignancy in these cases is about 60% (58). Most of these cases are determined to be PTC on definitive histologic analysis (63,68).

Surgery with intraoperative histologic examination is recommended (63). Frozen section may be performed to help guide surgical decision making (63,185). FNA biopsy may be repeated according to the clinician's or cytopathologist's opinion if more material is needed for ancillary studies (eg, immunocytochemistry or flow cytometry) (63,64).

7.5. Malignant Nodules by FNA Biopsy (Class 5)

Whenever possible, the type of carcinoma should be stated in the cytologic report (63,64). If cytologic results are compatible with a differentiated thyroid carcinoma, surgical intervention is necessary (62,186-188). If cancer is due to metastatic disease, efforts should be directed toward finding the primary lesion, which often precludes a thyroid surgical procedure. For anaplastic carcinoma and lymphoma, further diagnostic work-up is recommended before surgery (63).

Thyroid US and cytologic results should be reviewed with the patient and family, and treatment options should be discussed (62). Surgical excision should be recommended and its potential complications discussed. Consultation with a surgeon experienced in endocrine surgical procedures should be obtained as soon as possible (62). The surgical approach should be planned according to the clinical setting and imaging findings (188,189).

Preoperatively, in addition to evaluation by an anesthesiologist, patients with documented thyroid cancer should have a US examination of the neck, UGFNA biopsy of any concomitant suspicious nodule or lymph node, and vocal cord assessment (188). In case of suspicious US features, the metastatic nature of a malignant cervical mass should be confirmed with measurement of thyroglobulin or calcitonin in the washout of the needle used for UGFNA biopsy (43,188,190).

MRI and CT may be performed in selected cases, if needed for the assessment of nodal or airway involvement, substernal extension, or pulmonary metastatic disease (55,191,192).

Treatment and management of thyroid cancer are not covered by this guideline.

7.6. Key Recommendations

7.6.1. Nodules nondiagnostic by FNA biopsy (class 1)

- If initial FNA biopsy is nondiagnostic, it should be repeated with US guidance (grade B; BEL 3)
- Most persistently nondiagnostic solid nodules should be surgically excised (grade C; BEL 4)
- CNB may offer additional information in thyroid lesions with inadequate cytologic results of FNA biopsy (grade C; BEL 3)

7.6.2. Nodules benign by FNA biopsy (class 2)

7.6.2.1. Follow-up

- Cytologically benign nodules should be followed up (grade C; BEL 3)
- Repeat clinical and US examination and TSH measurement in 6 to 18 months (grade D)
- Repeat UGFNA biopsy in cases of appearance of clinically or US suspicious features (grade B; BEL 3)
- Repeat UGFNA biopsy in cases of a greater than 50% increase in nodule volume (grade B; BEL 3)
- Consider routine repeat UGFNA biopsy in 6 to 18 months, even in patients with initially benign cytologic results (grade D)

7.6.2.2. Levothyroxine therapy for benign nodules

- Routine levothyroxine therapy is not recommended (grade B; BEL 1)
- Levothyroxine therapy or iodine supplementation may be considered in young patients with small nodular goiter and no evidence of functional autonomy (grade B; BEL 1)
- Levothyroxine suppressive therapy is not recommended for preventing recurrence after lobectomy if TSH remains normal (grade B; BEL 1)

7.6.2.3. Surgical indications for benign nodules

- Presence of local pressure symptoms clearly associated with the nodule(s), previous external irradiation, progressive nodule growth, suspicious US features, or cosmetic issues (grade D)
- The preferred extent of resection for benign uninodular goiter is lobectomy plus isthmectomy and for MNG is (near) total thyroidectomy (grade D)

7.6.2.4. US-guided PEI

- PEI is effective in the treatment of benign thyroid cysts and complex nodules with a large fluid component (grade B; BEL 1)
- PEI should not be performed in solitary solid nodules, whether hyperfunctioning or not, or in MNGs (grade C; BEL 3)

7.6.2.5. Image-guided thermal ablation

- Laser ablation may be considered for the treatment of thyroid nodules causing pressure symptoms or cosmetic issues in patients who decline surgery or are at surgical risk. Its use should be restricted to specialized centers (grade C; BEL 2)
- RFA is not recommended in the routine management of thyroid nodules (grade C; BEL 3)

7.6.2.6. Radioiodine therapy for benign nodular goiter

7.6.2.6.1. Considerations

- Indications are hyperfunctioning and/or symptomatic goiter, previous thyroid surgery, or surgical risk (grade B; BEL 2)
- Before treatment, UGFNA biopsy should be performed per the recommendations given for nontoxic MNG (grade B; BEL 3)
- Avoid use of iodine contrast agents or iodinated drugs before administration of radioiodine; withdraw antithyroid drugs at least 1 week before treatment and consider resumption 1 week after radioiodine therapy (grade B; BEL 2)

7.6.2.6.2. Contraindications

- Radioiodine is contraindicated in pregnant or breastfeeding women (grade A; BEL 2)
- Always perform a pregnancy test before administration of radioiodine in women of childbearing age (grade A; BEL 2)

7.6.2.6.3. Follow-up after radioiodine therapy

- Regular thyroid function monitoring is mandatory (grade B; BEL 3)
- Consider repeating treatment in cases of persistent or recurrent hyperthyroidism or inadequate size reduction (grade C; BEL 3)

7.6.3. Follicular lesions (class 3)

7.6.3.1. Management

- Repeat FNA biopsy of follicular lesions is not recommended because it does not provide additional information (grade C; BEL 3)
- CNB is not recommended in the management of follicular lesions because it does not add additional information to FNA biopsy (grade D; BEL 4)
- Molecular and histochemical markers are currently not recommended for routine use; their use may be considered in selected cases (grade D; BEL 3)

7.6.3.2. Treatment

- Surgical excision is recommended for most follicular thyroid lesions (grade B; BEL 3)
- Intraoperative frozen section is not recommended as a routine procedure (grade D)
- Consider clinical follow-up in the minority of cases with favorable clinical, US, cytologic, and immunocytochemical features (grade D)

7.6.4. Management of FNA biopsy–suspicious nodules (class 4)

- Surgery is recommended (grade B; BEL 3)
- Intraoperative frozen section is useful (grade D)

7.6.5. Nodules malignant by FNA biopsy (class 5)

7.6.5.1. Management

- For a thyroid nodule with FNA biopsy results positive for differentiated thyroid carcinoma, surgical treatment is recommended (grade A; BEL 3)
- For anaplastic carcinoma, metastatic lesions, and lymphoma, further diagnostic work-up is recommended before surgery (grade B; BEL 3)

7.6.5.2. Preoperative evaluation

- Review US and cytologic results with the patient; discuss treatment options and obtain consultation with a surgeon experienced in endocrine surgery (grade D)
- US examination of the neck, UGFNA biopsy of any concomitant suspicious nodule or lymph node, and vocal cord assessment should be performed before surgery (grade B; BEL 3)
- In case of suspicious US features, the metastatic nature of a lymph node may be confirmed with measurement of thyroglobulin or calcitonin in the washout of the needle used for UGFNA biopsy (grade C; BEL 3)
- MRI and/or CT is useful in selected cases (grade D; BEL 3)

8. Pregnancy and Childhood

8.1. Thyroid Nodule During Pregnancy

Most cases of thyroid nodules during pregnancy are in patients with preexisting nodules who then become pregnant; occasionally, however, a thyroid nodule is detected for the first time during pregnancy. A thyroid nodule in a pregnant woman should be managed in the same way as in nonpregnant women, except for avoiding the use of radioactive agents for both diagnostic and therapeutic purposes (151,153). Thyroid nodule diagnosis during pregnancy necessitates FNA biopsy if findings are suspicious, regardless of the gestational age of the fetus (193).

Sharing of findings among the endocrinologist, obstetrician, thyroid surgeon, pathologist, and anesthesiologist is recommended. Furthermore, the patient's preferences should also be appropriately considered (62).

8.2. Effects of Pregnancy on Nodular Thyroid Disease

In one series, thyroid nodules were diagnosed in 34 of 221 pregnant patients, who had follow-up through 3 months after delivery (194). The volume of the single or dominant thyroid nodule increased from a mean of 60 mm³ at the beginning of pregnancy to 65 mm³ at the third trimester and to 103 mm³ 6 weeks after delivery. At 3-month postpartum follow-up, the volume was still increased from early in pregnancy (73 mm³). New thyroid nodules developed in 11.3% of women during pregnancy; this circumstance led to an increase in the incidence of nodular thyroid disease, from 15.3% at baseline to 24.4% 3 months after delivery. No new thyroid nodules discovered on US were palpable. These data indicate that pregnancy is associated with an increase in the size of preexisting nodules and with the appearance of newly developed thyroid nodules, possibly because of the negative iodine balance that frequently occurs during pregnancy (195).

8.3. Management and Therapy

8.3.1. *Benign thyroid nodule*

Although pregnancy is a risk factor for progression of nodular thyroid disease, no available evidence indicates that levothyroxine is effective in decreasing the size or arresting the growth of thyroid nodules during pregnancy (195). Hence, levothyroxine suppressive therapy for thyroid nodules is not advisable during pregnancy.

8.3.2. *Follicular or suspicious thyroid nodule*

Suspicious cytologic findings pose a difficult problem during pregnancy. Although pregnancy may cause a misleading diagnosis of follicular neoplasm because of a physiologic increase in follicular epithelium, the malignancy rate of follicular neoplasm in pregnant women is similar to that in nonpregnant women—about 14% (196). Therefore, deferring surgical treatment to the postpartum period seems reasonable.

8.3.3. *Malignant thyroid nodule*

Thyroid cancer is rarely diagnosed during pregnancy. If cancer is diagnosed during the first or second trimester, the patient may undergo surgical treatment during the second trimester, when anesthesia risks are minimal (193). However, women with no evidence of aggressive thyroid cancer may be reassured that surgical treatment performed soon after delivery is unlikely to adversely affect prognosis (197). If the cytologic diagnosis is made during the third trimester, the surgical procedure can be postponed until the immediate postpartum period (197).

8.4. Thyroid Nodules in Children

Although no epidemiologic studies are available on thyroid nodules in children, small cohort studies report prevalences of thyroid nodules in prepubertal children of up to 1.8% (198-200). A few small retrospective cohort studies report higher malignancy rates for thyroid nodules in children than in adults: a mean malignancy rate of 26% for operated thyroid nodules in children (201), and 9% to 18% malignant and suspicious results for children undergoing FNA biopsy (201-203). The lower prevalence of thyroid nodules in children, associated with higher malignancy rates than in adults (204), suggests a more frequent surgical approach for thyroid nodules in children.

Diagnostic and therapeutic practice patterns vary considerably for thyroid nodules in children (205). Sensitivity and specificity of FNA biopsy in children are 86% to 100% and 65% to 90%, respectively (15,202), and thyroid US criteria for malignancy seem to have a low predictive value in children (15,206).

Despite a high prevalence of positive lymph nodes and lung metastases at presentation, the prognosis of PTC in children is good (207). Young age is a major determinant of recurrence in children (208). Whereas thyroid carcinomas in children are mostly papillary, several case reports describe follicular thyroid carcinomas in patients with congenital hypothyroidism (195) who also have an increased incidence of thyroid nodules. Moreover, in contrast to adults, hot nodules in children seem to carry a substantial risk of malignancy (209).

8.5. Key Recommendations

8.5.1. *Management of thyroid nodules during pregnancy*

- Thyroid nodules in pregnant women should be managed in the same way as in nonpregnant women; in the presence of suspicious clinical or US findings, diagnosis necessitates FNA biopsy (grade C; BEL 3)
- Avoid use of radioactive agents for both diagnostic and therapeutic purposes (grade A; BEL 2)
- During pregnancy, suppressive levothyroxine therapy for thyroid nodules is not recommended (grade C; BEL 3)
- For a growing thyroid nodule during pregnancy, follow-up should include US and FNA biopsy (grade C; BEL 3)
- If FNA biopsy shows a follicular lesion, surgery may be deferred until after delivery (grade C; BEL 3)

8.5.2. *Management of FNA biopsy–malignant nodules during pregnancy*

- When a diagnosis of thyroid malignancy is made during the first or second trimester, thyroidectomy may be done during the second trimester, if recommended. Women with no

evidence of aggressive thyroid cancer may be reassured that surgical treatment performed soon after delivery is unlikely to adversely affect prognosis (grade C; BEL 3)

- When a diagnosis of thyroid malignancy is made during the third trimester, surgical treatment can be deferred until the immediate postpartum period (grade C; BEL 3)

8.5.3. Management of thyroid nodules in children

- Evaluation of nodular disease in children is similar to that in adults (grade C; BEL 3)
- Because of a higher prevalence of malignancy in children, surgery is often necessary for cold as well as hot nodules (grade C; BEL 3)

9. Methods

9.1. Development and Use of the Guidelines: Methods of Bibliographic Research

We searched for primary evidence to support the current guidelines by using a “clinical question” method. Each topic covered by the guidelines was translated to a related question. Accordingly, the bibliographic research was conducted by selecting the studies able to yield a methodologically reliable answer to each question.

The first step was to select pertinent published reports. The United States National Library of Medicine Medical Subject Headings (MeSH) database was used as a terminologic filter. Appropriate MeSH terms were identified, and care was taken to select them on a sensitive rather than a specific basis. The MeSH terms and their proper combination enabled us to retrieve the reports pertinent to a specific issue.

The second step was to select relevant published studies. Beginning with the pertinent reports indexed with the appropriate MeSH terminologic filters, we applied the PubMed clinical queries methodologic filters. The clinical queries were grouped into 4 categories: diagnosis, etiology, prognosis, and therapy. For each clinical question, a proper complex search string is available (210, 211). From the combination of terminologic (MeSH terms) and methodologic filters (clinical queries), the relevant studies, designed to provide a reliable answer to the question, were selected.

After the relevant published studies had been retrieved, the bibliographic research continued by looking for further evidence cited in the bibliography of each report and by following the Related Articles link listed next to each item in MEDLINE.

Meta-analyses were searched, both in MEDLINE and in the Cochrane Library. Three methods were used to search for meta-analyses in MEDLINE:

- Selection of “Meta-Analysis” from the “Publication Type” menu on the “Limits” tab of the PubMed main page
- Application of function “Find Systematic Reviews” on the “Clinical Queries” PubMed page

- Use of Hunt and McKibbon's complex string for systematic reviews (200): AND (meta-analysis [pt] OR meta-anal* [tw] OR metaanal* [tw]) OR (quantitative* review* [tw] OR quantitative* overview* [tw]) OR (systematic* review* [tw] OR systematic* overview* [tw]) OR (methodologic* review* [tw] OR methodologic* overview* [tw]) OR (review [pt] AND medline [tw])

The Cochrane Library was browsed by entering free terms in the search window.

Guidelines were searched in MEDLINE and in several guidelines databases. Two methods were used to search for guidelines in MEDLINE:

- Selection of "Practice Guidelines" from the "Publication Type" menu on the "Limits" tab of the PubMed main page
- Use of the following GIMBE-Gruppo Italiano Medicina Basata sulle Evidenze complex string for the guidelines: "guideline" [pt] OR "practice guideline" [pt] OR "health planning guidelines" [mh] OR "consensus development conference" [pt] OR "consensus development conference, nih" [pt] OR "consensus development conferences" [mh] OR "consensus development conferences, nih" [mh] OR "guidelines" [mh] OR "practice guidelines" [mh] OR (consensus [ti] AND statement [ti])

Guidelines were searched in the following databases: National Guideline Clearinghouse (USA); Agency for Healthcare Research and Quality (USA); Canadian Medical Association—Clinical Practice Guidelines; Canadian Task Force on Preventive Health Care; National Institutes of Health—National Heart, Lung, and Blood Institute (USA); National Health Service Research and Development Health Technology Assessment Programme (UK); National Institute of Clinical Excellence (UK); New Zealand Guidelines Group; PRODIGY Guidance—National Health Service (UK); and the Scottish Intercollegiate Guidelines Network.

9.2. Levels of Evidence and Grading of Recommendations

The American Association of Clinical Endocrinologists (AACE) protocol for standardized production of clinical practice guidelines was followed to rate the evidence level (EL) of each reference on a scale of 1 to 4 and to link the guidelines to the strength of recommendations on the basis of grade designations A (action based on strong evidence) through D (action not based on any evidence or not recommended) (Table 2) (212). The BEL, corresponding to the best conclusive evidence found, accompanies the recommendation grade (213). All recommendations resulted from a consensus among the AACE, Italian Association of Clinical Endocrinologists, and European Thyroid Association primary writers and were influenced by input from the Task Force members and reviewers.

Some recommendations were upgraded or downgraded on the basis of expert opinion. In these cases, subjective factors such as clinical experience, cost, risk, and regional availability of specific technologies and expertise took priority over the reported BEL (214).

Table 2. Strength-of-Evidence Scales Reported in the Medical Literature

Category	Description
Level of Evidence	
1	Well-controlled, generalizable, randomized trials Adequately powered, well-controlled multicenter trials Large meta-analyses with quality ratings All-or-none evidence
2	Randomized controlled trials; limited body of data Well-conducted prospective cohort studies Well-conducted meta-analyses of cohort studies
3	Methodologically flawed randomized clinical trials Observational studies Case series or case reports Conflicting evidence with weight of evidence supporting the recommendation
4	Expert consensus Expert opinion based on experience “Theory-driven conclusions” “Unproven claims”

Level of Recommendation	Description	Action
A	>1 Conclusive level 1 publications demonstrating benefit >> risk	Action recommended for indications reflected by published reports Action based on strong evidence Action can be used with other conventional therapy or as “first-line” therapy
B	No conclusive level 1 publication >1 Conclusive level 2 publication demonstrating benefit >> risk	Action recommended for indications reflected by the published reports Use if the patient declines or does not respond to conventional therapy; must monitor for adverse effects, if any

Table 2 (continued)

Level of Recommendation	Description	Action
		Action based on intermediate evidence Can be recommended as “second-line” therapy
C	No conclusive level 1 or 2 publication	Action recommended for indications reflected by the published reports
	<p>≥1 Conclusive level 3 publication demonstrating benefit >> risk</p> <p>OR</p> <p>No conclusive risk at all and no benefit at all</p>	<p>Use if the patient declines or does not respond to conventional therapy, provided there are no significant adverse effects;</p> <p>“No objection” to recommending their use</p> <p>OR</p> <p>“No objection” to continuing their use</p> <p>Action based on weak evidence</p>
D	No conclusive level 1, 2, or 3 publication demonstrating benefit >> risk	Not recommended Patient is advised to discontinue use
	Conclusive level 1, 2, or 3 publication demonstrating risk >> benefit	Action not based on any evidence

Adapted from Mechanick et al (212) and Mechanick et al (213). Used with permission.

10. Standards for Diagnostic and Therapeutic Procedures in Patients With Thyroid Nodules

10.1. Ultrasonography

US is the most valuable technique for evaluating thyroid anatomy because it provides accurate information about thyroid size, shape, and texture. In the vast majority of patients, US examination is considered the gold standard for detecting nodular thyroid disease; its high resolution currently can distinguish thyroid lesions as small as 1 or 2 mm in diameter. Hence, US examination has a pivotal role in localizing, counting, and measuring palpable and nonpalpable thyroid nodules. Tips for a good US examination are shown in Box 4. US evaluation of nodule margins, shape, texture, and vascularity may provide consistent clues for predicting the probability of malignancy, thus directing patient referral for FNA biopsy.

If the thyroid gland is grossly enlarged, the lower portions of both lobes may be located in the mediastinum and thus be partially or totally hidden to US assessment. Thyroid tissue extending behind the trachea also cannot be imaged by US. In these situations CT, MRI, or CT/positron emission tomography is indicated. Thyroid scintigraphy, although offering poor image definition, may be useful in detecting aberrant thyroid tissue such as lingual or thymic ectopia.

- | |
|---|
| 1. Use a good-resolution instrument with digital technology, a 10- to 14-MHz linear probe, and Doppler capability |
| 2. Sit comfortably in front of the ultrasound equipment with the controls within reach |
| 3. Have the patient in a supine position with neck hyperextended |
| 4. Hold the probe firmly in your hand; move it gently and slowly |
| 5. Examine the whole neck from clavicle to jaw |
| 6. Take transverse scans; rotate the probe clockwise to obtain longitudinal images |
| 7. Save images (printouts or digital) of standard projections, plus images of relevant findings. Indicate in each picture the placement of the probe by pictograms or written notes |
| 8. Measure 3 dimensions of nodular findings, using volume calculation for the best reproducibility of serial measurements |
| 9. Be concise but thorough in your report |
| 10. Be mentally neutral: expect unexpected findings |

Box 4. Ten tips for a good clinical ultrasonographic thyroid examination

10.1.1. Requirements for US equipment

US examination of the thyroid gland is usually performed with a 10- to 14-MHz linear transducer. Curved transducers may be useful for a large or mediastinal goiter. The US equipment needs to be

adjusted to operate at optimal frequency for clinical use, balancing resolution and beam penetration: deep targets can be evaluated with lower frequencies (5.0-7.5 MHz).

Color-flow Doppler and power Doppler US are highly useful for assessing the general vascularity of thyroid tissue and of single thyroid nodules, providing valuable information concerning their likelihood of malignancy. Doppler imaging is helpful in the evaluation of enlarged lymph nodes, and in the identification of parathyroid adenomas. A needle guide attachment is advisable for US-guided PEI or PLA treatments, to fit the needle exactly within the target under continuous observation during the procedure.

10.1.2. *Requirements for US training*

Training programs for thyroid US must include use of US for the whole neck region because thyroid diseases and tumors may extend beyond the anatomic boundaries of the thyroid gland. Most countries have no restrictions on who can perform thyroid and neck US; therefore, it is performed by general practitioners, endocrinologists, radiologists, nuclear medicine specialists, internists, general and neck surgeons, and others. On one hand, the widespread use of thyroid US allows for enhanced recognition of thyroid nodules and tumors; on the other hand, it may lead to inconsistent use of US equipment and interpretation of US images and inappropriate indications for and performance of FNA biopsies. National scientific societies for radiology, US, and endocrinology are the ideal settings for learning programs.

In the AACE Endocrine Certification in Neck Ultrasound program, the following 6 areas are emphasized:

- 1) Principles of US imaging
- 2) Neck anatomy
- 3) Thyroid pathology
- 4) Parathyroid pathology
- 5) Lymph node pathology
- 6) UGFNA

Minimum requirements for Endocrine Certification in Neck Ultrasound are performance of 100 to 125 US studies (70% diagnostic, 30% UGFNA) in the 12 months preceding certification. Candidates are also required to submit images and reports of diagnostic and UGFNA biopsy procedures. The Italian Society of Ultrasound requires 325 hours of training divided as follows: 20 hours theory; 105 hours tutorial practice in a certified US school in a hospital, university, or research institute; and 200 hours corresponding to 600 US examinations in private or hospital practice. This certification is not restricted to the neck but includes other endocrine glands.

The number of US studies or hours of practice does not automatically guarantee the quality of diagnostic performance. Thyroid US training is effective when the case mix is comprehensive,

including different types of tumor cases. The panel suggests the following minimums for expertise in thyroid US: at least 600 US examinations performed per year; at least 30 cases of thyroid tumors, metastatic lymph nodes, and local recurrences evaluated per year; at least 70% of the thyroid tumors, subsequently diagnosed by FNA biopsy and histology, suspected at first US examination; and at least 150 UGFNA biopsy procedures performed per year, with an inadequate sampling rate of less than 10%.

US is an operator-dependent imaging technique, and static images are unsatisfactory; therefore, endocrinologists should be trained to perform by themselves a complete sonographic evaluation of patients with nodular thyroid disease.

10.1.3. *Thyroid US method*

The US operator should sit comfortably in front of the screen of the US equipment, having the instrument keyboard at the right height for ease of handling. The patient is placed in the supine position for scanning, usually on the right side of the operator and US machine. A pillow under the patient's shoulders ensures that the neck is adequately hyperextended. A modest amount of gel is squeezed onto the probe footprint and, with the probe, is spread on the patient's neck. The probe is held gently with the right hand, ensuring complete probe contact without applying pressure. Excessive pressure may alter the anatomy and decrease venous blood flow, causing color Doppler mapping distortion.

During the procedure, both thyroid lobes should be imaged in at least 2 projections: axial (transverse) and sagittal (longitudinal) planes. It is advisable to start with a standard axial scan on the right lobe, then slowly rotate the probe 90° clockwise, without uncoupling the transducer from the neck. This method provides unlimited oblique images of the lobe, eventually obtaining the sagittal scan. The same procedure should be repeated on the left lobe. Thyroid images should include transverse scans of the superior, mid, and inferior portions of both thyroid lobes; longitudinal scans of the medial, mid, and lateral portions of both lobes; plus a transverse scan of the isthmus. Lobe size should be recorded in 3 dimensions (anteroposterior, transverse, and longitudinal), and the volume of each lobe may be calculated according to the ellipsoid formula. The thickness of the isthmus on the transverse view also should be recorded. The length and thickness of the thyroid lobes may vary considerably accordingly to body habitus; the more reliable index of thyroid size usually is lobe thickness. A thickness up to 2 cm is considered normal, and greater than 2.5 cm is definitely enlarged.

Thyroid abnormalities should be documented. Small focal alterations, including tiny 2- to 3-mm anechoic colloidal areas, isolated calcifications, and reflective bands of fibrous tissue are often seen as part of the normal range of thyroid texture appearance. The location, size, number, and characteristics of nodules should be recorded, including echogenicity (isoechoic, hypoechoic, markedly hypoechoic, hyperechoic, or anechoic), structure (solid, mixed, cystic), margins (regular,

irregular, halo), calcifications (coarse, eggshell, punctate), and vascularity (scanty, normal, high, peripheral, or central). Measurements should be made in 3 dimensions, reporting nodule volume according to the ellipsoid formula. Most US equipment allows automatic volume calculation.

US-assessed volume is the most reproducible indicator of nodule growth during serial clinical evaluations. Nodule thickness and length may change with variations in neck stretching or in probe pressure. Because nodule growth is one of the clinical criteria for recommending a repeat FNA biopsy or surgical excision, the use of a standardized, reproducible method of US measurement is of critical importance in clinical management of thyroid nodules. Significant nodule growth is usually defined as a volume increase of at least 50% or growth in 1 dimension of at least 20%.

US imaging should include the whole neck from clavicles to jaw. Abnormalities of soft tissues adjacent to the thyroid gland, such as abnormal lymph nodes or thrombosed veins, should be documented when encountered. Whenever possible, comparison should be made with previous US or other imaging studies.

Neck examination should be extended to the cervical lymph nodes. Enlarged lymph nodes (>0.5 cm) in the lateral and central compartments of the neck and their US characteristics (hilar line, microcalcifications, cystic necrosis, vascularity) should be studied.

During a US neck study, parathyroid adenomas may be seen incidentally.

10.1.4. *Preoperative US study of the neck*

All patients undergoing thyroid surgery should have a preoperative US study. When needed, other imaging studies may be performed (see section 3.6.).

Preoperative US study of thyroid nodules should include the thyroid gland and neck lymph nodes. For surgical planning, the following information should be provided to the surgeon:

- General appearance of the thyroid gland. In particular, coexistent chronic thyroiditis, because this may be associated with fibrosis and difficult gland dissection
- Site, side, and size of the nodule or nodules
- Number of nodules or suspected tumor foci, monolateral or bilateral
- Margins of the nodule, boundaries, suspicious extracapsular extension
- Suspicious lymph nodes in the central and lateral neck compartments
- Coexistent pathologic findings, including enlarged parathyroid gland or other masses

10.1.5. *Color and power Doppler US examination*

Color-flow and power Doppler US provide information regarding both the direction and velocity of blood flow. Power Doppler is currently the preferred technique for assessing nodule vascularity because of its high sensitivity in detecting the slow flows that occur in thyroid nodules. Doppler frequencies should be set to optimize flow detection. A low pulse-repetition frequency of 1 to 1.3 MHz

is generally used. The color or power Doppler box should be adjusted on the US screen according to the nodule size and position.

Thyroid nodules are categorized as having absent or present peripheral and intranodular blood flow. Most malignancies have increased central flow, and the finding of increased central vascularity should be reported when encountered. The negative predictive value of power Doppler analysis is low, and a negative study does not eliminate the need for FNA biopsy. Furthermore, the concept of absent or present intranodular flow requires reassessment in light of the increased Doppler sensitivity of newer US equipment. Intranodular blood flow is a generic indicator of viable tissue because it may be detected in virtually any solid nodule. True absence of intranodular vascularity is observed only in cysts, in tumor masses with areas of necrotic degeneration, or in small sclerotic microcarcinomas.

10.1.6. *US reporting*

The description of the US characteristics of the thyroid gland should provide all the information useful for clinical purposes. A definite diagnosis is not possible with US, but a mere descriptive report is not helpful if it does not include clinically useful details. Therefore, a US report should enable the reader to classify nodules on a malignancy risk scale.

Specific attention should be paid to several aspects of the US report:

- Aims of the report: Describe the US elements useful for a correct diagnosis and for the patient's knowledge of his or her own situation
- Contents of the report:
 - Thyroid gland: volume, echogenicity, and vascularity
 - Thyroid lesion(s): number of nodules and position within the thyroid gland; size, at least the maximal diameter, specifying whether it is longitudinal, anteroposterior, or laterolateral with respect to the lobe anatomy; echogenicity (anechoic, hypoechoic, isoechoic, or hyperechoic); presence and amount of a fluid component (mixed nodules); characteristics of the borders (regular, irregular, ill-defined) and presence of a peripheral halo; nodule shape ("more tall than wide"); presence of calcifications (micro, macro, egg shell); vascular pattern (presence of peripheral or intranodular flow)
 - Presence of suspicious lymph nodes or US signs suggestive of capsular invasion
 - If multiple nodules are present, a general description of the thyroid size and structure may be advisable, pointing out with detail the nodule (or nodules) bearing the US characteristics associated with malignant potential (see section 3.2.), rather than describing the largest ("dominant") nodule

The report should be typewritten and indicate the name of the operator and of the clinic or hospital. It should be stored in an archive or saved on a computerized database; it should be easily retrievable.

Stylistic suggestions for writing the US report:

- Be concise
- Point out the pathologic aspects
- Avoid writing too much about normal findings—describe normality only if a previous pathologic detail is no longer present (such as a cyst that disappeared) or if a normal report is clinically unexpected (for example, a thyroid nodule that is suspected by palpation but that is not shown with US)
- Do not use acronyms, and use technical or easily understandable terms, avoiding words with uncertain or multiple meanings

10.1.7. *Documentation*

In clinical practice, a standardized sketch of sonographic findings using a scheme of anatomic structures, at minimum, should be provided along with images. A permanent record of the US examination and its interpretation should be available. Images of all appropriate areas, whether normal or abnormal, should be archived along with measurements and labeled with all pertinent information (eg, patient and facility identification, date of examination, side imaged). The exact position of the probe by pictograms should appear on images. Images on printouts and/or digital media (CD-ROM, DVD) should be available along with the final report. US data should be stored and kept in accordance with the clinical needs and with legal and health care facility requirements.

10.1.8. *New technology*

The minimum US technology requirements for thyroid and neck examination are digital equipment, a high-frequency (10-14 MHz) linear probe, and color Doppler and power Doppler availability (see section 10.1.1.).

Image resolution and information may be integrated by several other options.

- Sensitive Doppler and B-flow technology. Sensitive and magnified visualization of blood reflectors permits unprecedented studies of nodular blood flow. As mentioned above (section 10.1.5.) the concept of absent or present intranodular flow should be reassessed
- Tissue harmonics. Additional frequency components over and above the incident frequency (ie, the first harmonic or fundamental frequency) may be detected and imaged (second or tissue harmonic). Tissue harmonics may be useful in detecting deep, ill-defined masses. Typically, tissue harmonics may enhance visualization of central compartment lymph nodes or deep parathyroid glands
- Extended view. Because of its small parts, the footprint of a linear probe is usually only 3.5 to 4 cm. This restricts the real-time visual field and prevents the visualization of a large mass in a single frame. Systems storing a continuous scan of the field of view can extend the real-time

image. Convex and miniconvex probes, with frequencies up to 8 MHz and more, may permit enlarged views, viewing large masses exceeding the linear probe footprint, and exploring the superior mediastinum in large goiters or suspicious metastatic central compartment lymph nodes

- Compound technology. Speckle noise is generated by rebound of the US beam on a transducer at a 90° angle. In traditional US equipment, thyroid tissues typically appear granular, which sometimes limits or hampers image resolution. Digital compound technology permits acquiring images generated by perpendicular and oblique US beam incidence, typically 90°±20°. The combined contributions of standard and steered US beams enable optimization of image quality, obtaining enhanced visualization of borders and interfaces, and smoothes speckle noise. True tissue architecture is thus visualized
- Volume US. Conventional 2-dimensional US images are reconstructed into 3-dimensional images by the operator's brain. The latest real-time 3- or 4-dimensional equipment acquires and constructs the volumetric dataset instantaneously, allowing for coronal, sagittal, and lateral scanning, as well as oblique planes to see anatomical relationships with rotating planes. Volume US is particularly promising in preoperative studies for surgical planning, as well as for evaluating the indications for and the results of minimally invasive ablation techniques
- Elastography. The basic principle of elastography is that tissue compression produces strain (displacement), which is smaller in harder tissues than softer tissues. It is scored measuring the degree of distortion of the US beam under the application of an external force, during the real-time US examination. The US elastogram is displayed over the B-mode image in a color scale that corresponds to tissue elasticity
- Contrast-enhanced imaging. Solutions of gas microbubbles are injected intravenously immediately before US examination. The lack of vascular signal is a useful tool for a precise definition of the size and margins of the area of thyroid necrosis induced by minimally invasive ablation procedures

10.2. Thyroid Biopsy

FNA biopsy is the most important diagnostic procedure in the initial evaluation of thyroid nodules, and its accuracy influences subsequent clinical management. The use of UGFNA biopsy is strongly recommended because its accuracy in diagnosing thyroid nodules exceeds that of conventional FNA. Because proper FNA biopsy technique and correct smear preparation are critical to ensure good diagnostic results, thyroid biopsy should be performed by experienced operators (see section 4.).

10.2.1. *Counseling, informed consent, and request form*

The FNA biopsy procedure should be clearly described to the patient beforehand, including reassurance of the absence of any major risks and the possibility of returning safely to work after its conclusion. The patient should be asked to cooperate with avoiding brisk movements, swallowing, speaking, or deep breathing during the procedure. The patient should be reassured that any neck pain during the procedure is transient and that it will be minimized by proper relaxation of the neck muscles.

A consent form should be signed by the patient before the procedure. The document should describe, in an easily comprehensible style, the procedure, the most frequent complications (neck pain, hematoma, skin bruising), and the possibility of a nondiagnostic sample or false-negative or false-positive results.

The request form accompanying the FNA biopsy specimen should include the following information: location of the nodule, size, suspicious or relevant US findings (eg, presence of microcalcifications or a completely cystic lesion), thyroid functional status and autoimmunity, drug treatment, previous radiation therapy, and personal or familial history of thyroid malignancy. Any suspicion for malignancy should always be described.

10.2.2. *Procedure for palpation-guided FNA biopsy*

Before FNA biopsy is performed, the thyroid gland should be palpated, and the nodule or nodules to be aspirated should be carefully identified. The patient then is placed supine on the examining table with the neck fully extended, supported by a pillow under the shoulders. Adequate lighting should be available. The skin is cleansed with alcohol; local anesthesia is not required. An assistant or a nurse is needed to help with the procedure, preparation and labeling of slides, and application of pressure over the puncture sites.

Commonly available 22- to 27-gauge, 1.5" (3.8 cm) needles attached to a 10- to 20-mL disposable plastic syringe are used. A mechanical syringe holder such as a syringe pistol may be used. The needle is inserted into the nodule without suction, and after the tip is in the nodule, suction is applied while the needle is moved back and forth within the nodule. This maneuver helps dislodge cellular material, which is then sucked into the needle; within a few seconds, the aspirate appears in the needle hub. At that point, suction is released, and the needle is withdrawn. Cytologic smears are prepared by removing the syringe from the needle and filling it with air by retracting the plunger. The needle is then reattached to the syringe, and with the bevel pointing down, one drop of aspirated material is placed on each of several glass slides. Smears are then prepared by using 2 glass slides, similar to those used to make blood smears. Prepared slides can be air-dried or wet-fixed by immediate submersion in 95% ethyl alcohol for Papanicolaou staining.

Some facilities use automated cytology systems, such as Thin Prep (Hologic, Inc, Bedford, Massachusetts), wherein the specimen is placed in the solution for the system and reviewed later in the laboratory. Usually, 2 to 4 aspirations are made from different sites in each nodule; for each aspiration, 2 to 4 slides are prepared. In general, 6 to 12 slides are prepared per biopsy.

Initial sampling may be performed with a 23-gauge needle; larger needles (up to 19 gauge) are used for drainage of a viscous sticky colloid. The use of thinner needles (\approx 27 gauge) without suction should be preferred in a vascularized or complex lesion to minimize blood contamination of the sample. For this nonaspiration (fine-needle capillary) biopsy, the hub of a 25-gauge needle is held in a pencil-grip fashion, the needle is inserted gently into the nodule, and, after aspirate flows into the hub, the needle is withdrawn. Cellular material in the needle shaft is expelled onto glass slides, and smears are prepared as described above.

Immediately after withdrawal of the needle, gentle pressure is applied to the aspiration site (or sites) to prevent hematoma formation. In the absence of problems and if the patient is comfortable, the patient is allowed to leave after a few minutes of observation.

FNA biopsy of a thyroid nodule often causes slight temporary pain and is occasionally associated with a minor hematoma. No serious adverse effects and no distant spreading of tumor cells have been reported. FNA biopsy is relatively safe even with use of aspirin or anticoagulants, but a 5-day withdrawal of aspirin and anticoagulants is recommended.

10.2.3. Procedure for UGFNA biopsy

UGFNA biopsy, regardless of nodule dimension, provides a significant improvement in the rate of satisfactory cellular yields for cytologic interpretation compared with conventional FNA biopsy.

The operator plus a trained assisting nurse are sufficient to perform UGFNA biopsy, provided that needle guides are used. If guides are not used, a sonographer is required who holds the US probe for real-time imaging while the operator is performing UGFNA biopsy.

The room should be large enough to move around the operating bed and all the US equipment. Although the procedure does not require a sterile field, aseptic technique should be used. Therefore, the room should be clean, all materials should be disposable, and sterile gloves, appropriate dressing, and a cap should be worn by the operators.

The US machine should have at least a linear transducer that has a 3.5- to 4.0-cm footprint and multiple frequency settings ranging between 7.5 and 14 MHz, plus Doppler capability. Small curvilinear transducers may be useful for imaging difficult locations, especially in the low neck.

A large adjustable operating table or bed, about 50 cm, is preferable to ease the operator's movements. An auxiliary monitor mounted on a tower is helpful. The monitor should be placed in front of the operator, allowing a straight, comfortable view. A setup tray should be placed well over the patient's legs. The tray should include all materials required for topical cleansing, as well as sterile

transducer covers, coupling gel, syringes, and needles. Ten- to 20-mL syringes with a slip-on tip or Luer lock with an eccentric tip that enhances visibility of the syringe hub are preferred, as are bevel-tip needles. The use of spinal or stylet-type needles permits crossing the coupling gel and the thyroid parenchyma while advancing the needle into the lesion of interest and prevents the uploading of gel or thyroid cells into the needle. Moreover, the stylet stiffens the needle, making it easier to maneuver before aspirating the nodule. An assortment of small needles (25- to 27-gauge), medium needles (21- to 23-gauge), and special needles such as the 22-gauge Westcott needle (BD, Franklin Lakes, New Jersey), which has a lateral scraping window for collecting material from hard lesions, should be part of the routine setup. Needle length is variable. Spinal needles inserted through the needle guide should be 75 to 90 mm long. Shorter needles may prevent the use of guide attachment. Pistol grip holders allow the operator to use the left hand to hold the transducer for direct control of the target view and the right hand to fit and move the needle. This procedure enhances the real-time view of the target. A detachable needle guide adapted for the transducer permits the operator to act independently, with the assistance of a nurse.

The operator stands on the left side of the recumbent patient and the nurse or assistant stands on the right. The US equipment is placed on the right side of the patient at head level. The nurse handles the basic US machine switches, such as freeze, depth, gain, color, and power color adjustments. The operator, in a comfortable standing position, holds the transducer with the left hand and watches images in the auxiliary monitor in front of her or him, aims the guide to the target, and inserts the needle using the right hand.

A 110-cm high bench equipped with glass slides and fixing materials (95% alcohol solution for slide glass immersion or isofix spray) should be available for immediate smearing and fixation. The bench should be illuminated by spotlights for optimal vision during the production of direct smears of aspirated material. If the physician has poor technical skill in slide production, the entire procedure is at risk of inadequacy. This problem may be overcome by diluting material in transport media for subsequent cytopspin or cell block preparations.

Waste boxes to dispose of needles and biologic material should be on hand. Two 4-mL tubes containing 1-mL normal saline solution should be available for hormone measurement in the needle washout (thyroglobulin, calcitonin, parathyroid hormone, other markers).

With UGFNA biopsy, the operator is able to choose the biopsy site on the basis of US evaluation. The recommended biopsy sites are as follows:

- In large nodules, the peripheral part of the lesion is recommended rather than the central area, because of frequent degenerative changes
- In entirely cystic areas, the center of the lesion should be reached to drain the fluid content completely. Cystic fluid should be submitted to the laboratory for evaluation. Most colloid fluids are clear yellow; clear-colorless fluid suggests parathyroid origin, and material should be

submitted for measurement of parathyroid hormone. Hemorrhagic fluid suggests increased malignant potential

- In mixed or mostly fluid complex lesions, the needle should be addressed to the root of hubs or pedicles growing into the cystic lumen (the inner area of the pedicle facing the lumen usually contains necrotic debris and cells with degenerative changes). After complete drainage of the fluid, both the solid areas and the peripheral borders of the lesion should be sampled

10.2.4. *Hormone determination on FNA biopsy washout*

UGFNA biopsy is a sensitive technique for identifying malignant lymph nodes, but 5% to 10% of smears are nondiagnostic.

In patients with lymph node metastatic lesions or local neck recurrence from differentiated thyroid cancer, the combination of cytologic analysis and measurement of thyroglobulin in the needle washout increases the diagnostic sensitivity and specificity of FNA biopsy to nearly 100%. Washout is performed by rinsing the needle with 1 mL of normal saline solution immediately after smear preparation. In patients with metastatic lymph nodes from differentiated thyroid cancer, thyroglobulin levels before radioiodine ablation are much higher (100-100,000 ng/mL) in the washout than in serum. In patients who have undergone thyroidectomy plus radioiodine therapy and have undetectable serum thyroglobulin levels, US may sometimes detect small suspicious neck recurrences. After radioiodine administration, metastatic lymph nodes may be atrophic and yield nondiagnostic cytology. In these cases, the detection of even low thyroglobulin levels by UGFNA should be considered suspicious for malignancy.

In patients with MTC, measurement of calcitonin in the needle washout with the same technique may aid cytologic diagnosis of the tumoral mass. Calcitonin levels greater than 100 ng/mL by UGFNA should be regarded as suspicious for MTC. This indicator is particularly useful in decision making for patients with borderline serum calcitonin levels, either basal or pentagastrin stimulated. In such patients, the cytologic findings can be nondiagnostic, especially in those with MNG.

Because surgery is the only available cure for MTC, surgical planning for MTC is of particular importance. When metastatic lymph nodes from MTC are suspected on the basis of US examination, either before or after surgery, measuring calcitonin in the UGFNA washout increases the diagnostic sensitivity and specificity of cytologic analysis. Calcitonin levels greater than 50 ng/mL by this method should be regarded as suspicious, and a value greater than 100 ng/mL is nearly diagnostic of metastasis or recurrence of MTC.

During neck US examination for thyroid nodules, parathyroid masses may be detected. Sometimes it is not clear whether these masses are metastatic lymph nodes or parathyroid glands. Cytologic evaluation frequently yields a poor diagnosis in parathyroid adenomas. The washout technique allows

measurement of parathyroid hormone from UGFNA and permits the diagnosis of parathyroid adenomas with a near-100% sensitivity and specificity.

10.2.5. LNB and CNB

LNB is a thyroid sampling procedure performed with multiple passes of a large-bore needle (16- to 19-gauge). The slight increase in diagnostic accuracy with LNB, however, is largely outweighed by the need for local anesthesia, the increase in patient discomfort, and the risk of local bleeding. LNB currently has no place in the diagnostic work-up of thyroid lesions.

CNB is aimed at obtaining a small tissue sample for histologic evaluation by means of a 19- to 21-gauge, 10- to 12-cm cutting needle that is usually a disposable, spring-activated device. CNB must be performed under US guidance by an experienced operator. Local anesthesia of the subcutaneous and muscle layers of the neck with 2% lidocaine is requested to decrease local pain and discomfort.

For a safe procedure, a longitudinal (craniocaudal) rather than a transverse approach is recommended. With this approach, the needle track never aims toward the large neck vessels or trachea, as could happen with a transverse approach. With the bevel pointed up, the needle is inserted into the lesion under continuous US real-time imaging. The needle tip should never break outside the lesion. Absence of bleeding during insertion should be ensured through US images. After careful placement, the needle is triggered and rapidly extracted. Pressure and an ice pack should be immediately placed on the biopsy site to prevent bleeding.

10.3. Cytologic Diagnosis and Reporting

10.3.1. *Preparation of FNA biopsy material for routine evaluation*

In preparing cytologic samples, the selection of a particular technique should be based on the experience of the technical staff, laboratory facilities, and personal preference of the cytopathologist. The appearance of the cytologic details will vary according to the adopted method, but correct processing technique is a prerequisite for a reliable diagnosis. In palpable nodules, the procedure also may be effectively performed by pathologists experienced in thyroid FNA biopsy, which may immediately control the adequacy of smeared specimens.

10.3.1.1. Direct smears on slides

This method has been used since the field of diagnostic cytology began and is still the most widely used. Standard diagnostic criteria have been established on the basis of this relatively simple, rapid, and inexpensive method. Artifacts may be produced if excessive pressure is applied to the slides when preparing the smear and if the fixation of material is even slightly delayed, because it prevents a

reliable evaluation of nuclear morphology with Papanicolaou staining. Direct smears are essential for immediate on-site interpretation and assessment of sample adequacy.

10.3.1.2. Liquid-based cytology

This technique was introduced for automated screening systems for gynecologic samples and was extended to thyroid cytology. The specimen is put into a methanol-based preservative solution and sent to a laboratory where a dedicated machine centrifuges, lyses, and disperses cells. A sample of this cell material is then transferred in a monolayer on a glass slide.

The advantages are ease of use for the clinician, who avoids preparing smears; need to screen only 1 slide; availability of a clear monolayer smear; and possibility of saving material for subsequent ancillary studies. The drawbacks are loss of architectural features; different aspects of the colloid (quality and quantity); decreased evidence of inflammatory cells; and its high cost versus its effectiveness. Pathologists need dedicated training in this technique because cytomorphology appears different from that on conventional direct smears.

Because of the role of cell architecture and colloid in diagnostic accuracy, liquid-based cytology is not widely used for thyroid FNA biopsy. However, this method may be used as a supplement to direct smears to save the material for possible ancillary studies.

10.3.1.3. Cell block

The cell block technique refers to cytologic specimens that are processed by paraffin embedding and hematoxylin-eosin staining. The sample is directly immersed in fixative and sent to the laboratory, where it is processed. This method does not require the clinician to prepare the smear and allows sequential sections to be obtained from paraffin blocks for immunohistochemical or molecular studies. Cytomorphologic details in these samples, however, are different from those observed in direct smears. For this reason, the cell block technique does not replace cytologic smears but is an additional resource for possible ancillary investigations. A dedicated sample of cytologic material for cell block preparation is recommended.

10.3.2. *Classification schemes for cytologic diagnosis*

The main goal of thyroid FNA biopsy is to distinguish patients who may benefit from medical treatment from those who should undergo surgery. The cytologic report should be descriptive, but, whenever possible, a diagnosis should be made. A numeric code should be added identifying the category of lesion for risk of malignancy and therapeutic options.

Currently, several classification schemes are used for cytologic diagnosis of thyroid lesions. The 4-category 2006 American Thyroid Association and AACE/Italian Association of Clinical Endocrinologists classifications combine in 1 class both follicular lesions that are mostly benign but

that cannot be definitely diagnosed on cytologic grounds and nodules suspicious for malignancy, which are primarily carcinomas on definitive histologic evaluation. Because the risk of malignancy is quite different in these 2 types of lesions, it seems inappropriate to combine them, with a similar risk evaluation and the same operative recommendation (surgery without frozen section evaluation). In contrast, the National Cancer Institute's proposal of splitting the former "indeterminate" category into 3 new classes (follicular lesion, follicular neoplasm, and suspicious for malignancy) is accurate, but its widespread use may not be easy in nonspecialized centers because of its relative complexity and the lack of clear-cut cytologic criteria for distinguishing follicular lesions from follicular neoplasms. We propose the use in clinical practice of 5 cytologic diagnostic categories, as a trade-off between a simpler but less accurate 4-class scheme and the more accurate 6-class categorization that may be more difficult to use (Tables 3 and 4).

Table 3. AACE/AME/ETA Classification for Cytologic Diagnosis of Thyroid Nodule^a

Diagnostic Category	Suggested Action
Class 1. Nondiagnostic (inadequate-insufficient)	Repeat US-guided FNA biopsy, unless pure cyst
Class 2. Benign (non-neoplastic)	Clinical and US follow-up FNA biopsy repetition if nodule size increases or according to clinician's or cytopathologist's judgment
Class 3. Follicular lesion/neoplasm	Surgery for most Frozen section usually not recommended In some cases follow-up on the basis of a multidisciplinary team evaluation
Class 4. Suspicious	Surgery Frozen section recommended Repeat FNA biopsy only if more material is needed
Class 5. Positive for malignant cells	Surgery for differentiated carcinomas Further diagnostic work-up for anaplastic carcinomas, lymphomas, and metastatic lesions

^a This proposed classification is based on the results of the British Thyroid Association Thyroid Cancer Guidelines (2002-2006) (62) and the Italian SIAPEC-IAP Working Group for the Consensus on Classification of Thyroid Cytology (2008) (63).

Table 4. Summary of Current Classification Schemes for Cytologic Diagnosis on the Basis of Thyroid Fine-Needle Aspiration Biopsy

AAACE/AME/ETA,			
2010	ATA, 2006	BTA, 2007	NCI, 2008
1. Nondiagnostic	Nondiagnostic/inadequate	Nondiagnostic	Unsatisfactory
2. Benign	Non-neoplastic	Benign	Benign
3. Follicular lesion	Indeterminate	Follicular lesion	Follicular lesion Follicular neoplasm
4. Suspicious		Suspicious	Suspicious
5. Malignant	Malignant	Malignant	Malignant

Abbreviations: ATA, American Thyroid Association; BTA, British Thyroid Association; NCI, National Cancer Institute. Data from (62-64).

10.3.2.1. Class 1: nondiagnostic

“Nondiagnostic” reports should not exceed 15% of FNA biopsy samples. They can be classified as inadequate (presence of processing problems), insufficient, or both. A sample is *inadequate* when biased by smearing, fixing, or staining errors, whereas a sample is defined as *insufficient* (or nonrepresentative) when the number of cells collected from the lesion is insufficient for a definite diagnosis. The pathologist should indicate the inadequacy or insufficiency of the sample and the possible causes in the cytologic report.

A sample correctly smeared, fixed, and stained is defined as *adequate*. Although the evaluation of adequacy is difficult to standardize, a sample with at least 6 groups of 10 to 20 well-preserved epithelial cells from the lesion is considered sufficient.

Some insufficient cases may be included in the benign category (class 2) in specific clinical settings, such as samples with:

- Presence of abundant and homogeneous colloid with scattered thyrocytes or follicular cells aspirated from colloid nodules or cysts, if the lesion lacks a solid component or the nodule collapses after aspiration
- Presence of lymphocytes only, in clinically and US-diagnosed Hashimoto thyroiditis
- Presence of red blood cells, necrosis, and macrophages from hemorrhagic pseudocysts

If, however, the sample contains only a few cells that demonstrate unambiguous neoplastic features, the sample is not considered insufficient and should be classified as suspicious (class 4).

If a solid part remains after drainage of a cystic lesion, it should be immediately reaspirated. US guidance is needed to guide the needle into the solid component of the nodule.

Operative suggestion:

- Repeat FNA biopsy at least 1 month after the previous procedure, according to the clinician’s opinion

- Always use UGFNA biopsy

10.3.2.2. Class 2: benign

This category usually accounts for 60% to 75% of the cytologic reports. It includes colloid goiter, hyperplastic nodule, autoimmune (Hashimoto) thyroiditis, and granulomatous (de Quervain) thyroiditis.

Operative suggestion:

- Follow-up clinically and with US
- Repeat FNA biopsy, according to the clinician's or cytopathologist's judgment, to decrease false-negative results
- Always repeat UGFNA biopsy in case of nodule growth or suspicious US findings and in relapsing cystic lesions

10.3.2.3. Class 3: follicular lesion

This category encompasses all follicular-patterned lesions: adenomatoid hyperplasia, adenoma, follicular carcinoma, oxyphilic cell lesions, and some cases of the follicular variant of PTC. In these cases, only histology (and not cytology alone) provides a diagnostic conclusion. This category usually accounts for 20% of thyroid cytologic reports.

At histologic examination, about 80% of the class 3 diagnoses are benign lesions, whereas only 20% of them are malignant tumors.

Evaluation of some immunohistochemical markers, such as galectin-3, HBME-1, and cytokeratin 19, may improve the accuracy of the cytologic diagnosis. Although they do not have definitive predictive value, they can be used as an aid to determine the few cases that can be followed up without surgery. The molecular markers PPARgamma and Ras have shown promise, but larger studies are needed to prove their efficacy.

Some cases characterized by cytologic alterations that are too mild to be included in the class 4 category (suspicious), but that are too marked to be included in the benign category (class 2), may be designated class 3. The choice of including such samples in the "follicular lesion" category must be supported by an adequate description in the report.

Operative suggestion:

- Perform surgical excision and histologic examination of the lesion in most cases
- Consider clinical follow-up without immediate diagnostic surgery in cases with favorable clinical, cytologic, and US features
- Provide information about the uncertain nature of the nodule to the patient
- Do not obtain frozen section

10.3.2.4. Class 4: suspicious

This category includes 1) samples with cellularity characterized by cytologic features suggesting malignancy but that do not fulfill the criteria for a definite diagnosis and 2) samples with insufficient cellularity but with cellular features strongly suggesting malignancy.

Most cases are determined to be PTC on definitive histologic analysis. This class accounts for about 5% of cytologic diagnoses.

Operative suggestion:

- Perform surgery with intraoperative histologic examination
- Repeat FNA biopsy, according to the clinician's or cytopathologist's opinion, if more material is needed for ancillary studies (eg, immunocytochemistry, molecular studies, or flow cytometry)

10.3.2.5. Class 5: malignant

All cases with a diagnosis of malignant neoplasm (papillary, medullary, and anaplastic carcinoma; lymphoma; and metastasis) are included in this category. It accounts for 5% to 15% of cytologic diagnoses. The report should contain an adequate cytologic description.

Operative suggestion:

- Perform surgery for differentiated carcinomas
- Plan the surgical approach according to the clinical setting and imaging findings
- Perform further diagnostic work-up before surgery for anaplastic carcinomas, lymphomas, and metastatic lesions

10.3.3. *Additional studies*

With the application of gene analyses to FNA biopsy material, it is possible to identify several specific gene alterations (eg, in p53, Ras, met, erb2, retinoblastoma, p27, cyclin D1), but these are not yet used in daily cytologic practice. However, some mutations (eg, *RET* in MTCs) or gene alterations (eg, *RET* and *BRAF* in PTC) currently can be investigated in FNA biopsy material. To date, only histochemistry (Congo red stain for amyloid) and immunohistochemistry are widely used techniques. These techniques can be easily applied to smears (either fresh or destained) or cell blocks. An important drawback of immunohistochemistry is the risk of false-positive interpretations due to detection of endogenous biotin, particularly in oncocytic (oxyphilic) cell lesions. However, procedures based on a biotin-free detection system block endogenous biotin reactivity and generally provide reliable results.

Immunocytochemical markers are indicated for diagnostic purposes, but they are not yet used routinely as prognostic or therapeutic markers (eg, cell cycle proteins, oncogenes, receptors).

Calcitonin, carcinoembryonic antigen, and chromogranin A are useful MTC markers. Pancytokeratin is helpful to distinguish anaplastic carcinoma from sarcomas and lymphomas. Flow cytometry or immunocytochemical detection of the different lymphocytic lineage markers may assist in the characterization of non-Hodgkin lymphomas. Parathyroid hormone reactivity identifies intrathyroidal nodules of parathyroid origin. Finally, thyroglobulin and thyroid transcription factor 1 are useful initial markers in the diagnostic work-up of suspected metastasis to the thyroid (excluding those of lung origin). A peculiar Ki67 reactivity pattern at the membrane level is specifically reported in hyalinizing trabecular tumor and may be useful for distinguishing this rare tumor from the more common PTCs and MTCs (that may share both nuclear and architectural features with hyalinizing trabecular tumor).

The topic of follicular (or indeterminate) cytology (follicular-patterned nodules including follicular adenoma, follicular carcinoma, and the follicular variant of PTC) has been the subject of numerous studies exploring the sensitivity and specificity of different markers of malignancy of follicular thyroid tumors. The results are still controversial, but no single marker is specific and sensitive enough to replace the conventional morphologic diagnosis of "indeterminate follicular neoplasm." The most common and explored markers of follicular tumors include: 1) the fusion gene product PAX8-PPARgamma (expressed by follicular carcinoma but not by adenoma nuclei); 2) HBME-1 (a marker of mesothelial cells and tumors, which is overexpressed by thyroid follicular and papillary carcinomas); 3) thyroid peroxidase; 4) cytokeratin 19 (strongly expressed in PTC as opposed to benign lesions); 5) RET (gene rearrangements are markers of some PTCs); and 6) galectin-3 (a β -galactoside-binding lectin, widely distributed in human macrophages, endothelial cells, and several epithelia, including those of malignant follicular-derived thyroid tumors).

Since none of the above markers are 100% sensitive and specific, a panel of markers may provide the highest diagnostic accuracy in cytologic diagnosis. The combination of HBME-1, cytokeratin 19, and galectin-3 seems to provide the highest sensitivity and specificity in thyroid lesions. The demonstration of a *BRAF* mutation is of relevant importance for clinical management of a thyroid lesion because it confirms the presence of a PTC and may predict an aggressive tumor.

10.4. Laboratory Standards

10.4.1. Thyrotropin

Baseline serum TSH determination is the single best test of thyroid status. The use of anti-TSH monoclonal antibodies eliminates cross-reactivity with other glycoprotein hormones. Pituitary extracts used for assay standardization (Medical Research Council 80/558) contain various TSH isoforms, but the isoforms that circulate in the blood may be different from those used for calibration of the assay. The different abilities of the antibodies used in TSH assays to detect these various isoforms may be the basis of assay bias. Moreover, falsely high results can be caused by heterophilic antibodies.

Interference can be ruled out by measuring TSH concentration in the sample specimen using the method of a different manufacturer and checking for discordance between the 2 results.

In samples with TSH concentrations less than 0.2 mIU/L, different methods may give different results. The functional sensitivity indicates the concentration showing a 20% between-run coefficient of variation. Laboratories should use a reliable and sensitive method for measuring TSH with a functional sensitivity of less than 0.02 mIU/L. Because some but not all commercial methods have a functional sensitivity less than 0.02 mIU/L, it is mandatory that each laboratory report contains detailed and clear information about the method used by the laboratory and its functional sensitivity. This information helps the clinician to better evaluate the accuracy of the reference intervals provided by the manufacturer, because in some cases a reference interval inconsistent with the characteristics of the method may be reported.

10.4.2. *Plasma total thyroxine and total triiodothyronine*

Increase or decrease in serum thyroid hormone binding proteins will result in changes in total thyroxine or triiodothyronine values, or both. Therefore, measurement of total thyroid hormones in serum is of limited clinical value.

10.4.3. *Free thyroxine and free triiodothyronine*

Free thyroid hormones, unaffected by binding proteins, make up the active portion of the total pool of thyroid hormones. In practice, measurement of free thyroxine and free triiodothyronine is a more reliable test of thyroid function than measurement of total hormone levels. Nevertheless, free thyroxine and triiodothyronine measurements are fraught with technical problems, and results must be interpreted cautiously. Free thyroxine measurement by equilibrium dialysis is more reliable but technically demanding and is not performed routinely by commercial laboratories.

Because of the problematic nature of free hormone measurement, laboratories should 1) obtain from kit manufacturers information regarding both the effect of sample dilution on the free hormone assay and how the kit assay compares with equilibrium dialysis; 2) acknowledge differences in assay performance in different situations such as during pregnancy, with nonthyroidal illness, and with use of some medications such as heparin, phenytoin, furosemide, carbamazepine, and salicylate; and 3) inform endocrinologists about the limitations of the method.

10.4.4. *Anti-thyroid peroxidase antibodies*

Many assays for thyroid peroxidase are now standardized against the World Health Standard National Institute for Biological Standards and Control 66/387. A set of calibrators can be used to construct a curve for calculation of antibody values (in IU/mL) and is used as the reference for establishing the concentrations of the calibrators.

TPOAb can be detected using complement fixation, agglutination test, or by immunofluorescence on thyroid tissue sections, but concentrations are most commonly estimated using enzyme-linked immunosorbent assay or other sensitive and specific (manual or automated) immunoassays.

Functional sensitivity should be determined for TPOAb. Clinicians and laboratorians should recognize that TPOAb results are method dependent.

10.4.5. *Antithyroglobulin antibodies*

Antithyroglobulin antibodies interfere with the accurate measurement of serum thyroglobulin. Iodination of thyroglobulin may alter the epitope-binding patterns, and this results in multiple naturally occurring molecular configurations that are compatible with adequate hormone synthesis (ie, iodination of thyroglobulin results in conformational changes in the molecule and the antigenic epitopes). The epitope specificity of the antithyroglobulin antibody methods used for patients with thyroid cancer should be broader than the restricted epitope specificity typically associated with autoimmune thyroid disease. Assays for antithyroglobulin antibodies include simple hemagglutination techniques, immunofluorescence on thyroid tissue sections, enzyme-linked immunosorbent assays, and radioimmunoassays. Serial antithyroglobulin antibody monitoring necessitates the use of the same method each time, because assays vary in sensitivity, specificity, and absolute values despite claiming standardization against the International Reference Preparation MRC 65/93.

10.4.6. *Anti-TSH receptor antibodies*

Two methods are used for estimation of TRAb concentrations. One is a bioassay based on cultured cells to measure the stimulating antibodies, and the other involves a receptor assay based on the measurement of ^{125}I -labelled TSH. Radioreceptor assays using isolated, solubilized, or, more recently, recombinant human TSH receptor have been used to develop thyroid-binding inhibition immunoglobulin assays that detect both classes of antibody (stimulating and blocking).

Intermethod differences for TSH-binding inhibitory immunoglobulin methods are wide, and the interassay precision is so poor (coefficients of variation $\approx 20\%$) that it is difficult to compare values from different methods.

The antibodies measured using these commercial methods that quantify the inhibition of TSH binding to porcine or human TSH receptors are referred to by different names, including thyroid-binding inhibiting immunoglobulins and TRAbs. The assays do not distinguish between stimulatory or blocking properties of the antibodies. Only a few research laboratories offer assays that assess the stimulating ability of TRAbs by quantifying cyclic AMP production in cultured thyrocytes or cell lines that express the TSH receptor.

10.4.7. *Thyroglobulin*

Marked bias differences occur among thyroglobulin assays, with an up-to-4-fold difference between the highest- and lowest-biased assays. Assays for thyroglobulin should be standardized using the International Standard Certified Reference Material 457. Thyroglobulin values measured by immunometric methods have more pronounced variation than those determined by radioimmunologic methods. This difference is probably due to the use of polyclonal antibodies with a broad epitope specificity for radioimmunologic determinations. This makes them able to measure a wider range of abnormal tumor-derived thyroglobulin isoforms than immunometric assay methods that use monoclonal antibodies with a limited epitope specificity.

The use of a reference range derived from normal subjects is not recommended. The laboratory should ensure that users are aware that patients receiving thyroxine suppressive therapy should ideally have a thyroglobulin value less than 2 µg/L or a bias-adjusted cut-off as advised by the laboratory. Laboratories and manufacturers should determine and quote the minimum detection limit of their thyroglobulin assay based on functional sensitivity derived from patient samples. The minimum detection limit should ideally be 0.2 µg/L or less. It is important that the laboratory is consulted to determine the appropriate bias-adjusted cut-offs for use in clinical practice.

Laboratories and manufacturers should identify the analytical range of their thyroglobulin assay and adopt procedures to identify samples with “hook effects.” They should inform clinicians of the possibility of interference due to endogenous antithyroglobulin antibodies and should indicate the nature of the possible interference (false increase or decrease in measured thyroglobulin).

Identification of possible assay interference is best achieved using either antithyroglobulin antibody measurements or the discordance between the thyroglobulin results obtained using both immunometric assays and radioimmunoassays. Recovery experiments alone are not recommended to identify assay interference.

For any thyroglobulin method, it is appropriate to make available the results of a clinical assessment of the assay performance. The clinical sensitivity and specificity (ie, positive and negative predictive values) of the assay should always be quoted.

10.4.8. *Calcitonin*

Calcitonin assays should be standardized using World Health Organization International Standard IS 89/620, and laboratories can choose whether to use a method that recognizes primarily monomeric calcitonin (immunometric assay) or a method with a broader specificity (radioimmunoassay).

Laboratories should quote the minimum detection limit of their assay on the basis of a precision profile derived from patient samples. For any method of measuring calcitonin, the results of a clinical assessment of assay performance should be available. The clinical sensitivity and specificity of the assays should be quoted.

Even though calcitonin is a marker for MTC, calcitonin levels may also be increased, although infrequently, in other clinical conditions such as C-cell hyperplasia, pulmonary and pancreatic neuroendocrine tumors, renal failure, and hypergastrinemia (use of proton-pump inhibitors). Higher calcitonin levels are measured in males. Depending on the method used, smoking may either increase or decrease calcitonin concentration.

On the basis of the above considerations, slightly increased levels of calcitonin should be verified by a pentagastrin stimulation test, but this, unfortunately, is not universally available. Pentagastrin (Cambridge Laboratories, Cambridge, United Kingdom), 500 µg/2 mL vials, should be administered at a dose of 0.5 µg/kg by rapid intravenous injection. Blood samples should be drawn at baseline and 2, 5, and 15 minutes. Calcium infusion, although less sensitive than pentagastrin, is a practical and attractive alternative.

10.5. Radioiodine Treatment

The amount of radioiodine to be administered can be fixed (300-1,800 MBq), without any dose calculation or adjustment, based on clinical criteria such as goiter size or uptake determination. This approach is simple, minimizes cost, and performs no worse than using a cumbersome calculation method. Alternatively, an individual computation of the desired concentration of radioiodine (2.96-7.4 MBq/g) or of retained radioactivity (300-400 Gy) at the target (ie, autonomous tissue) can be performed. For absorbed dose estimation, the following formula can be used:

$$A_0 = 5,829 \times ([DT \times m] / [U_{\max} \times T_{1/2\text{eff}}])$$

where A_0 = administered activity of ^{131}I (MBq); DT = prescribed absorbed dose (cGy); m = autonomous tissue mass (g); U_{\max} = maximal thyroid uptake (%); and $T_{1/2\text{eff}}$ = ^{131}I effective half-life in target tissue (h).

Maximal thyroid uptake and effective half-life are measured as tracer activity after administration of ^{131}I , and the target volume can be estimated by US or thyroid scan. In cases of multifocal autonomy, the thyroid gland as a whole can be used as the target volume, and the desired absorbed dose decreased to 150 to 200 Gy.

Individualized approaches can decrease the number of ineffective treatments or help to avoid administration of too much radioiodine, but individualizing therapy is more complex and expensive. Neither approach has been proved definitively superior to the other. Radioiodine therapy is usually defined as successful when the posttreatment TSH value exceeds 0.5 IU/mL. Technetium thyroid uptake under suppression can also be used to evaluate the success of radioiodine treatment. If hyperthyroidism is not definitively cured, radioiodine treatment can be repeated after 3 to 6 months.

Until approval by the United States Food and Drug Administration, the use of rhTSH to augment radioiodine treatment of MNG is considered off label. Its use should be considered in elderly patients or in those with comorbid conditions that preclude anesthesia and surgical treatment.

Radioiodine should not be used if the presence of a malignant lesion is suspected, and UGFNA biopsy should precede treatment.

Treatment of patients with an adrenergic blocking agent or calcium-channel blocker during ^{131}I treatment should be considered. After treatment, patients should undergo follow-up for the development of hypothyroidism or hyperthyroidism.

10.6. US-Guided Interventional Procedures

10.6.1. PEI of cystic lesions

For PEI, a real-time US system with a 7.5- to 14.0-MHz probe, 95% sterile ethanol, a spinal needle, and a disposable plastic syringe are needed. A 22-gauge, 75-mm spinal needle is used because it is a flexible needle, is fitted with a mandrel, and is long enough to cross the steering device and reach deep thyroid nodules. Guidance devices may be helpful, but experienced operators may prefer manual needle placement in order to fit needles according to the variable anatomy of the nodule and the neck.

Near-complete fluid removal is performed to facilitate clear visualization of the needle in the cavity. Without removal of the needle, a syringe containing ethanol is substituted for the aspirating syringe. The ethanol is slowly injected in amounts of 1 to 10 mL, depending on the volume of the aspirated fluid. PEI can be performed by 1 operator, inserting the needle through a guiding device connected to the probe, or by 2 operators, one handling the probe and the other the needle.

PEI is performed on outpatients; the procedure is rapid (not exceeding 10 minutes) and no general anesthesia is needed. There is no evidence that the serum ethanol level increases after PEI, so the patient can drive after the procedure. There is no need for a sterile field, but aseptic technique and disposable items should be used.

The procedure should be performed by experienced operators with adequate training. Adverse effects of PEI are generally mild and transient. Local infiltration with lidocaine 2% (2-4 mL), best seen under real-time US imaging, generally prevents local pain. Should mild local pain occur, it can be controlled with low doses of nonsteroidal antiinflammatory drugs for 1 to 2 days.

Transient dysphonia is quite rare after PEI treatment of cystic lesions. Real-time US monitoring during PEI allows verification of the correct position of the needle tip within the nodule and assessment of the distribution of the injected ethanol, which is recognizable as an expanding hyperechoic area within the cystic cavity. Ethanol seeping outside the cystic nodule is always attributable to incorrect procedure (usually the displacement of the needle tip) and may induce chemical damage to the recurrent laryngeal nerve. If unilateral vocal cord paresis is confirmed with laryngoscopy, corticosteroid therapy (betamethasone, 1.5 mg daily) can be administered for a few days. The patients should be reassured that, in most cases, a complete recovery from vocal cord paresis usually occurs within 1 to 2 months.

In patients with thyrotoxicosis (rare in cystic AFTN), the procedure may be followed by transient exacerbation of thyrotoxic symptoms. In most cases, only a slight, transient, and asymptomatic increase in serum thyroid hormone levels is observed.

Subcutaneous and intracapsular hematomas are rare and self-resolving complications. Antiplatelet agents and anticoagulants should be withdrawn before PEI to ensure normal coagulation tests.

10.6.2. *Thermal ablation procedures*

10.6.2.1. Percutaneous laser thermal ablation

PLA is a minimally invasive procedure proposed as an alternative to surgical ablation of benign thyroid lesions causing compressive symptoms or cosmetic concerns. The flat-tip technique is based on inserting a 300- μ m plane-cut optic fiber through the sheath of a 21-gauge Chiba needle and placing the bare fiber in direct contact with thyroid tissue for a length of 7 to 10 mm, according to the size of the lesion. Fiber locks allow the tip of the fiber to remain still inside the lesion for the appropriate time. A single optic fiber, maintained in a still position, destroys only a small amount of tissue (about 1 mL) when an energy of 1,600 to 1,800 J is delivered with an output power of 2 to 4 W. Therefore, simultaneous insertion of multiple fibers is generally needed. Parallel insertion of 2 to 4 fibers allows an ellipsoid ablation, suitable for the ellipsoid shape of most benign thyroid nodules. This multiple parallel fiber technique obtains ablation diameters of up to 40 to 45 mm wide and 18 to 22 mm thick. Fiber pull-back from the bottom toward the upper part of the nodule, along the craniocaudal axis, achieves further tissue destruction. A maximum of 30 mL of nodular tissue may be destroyed in a single session.

Anticoagulants or antiplatelet drugs should be withdrawn before the PLA procedure to allow for normal coagulation tests. PLA is an office-based procedure, but precautions should be established for patient safety. A multiple-channel monitor showing vital functions is connected to the patient. A venous catheter is inserted in a peripheral forearm vein to ensure continuous venous access. Emergency care facilities and materials, including a defibrillator, should be on hand in the operating room, and an anesthesiologist should be available in case of emergency.

A sterile operative setting is arranged, and the operator and assistants are dressed with sterile disposable coats, masks, caps, and laser-protection glasses. A sensitive color Doppler US machine is required, equipped with 7.5- to 15-MHz multifrequency linear probes with a 3.5- to 4.5-cm footprint. The patient is placed on the adjustable operating bed in the supine position with neck hyperextended. For the patient's comfort, pillows are placed under the shoulders and back. Patients wear laser-protection glasses. Light sedation is obtained with intravenous diazepam (2-3 mg, repeatable during the procedure if necessary). Local anesthesia with lidocaine subcapsular and subcutaneous infiltration is performed under US assistance with thin needles (29- to 30-gauge). Sedation decreases

patient anxiety, swallowing, cough, and other untoward movements. Local analgesia prevents or minimizes local discomfort and pain.

Chiba needles (21-gauge), up to 4 simultaneously, are inserted along the craniocaudal nodule axis in parallel planes at a distance of 8 to 10 mm. Guidance devices may be helpful. However, experienced operators may prefer manual needle placement in order to fit needles according to the variable anatomy of the nodule.

Accurate needle placement is critical for procedure success and prevention of adverse effects. After needle placement, stylets are removed, fibers are inserted through the needle sheath into the nodule, and laser firing starts. A continuous, real-time, US view, with axial, longitudinal, and multiplanar scans, is ensured by having an expert sonographer assist the operator. A highly echogenic area resulting from tissue heating and vaporization slowly enlarges over time. Appropriate energies (500-700 J/mL with mean output power of 3 W) are delivered in 10 to 30 minutes. The laser is switched off and, at the same time, the fibers and needle are extracted. At the end of the procedure, an oval-shaped hypoechoic area shows the zone of presumed tissue ablation, and no blood flow is observed inside the destroyed tissue. The ablation area can be seen more definitively by US several hours or the day after the procedure, after the vapors infiltrating the tissue have disappeared.

Necrotic tissue will be reabsorbed over several months after PLA, with consequent nodule shrinkage proportional to the amount of destroyed nodular tissue. After PLA intervention, most patients are able to leave the operating room without assistance. However, because of residual sedation, they must wait in the recovery room for a few hours, and before going home they are checked clinically and by US examination.

Pain may be the principal adverse effect after PLA. Local anesthesia prevents pain during needle fitting and laser exposure. Pain after the procedure is minimized by immediate intravenous administration of 20 mg methylprednisolone and 100 mg ketoprofen. Intranodular bleeding during needle fitting should not prevent PLA because laser firing stops the bleeding. Rare subcapsular hematomas are spontaneously reabsorbed in 2 weeks. Skin burn is due to an incorrect procedure with excessive fiber pull-back and subdermal heating. Swelling due to colliquation of the ablated nodule may occur uncommonly after 1 to 2 weeks if high energies with multiple needles have been used. In these cases, drainage ensures immediate relief. Accurate procedure, allowing at least 7 mm between fiber tip and the trachea/cricoid cartilage corresponding to the recurrent laryngeal nerve position, guards against nerve damage and vocal palsy. Late vocal palsy, occurring minutes or hours after PLA, is extremely rare. It is due to nodule swelling and pressure on the laryngeal nerve and is reversible in 4 to 6 weeks with corticosteroid treatment. Immediately and for 7 to 10 days after the PLA procedure, the nodule swells by an average of 7% due to edema and may cause a sense of pressure spontaneously subsiding. Beginning the day after PLA, patients receive prednisone 25 mg for 3 days and 5 mg for 4 days. Proton-pump inhibitors (lansoprazole 30 mg) are simultaneously administered for 10 days.

The mean TSH level decreases and free thyroxine increases the day after PLA, returning to baseline within 1 to 3 months. Antithyroglobulin antibody and TPOAb levels may increase in some patients, returning to baseline in a year. These laboratory changes are not symptomatic.

10.6.2.2. Other thermal ablation procedures

RFA has been proposed for the debulking of large benign thyroid nodules. RFA is based on percutaneous insertion of large needle electrodes (14-18 gauge) or hook needles and is usually performed with local anesthesia or under conscious sedation. A high-frequency electrical current moves from the electrodes into the tissues, and the alternate movement of ions results in frictional heating of the target tissue. Monopolar probes produce heat by ionic agitation within a 2-mm radius; tissue heating beyond this zone is due to heat conduction.

Due to the cost of the device, the cumbersome technique, and the absence of prospective randomized trials, RFA is currently not recommended in the routine management of thyroid nodules. Highly focused US ablation was proposed for the debulking of thyroid nodules in experimental models. Ultrasound energy is focused through the skin, achieving precise destruction of small sections ($2 \times 2 \times 10$ mm) of the target tissue. The procedure is noninvasive and allows continuous on-line US targeting. The technique has not yet been tested in controlled clinical trials.

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12. Appendix

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13. Executive Summary

This is a summary constituting part of the Thyroid Nodule Guidelines document published by the AACE, Italian Association of Clinical Endocrinologists (AME), and European Thyroid Association (ETA).

These guidelines cover the diagnostic and therapeutic aspects of thyroid nodular disease but not thyroid cancer management.

The AACE protocol for standardized production of clinical practice guidelines was followed to rate the EL of each reference (1-4) and to link the guidelines to the strength of recommendations on the basis of grade designations A (action based on strong evidence) through D (action not based on any evidence or not recommended). The BEL, corresponding to the best conclusive evidence found, accompanies the recommendation grade. All recommendations resulted from a consensus among the AACE, AME, and ETA primary writers and were influenced by input from the Task Force members and reviewers. Some recommendations were upgraded or downgraded on the basis of expert opinion. In these cases, subjective factors such as clinical experience, cost, risk, and regional availability of specific technologies and expertise took priority over the reported BEL.

These guidelines reflect the state of the field at the time of publication. Because rapid changes in this area are expected, periodic revisions are inevitable. We encourage medical professionals to use this information in conjunction with their best clinical judgment. Any decision by practitioners to apply these guidelines must be made in light of local resources and individual patient preferences.

1. The Scope of the Problem

Thyroid nodules are common, with an estimated prevalence ranging from 3% to 7% on the basis of palpation. The prevalence of incidental thyroid nodules detected by US is estimated to be 20% to 76% in the general population. Moreover, 20% to 48% of patients with 1 palpable thyroid nodule are found to have additional nodules when investigated by US.

2. Clinical Evaluation and Diagnosis

2.1. History

- Record the following information (grade B; BEL 2):
 - Age
 - Family history of thyroid disease or cancer
 - Previous head or neck irradiation
 - Rate of growth of the neck mass
 - Dysphonia, dysphagia, or dyspnea
 - Symptoms of hyperthyroidism or hypothyroidism

- Use of iodine-containing drugs or supplements
- The vast majority of nodules are asymptomatic, and absence of symptoms does not rule out malignancy (grade C; BEL 3)

2.2. Physical Examination

- A careful physical examination of the thyroid gland and cervical lymph nodes is mandatory (grade A; BEL 3)
- Record (grade C; BEL 3):
 - Location, consistency, and size of the nodule(s)
 - Neck tenderness or pain
 - Cervical adenopathy
- The risk of cancer is similar in patients with a solitary nodule or with MNG (grade B; BEL 2)

3. US and Other Diagnostic Imaging Studies

3.1. Ultrasonography

3.1.1. *When to perform thyroid US*

- US evaluation is not recommended as a screening test in the general population nor in patients with a normal thyroid on palpation and a low clinical risk of thyroid cancer (grade C; BEL 3)
- US evaluation is recommended for (grade B; BEL 3):
 - Patients at risk for thyroid malignancy
 - Patients with palpable thyroid nodules or MNGs
 - Those with lymphadenopathy suggestive of a malignant lesion

3.1.2. *How to describe US findings*

- Report should focus on risk stratification for malignancy (grade C; BEL 4)
- Describe position, shape, size, margins, content, echogenic pattern, and vascular features of the nodule(s) (grade C; BEL 3)
- For multiple nodules, detail the nodule(s) bearing the US characteristics associated with malignancy (hypoechoic pattern and/or irregular margins, a more-tall-than-wide shape, microcalcifications, or chaotic intranodular vascular spots) rather than describing the largest (“dominant”) nodule (grade C; BEL 3)

3.2. Indications for FNA Biopsy

3.2.1. *How to select nodule(s) for FNA Biopsy (grade B; BEL 3):*

- FNA biopsy is recommended for nodule(s):
 - Of diameter >10 mm, and solid, hypoechoic on US
 - Of any size with US findings suggestive of extracapsular growth or metastatic cervical lymph nodes
 - Of any size with: patient history of neck irradiation in childhood or adolescence; PTC, MTC, or MEN2 in first-degree relatives; previous thyroid surgery for cancer; increased calcitonin levels in the absence of interfering factors
 - Of diameter <10 mm along with US findings associated with malignancy; the coexistence of 2 or more suspicious US criteria greatly increases the risk of thyroid cancer
- Nodules that are hot on scintigraphy should be excluded from FNA biopsy

3.2.2. *FNA biopsy of multinodular glands*

- It is rarely necessary to biopsy more than 2 nodules when they are selected on the basis of previously described criteria (grade D)
- If a radioisotope scan is available, do not biopsy hot areas (grade B; BEL 4)
- In the presence of suspicious cervical lymphadenopathy, FNA biopsy of both the lymph node and suspicious nodule(s) is essential (grade B; BEL 4)

3.2.3. *FNA biopsy of complex (solid-cystic) thyroid nodule(s)*

- Always sample the solid component of the lesion by UGFNA biopsy (grade B; BEL 4)
- Submit both the FNA biopsy specimen and the drained fluid for cytologic examination (grade B; BEL 4)

3.2.4. *FNA biopsy of thyroid incidentalomas*

- Thyroid incidentalomas should be managed according to previously described criteria for nodule diagnosis (grade C; BEL 3)
- Incidentalomas detected by CT or MRI should undergo US evaluation before consideration for UGFNA biopsy (grade C; BEL 3)
- Incidentalomas detected by positron emission tomography with ¹⁸F-fluorodeoxyglucose should undergo US evaluation plus UGFNA biopsy because of the high risk of malignancy (grade C; BEL 3)

3.3. Other Diagnostic Imaging Techniques

- MRI and CT are not indicated for routine thyroid nodule evaluation (grade D)
- MRI and CT are of value for assessment of size, airway compression, or substernal extension of a nodular goiter (grade C; BEL 3)

3.4. Novel US Techniques

- Elastography and US contrast media currently are not used routinely in the evaluation of thyroid nodules (grade C; BEL 3)

4. Thyroid Biopsy

4.1. Thyroid FNA Biopsy

- Clinical management of thyroid nodules should be guided by the combination of US evaluation and FNA biopsy (grade A; BEL 3)
- Cytologic diagnosis is more reliable and the nondiagnostic rate is lower when FNA biopsy is performed with US guidance (grade B; BEL 3)

4.2. Cytologic Reporting

- Thyroid smears or liquid-based cytology should be reviewed by a cytopathologist with a special interest in thyroid disease (grade C; BEL 3)
- The request form accompanying the cytologic specimen should include all the relevant clinical and US information (grade D)
- The cytologic report should be descriptive, and, whenever possible, a diagnosis should be made (grade B; BEL 4)

4.3. Cytologic Diagnosis

FNA biopsy results may be diagnostic (satisfactory) or nondiagnostic (unsatisfactory). Even if the evaluation of adequacy is difficult to standardize, the specimen is labeled “diagnostic” if it contains a minimum of 6 groupings of well-preserved thyroid epithelial cells, consisting of at least 10 cells per group (grade D; BEL 4).

Cytologic diagnoses should be organized into 5 classes (grade B; BEL 3):

- **Class 1. Nondiagnostic** (inadequate or insufficient): samples with processing errors or an insufficient number of follicular cells
- **Class 2. Benign** (or negative for malignancy): includes colloid or hyperplastic nodules, Hashimoto or granulomatous thyroiditis, and cysts

- **Class 3. Follicular lesions:** all follicular-patterned lesions, including follicular neoplasms, Hürthle cell lesions, and the follicular variant of PTC. In centers with specific experience in thyroid cytology, follicular cytology may be further subdivided into “follicular lesion/atypia of undetermined significance” and “follicular neoplasm.” This distinction separates 2 cytologic groups at different risk for thyroid malignancy but with the same operative indications
- **Class 4. Suspicious:** samples that suggest a malignant lesion but do not completely fulfill the criteria for a definite diagnosis
- **Class 5. Malignant** (or positive): samples characterized by malignant cytologic features that are reliably identified by the cytopathologist and are diagnostic of primary or metastatic tumors

4.4. Pitfalls in FNA Biopsy

- False-negative results are usually due to inadequate sampling or inappropriate target selection (grade D)
- False-positive results are usually due to specimens with suspicious findings (grade D)
- Gray zones in cytologic reports are follicular lesions and cytologic findings suggestive of but not diagnostic for PTC (grade D)
- In follicular lesions, consider performing thyroid scintigraphy to exclude a hot nodule at very low risk for malignancy (grade B; BEL 3)

4.5. Ways to Minimize False-Negative Results

- Use UGFNA biopsy (grade C; BEL 3)
- Aspirate multiple nodule sites (grade C; BEL 4)
- For multiple nodules, prioritize the nodule to biopsy according to US findings (grade B; BEL 3)
- For cystic lesions, sample solid areas with UGFNA biopsy and submit cyst fluid for examination (grade C; BEL 4)
- Review slides with an experienced cytopathologist (grade D)
- Follow up cytologically benign nodules (grade D)
- Consider repeat UGFNA biopsy for benign nodules (grade C; BEL 3)

4.6. Core-Needle Biopsy

- CNB performed under US guidance may offer additional information in selected cases with thyroid or neck masses and inadequate FNA biopsy cytologic results (grade C; BEL 3)

5. Laboratory Evaluation

5.1. Laboratory Evaluation in Patients With Thyroid Nodules

- Always measure serum TSH (grade A; BEL 3)
- If TSH level is decreased, measure free thyroxine and total or free triiodothyronine; if TSH level is increased, measure free thyroxine and TPOAb (grade B; BEL 3)
- Testing for antithyroglobulin antibodies should be restricted to patients with US and clinical findings suggestive of chronic lymphocytic thyroiditis when serum levels of TPOAb are normal (grade C; BEL 3)
- Assessment of serum thyroglobulin is not recommended in the diagnosis of thyroid nodules. In patients undergoing surgery for malignancy, serum thyroglobulin measurement is useful to detect potential false-negative results (grade C; BEL 3)
- TRAb measurement should be performed in patients with TSH levels below normal (grade D)

5.2. Calcitonin

- Measurement of basal serum calcitonin level may be a useful test in the initial evaluation of thyroid nodules (grade B; BEL 3)
- Measurement of nonstimulated serum calcitonin level may be considered before thyroid surgery for nodular goiter (grade B; BEL 3)
- Measurement is mandatory in patients with a family history or clinical suspicion of MTC or MEN2 (grade A; BEL 2)
- If calcitonin level is increased, the test should be repeated and, if confirmed in the absence of modifiers, a pentagastrin or calcium stimulation test will increase the diagnostic accuracy (grade B; BEL 3)

5.3. Other Tests

- Measure serum calcium, parathyroid hormone, or both if a nodular lesion is suspicious for intrathyroidal parathyroid adenoma on US examination (grade D)

6. Radionuclide Scanning

6.1. When to Perform Thyroid Scintigraphy

- Perform scintigraphy for a thyroid nodule or MNG if the TSH level is below the lower limit of the normal range or if ectopic thyroid tissue or a retrosternal goiter is suspected (grade B; BEL 3)

- In iodine-deficient regions, consider performing scintigraphy for a thyroid nodule or MNG even if TSH is normal to exclude autonomy (grade C; BEL 3)

6.2. How to Perform Thyroid Scintigraphy

- Either ^{123}I or $^{99\text{m}}\text{TcO}_4^-$ (sodium pertechnetate) can be used for thyroid scintigraphy (grade B; BEL 3)
- ^{131}I thyroid uptake is not recommended for routine diagnostic use unless low-uptake thyrotoxicosis is suspected (grade A; BEL 3)

7. Management and Therapy

7.1. Nodules Nondiagnostic by FNA Biopsy (Class 1)

- If initial FNA biopsy is nondiagnostic, it should be repeated with US guidance (grade B; BEL 3)
- Most persistently nondiagnostic solid nodules should be surgically excised (grade C; BEL 4)
- CNB may offer additional information in thyroid lesions with inadequate cytologic results of FNA biopsy (grade C; BEL 3)

7.2. Nodules Benign by FNA Biopsy (Class 2)

7.2.1. Follow-up

- Cytologically benign nodules should be followed up (grade C; BEL 3)
- Repeat clinical and US examination and TSH measurement in 6 to 18 months (grade D)
- Repeat UGFNA biopsy in cases of appearance of clinically or US suspicious features (grade B; BEL 3)
- Repeat UGFNA biopsy in cases of a greater than 50% increase in nodule volume (grade B; BEL 3)
- Consider routine repeat UGFNA biopsy in 6 to 18 months, even in patients with initially benign cytologic results (grade D)

7.2.2. Levothyroxine therapy for benign nodules

- Routine levothyroxine therapy is not recommended (grade B; BEL 1)
- Levothyroxine therapy or iodine supplementation may be considered in young patients with small nodular goiter and no evidence of functional autonomy (grade B; BEL 1)
- Levothyroxine suppressive therapy is not recommended for preventing recurrence after lobectomy if TSH remains normal (grade B; BEL 1)

7.2.3. *Surgical indications for benign nodules*

- Presence of local pressure symptoms clearly associated with the nodule(s), previous external irradiation, progressive nodule growth, suspicious US features, or cosmetic issues (grade D)
- The preferred extent of resection for benign uninodular goiter is lobectomy plus isthmectomy and for MNG is (near) total thyroidectomy (grade D)

7.2.4. *US-guided PEI*

- PEI is effective in the treatment of benign thyroid cysts and complex nodules with a large fluid component (grade B; BEL 1)
- PEI should not be performed in solitary solid nodules, whether hyperfunctioning or not, or in MNGs (grade C; BEL 3)

7.2.5. *Image-guided thermal ablation*

- Laser ablation may be considered for the treatment of thyroid nodules causing pressure symptoms or cosmetic issues in patients who decline surgery or are at surgical risk. Its use should be restricted to specialized centers (grade C; BEL 2)
- RFA is not recommended in the routine management of thyroid nodules (grade C; BEL 3)

7.2.6. *Radioiodine therapy for benign nodular goiter*

7.2.6.1. Considerations

- Indications are hyperfunctioning and/or symptomatic goiter, previous thyroid surgery, or surgical risk (grade B; BEL 2)
- Before treatment, UGFNA biopsy should be performed per the recommendations given for nontoxic MNG (grade B; BEL 3)
- Avoid use of iodine contrast agents or iodinated drugs before administration of radioiodine; withdraw antithyroid drugs at least 1 week before treatment and consider resumption 1 week after radioiodine therapy (grade B; BEL 2)

7.2.6.2. Contraindications

- Radioiodine is contraindicated in pregnant or breastfeeding women (grade A; BEL 2)
- Always perform a pregnancy test before administration of radioiodine in women of childbearing age (grade A; BEL 2)

7.2.6.3. Follow-up after radioiodine therapy

- Regular thyroid function monitoring is mandatory (grade B; BEL 3)
- Consider repeating treatment in cases of persistent or recurrent hyperthyroidism or inadequate size reduction (grade C; BEL 3)

7.3. Follicular Lesions (Class 3)

7.3.1. Management

- Repeat FNA biopsy of follicular lesions is not recommended because it does not provide additional information (grade C; BEL 3)
- CNB is not recommended in the management of follicular lesions because it does not add additional information to FNA biopsy (grade D; BEL 4)
- Molecular and histochemical markers are currently not recommended for routine use; their use may be considered in selected cases (grade D; BEL 3)

7.3.2. Treatment

- Surgical excision is recommended for most follicular thyroid lesions (grade B; BEL 3)
- Intraoperative frozen section is not recommended as a routine procedure (grade D)
- Consider clinical follow-up in the minority of cases with favorable clinical, US, cytologic, and immunocytochemical features (grade D)

7.4. Management of FNA Biopsy–Suspicious Nodules (Class 4)

- Surgery is recommended (grade B; BEL 3)
- Intraoperative frozen section is useful (grade D)

7.5. Nodules Malignant by FNA Biopsy (Class 5)

7.5.1. Management

- For a thyroid nodule with FNA biopsy results positive for differentiated thyroid carcinoma, surgical treatment is recommended (grade A; BEL 3)
- For anaplastic carcinoma, metastatic lesions, and lymphoma, further diagnostic work-up is recommended before surgery (grade B; BEL 3)

7.5.2. Preoperative evaluation

- Review US and cytologic results with the patient; discuss treatment options and obtain consultation with a surgeon experienced in endocrine surgery (grade D)

- US examination of the neck, UGFNA biopsy of any concomitant suspicious nodule or lymph node, and vocal cord assessment should be performed before surgery (grade B; BEL 3)
- In case of suspicious US features, the metastatic nature of a lymph node may be confirmed with measurement of thyroglobulin or calcitonin in the washout of the needle used for UGFNA biopsy (grade C; BEL 3)
- MRI and/or CT is useful in selected cases (grade D; BEL 3)

8. Pregnancy and Childhood

8.1. Management of Thyroid Nodules During Pregnancy

- Thyroid nodules in pregnant women should be managed in the same way as in nonpregnant women; in the presence of suspicious clinical or US findings, diagnosis necessitates FNA biopsy (grade C; BEL 3)
- Avoid use of radioactive agents for both diagnostic and therapeutic purposes (grade A; BEL 2)
- During pregnancy, suppressive levothyroxine therapy for thyroid nodules is not recommended (grade C; BEL 3)
- For a growing thyroid nodule during pregnancy, follow-up should include US and FNA biopsy (grade C; BEL 3)
- If FNA biopsy shows a follicular lesion, surgery may be deferred until after delivery (grade C; BEL 3)

8.2. Management of FNA Biopsy–Malignant Nodules During Pregnancy

- When a diagnosis of thyroid malignancy is made during the first or second trimester, thyroidectomy may be done during the second trimester, if recommended. Women with no evidence of aggressive thyroid cancer may be reassured that surgical treatment performed soon after delivery is unlikely to adversely affect prognosis (grade C; BEL 3)
- When a diagnosis of thyroid malignancy is made during the third trimester, surgical treatment can be deferred until the immediate postpartum period (grade C; BEL 3)

8.3. Management of Thyroid Nodules in Children

- Evaluation of nodular disease in children is similar to that in adults (grade C; BEL 3)
- Because of a higher prevalence of malignancy in children, surgery is often necessary for cold as well as hot nodules (grade C; BEL 3).

PHOSPHODIESTERASE 8B (*PDE8B*) GENE VARIANTS AND TSH LEVELSStefano Mariotti¹, Silvia Naitza², Antonio Cao²¹*Dipartimento di Scienze Mediche “M. Aresu”, Università di Cagliari;* ²*Istituto di Neurogenetica e Neurofarmacologia, Consiglio Nazionale delle Ricerche, Cagliari, Italy**Reviewing Editor: Luca Persani**Conflict of interest declaration: None declared**Correspondence to: Stefano Mariotti, MD, Professor of Endocrinology, Dipartimento di Scienze Mediche “M. Aresu” SS 554 I-09042 Monserrato CA, Italy e-mail: mariotti@medicina.unica.it***ABSTRACT**

It has been estimated that 40-60% of the variation of serum TSH levels is under genetic control. In keeping with this notion, polymorphisms of several genes potentially involved in the control of thyroid function have been linked to serum TSH concentrations. Genome-wide association scan (GWAS) is a powerful tool to simplify genetic analysis of complex traits and diseases. By genotyping >360,000 single nucleotide polymorphisms (SNP) in a large cohort of 4,300 Sardinian subjects, we recently identified a strong association ($p = 1.3 \times 10^{-11}$) between alleles of the SNP rs4704397 and serum TSH. This association was confirmed in two genetically unrelated cohorts from Tuscany and the Old Order Amish and contributed to the 2.3% of the total variation of circulating TSH concentration. The rs4704397 SNP is located in intron 1 of the phosphodiesterase 8B (*PDE8B*) gene, encoding a high-affinity cAMP-specific phosphodiesterase abundantly expressed in thyroid tissue. This suggests that different *PDE8B* variants may modulate c-AMP-dependent thyroid hormone secretion and affect by feed-back pituitary TSH production. So far at least one independent study confirmed that the minor A allele of the rs4704397 SNP is associated with higher serum TSH in a cohort of pregnant women.

In conclusion *PDE8B* is an important gene involved in controlling serum TSH concentration in normal individuals. Further studies are needed to ascertain whether and to what extent *PDE8B* may also represent a candidate gene for thyroid dysfunction and/or response to treatment.

Key-words: TSH; cyclic AMP (cAMP); phosphodiesterase (PDE); thyroid hormone secretion.

Introduction

TSH secreted by anterior pituitary is the key regulator of thyroid function and its secretion is in turn strictly controlled through negative feed-back by circulating thyroid hormone concentrations. In the absence of hypothalamus-pituitary failure, serum TSH is therefore a sensitive indicator of thyroid function, with high and low levels indicating hypo- and hyperfunction of the thyroid gland. “Normal” reference range of serum TSH concentrations as derived from population studies carried out in euthyroid subjects without clinical, ultrasound or serological evidence of subtle underlying thyroid disease is generally referred between 0.4 and 4.0 mU/ml. However, recently both lower (2.5 mU/ml)

(1) and higher (6-7 mU/ml) serum TSH concentrations (2) have been proposed as upper normal range values for adult young-to-middle aged and elderly (> 80 years) subjects, respectively. Independently from the criteria employed to calculate TSH normal reference levels, it is clear that, together with serum thyroid hormone, in a given population intra-individual circulating TSH variability is comprised in a narrower range when compared to the general inter-individual variability (3). The precise mechanisms underlying the remarkable inter-individual variation of serum TSH have not been fully elucidated. It is believed that very mild thyroid dysfunction, together with other environmental factors such as diet, smoking and medication concur with the genetic background to determine the individual “set-point” of the hypothalamus-pituitary thyroid axis (4). As detailed in the next paragraph, evidence has been provided that polymorphisms of several key genes involved in thyroid function control may account for differences in serum TSH concentration. In this brief review, attention will be focused on the phosphodiesterase 8B (*PDE8B*) gene, recently identified as important controller of serum TSH concentration by genome-wide association scan (GWAS) on different unrelated populations in the context of the *Progenia* study coordinated by Silvia Naitza, Manuela Uda and Antonio Cao in Cagliari (Italy) and by David Schlessinger in Baltimore (U.S.A.).

Genetic control of serum TSH concentration: studies of candidate gene known to affect thyroid hormone secretion activity and metabolism

On the basis of studies carried out on twins, the heritability of serum TSH levels has been estimated between 40-65% (4). To identify the genes involved in the control of serum TSH several studies have been initially focused on candidate genes involved in different known thyroid hormone pattern.

Table 1. Common polymorphisms related to serum thyroid hormones and TSH variation

Gene	Polymorphism	Effect on serum					
		TSH	T4	T4/T3	T3	rT3	T3/rT3
<i>TSHR</i>	Asp727Glu	↓	=	=	=	=	=
	rs10149689 A/G*	↑	=	=	=	=	=
	rs12050077 AG	↑	=	=	=	=	=
<i>DIO1</i>	D1a- C/T	=			↓	↑	↓
	D1b- A/G	=			↑	↓	↑
	rs2235544 C/A	=			↑	↓	↑
<i>DIO2</i>	D2-ORFa-Asp3	=	↑ ¹	=	=	=	=
	Thr92Ala	=	=	=	=	=	= ²
	rs225014 C/T	=	=	=	=	=	= ³
<i>THRB</i>	TRHB-in9. A/G	(↑)	=	=	=	=	=
<i>PDE8B</i>	rs4704397 A/G	↑	=	=	=	=	=

* Alleles associated with the specified trait are reported in **bold**

¹ Only in young subjects

² Influence L-T4 dose needed to normalize serum TSH in hypothyroid patients

³ Influence psychological well-being of hypothyroid patients on L-T4 therapy

As summarized in Table 1, the results obtained provided evidence that polymorphisms of TSH receptor, deiodinases, thyroid hormone transporters and thyroid hormone receptor do account for significant serum TSH variability.

TSH receptor gene (*TSHR*) polymorphisms

A common *TSHR* polymorphism (Asp727Glu) has been firstly associated to lower serum TSH levels in a small cohort of 156 healthy Dutch Caucasian blood donors (5), and this finding was confirmed in a subsequent study on 756 Dutch twin pairs (6). In the latter study, however, evidence was provided that the contribution of *TSHR* Asp727Glu to the genetic variability was very small. Quite recently, two single nucleotide polymorphisms (SNPs) in the promoter/enhancer region of the *TSHR* gene (rs10149689 G and rs12050077 A) were found associated with increased serum TSH (7). Interestingly, the allelic frequency of these 2 SNPs as well as that of the GA haplotype was significantly increased in Ashkenazi Jewish centenarians and in their offspring compared to controls (7), suggesting that in humans a heritable phenotype characterized by higher serum TSH is associated with longevity. Loss-of-function mutations in the *TSHR* gene are well known cause of increased serum TSH and different degrees of hypothyroidism in single patients and in families (8), but recently two inactivating *TSHR* gene mutations were found to be responsible of the high frequency of hyperthyrotropinemia in an Israeli Arab-Muslim consanguineous community (9). It is therefore conceivable that unrecognized minor loss-of-function mutations may also contribute to the general variation of serum TSH levels.

Deiodinases genes (*DIO1*, *DIO2*, *DIO3*) polymorphisms

Several polymorphisms of the deiodinase genes have been found associated with different levels/ratios of circulating thyroid hormones, and to a lesser degree, TSH (10). Both type 1 (D1) and type 2 (D2) deiodinase activate the prohormone T4 by conversion to T3 through 5'-deiodination, while type 3 (D3) deiodinase acts as inactivating enzyme through 5-deiodination of T4 to inactive rT3. The gene most consistently associated to serum thyroid hormone concentration is *DIO1*, encoding for D1 protein. Two polymorphisms of the *DIO1* gene (D1a-C/T and D1b-A/G) have been extensively studied (11-13): carriers of the T allele of D1a-C/T had higher serum rT3, lower T3 and lower T3/rT3 ratio (indicative of lower 5'-deiodinating activity), while the D1b-G allele was associated with higher serum T3 and T3/rT3 ratio (indicative of higher 5'-deiodinating activity) (13). A similar circulating thyroid hormone pattern suggestive of increased 5'-deiodinating activity has been found associated with C-allele carriers of a further *DIO1* gene SNP (rs2235544 C/A (14). Increased serum T4 levels were found to be associated to a *DIO2* gene polymorphism of (D2-ORFa-Asp3) in young but not in elderly (11) subjects, possibly as a consequence of the age-dependent decrease in the D2/D1 activity ratio in the 5'-deiodination process of T4 (12). None of the above polymorphisms were found to be associated with significant changes in serum TSH concentration. On the other hand, and more relevant to the purpose of the present review, a common *DIO2* gene variant (Thr92Ala) has been found involved in determining the dose of L-T4 needed to normalize serum TSH in thyroidectomized patients, since

Ala/Ala homozygous patients needed higher L-T4 dose as compared with patients carrying the Thr92 variant (15). Moreover, another common *DIO2* SNP (rs225014), although not associated with circulating thyroid hormone level, appears to influence the clinical effectiveness of L-T4 therapy (16). In particular, the carriers of the rarer genotype CC showed impaired psychological well-being on L-T4 and enhanced response to combined L-T3/L-T4 therapy, which was not observed in patients carrying the more common TT genotype.

Thyroid hormone transporters (MCT8, MTC10, OATP1B1) gene polymorphisms

Polymorphisms of thyroid hormone transporters genes MCT8, MTC10, OATP1B1 have been studied in rather small populations (10). No effect on serum TSH and inconstant effects on circulating T3, T4, rT3 have been reported so far (17).

Thyroid hormone receptor gene polymorphisms

The consequences of polymorphisms in the thyroid hormone receptor (THR) alpha (*THRA*) and beta (*THRB*) genes on serum thyroid hormone and TSH levels have been thoroughly investigated (18). No association was found between SNPs and serum thyroid hormone (T4, T3 And rT3) concentrations, while only the intronic SNP *THRB*-in9-G/A was inconsistently associated to serum TSH levels, with higher concentrations found in carriers of the A/A genotype.

Taken together the above studies provide evidence that subtle differences in most of the key genes involved in thyroid hormone secretion, metabolism and action on peripheral tissues may be relevant in determining the individual “set-point” of the hypothalamus-pituitary-thyroid axis. Further insight on the mechanisms involved in determining circulating TSH level may be derived from genome-wide scan studies which may lead to the identification of genes unknown or not previously related to the control of thyroid function. The recent identification of *PDE8B* as a significant determinant of serum TSH levels is an example of the importance of this approach and shall be discussed in detail in the next paragraph.

Genetic control of serum TSH concentration: genome-wide scan studies and the identification of *PDE8E* as determinant of serum TSH levels.

The SardiNIA team of the *Istituto di Neurogenetica e Neurofarmacologia, Consiglio Nazionale delle Ricerche* of Cagliari, Italy in collaboration with Laboratory of Genetics, National Institute on Aging of Baltimore, USA has recently carried out several extensive genome-wide association scan (GWAS) studies in a large Sardinian cohort (Progenia study) in whom the founder-population structure can simplify genetic analyses of complex traits and diseases (19). This approach lead to identification of several genes associated with susceptibility to asthma, obesity-related traits, uric acid and lipid levels, height and severity of β -thalassemia (see (20) for detailed references). To evaluate the genes involved in serum TSH control, a total of 4,300 subjects selected from a sample of 6,148 individuals to represent the largest available families, were genotyped with the 500K Affymetrix mapping Array Set or the 10K Mapping Array System. A total of >360,000 SNPs were tested for

association with serum TSH levels with an additive model (20). The results obtained, recently published in the American Journal of Human Genetics (20), revealed 3 SNPs (rs4704397, rs6885099 and rs2046045) with genome-wide significance ($p < 10^{-10}$ association) at a single chromosome 5 locus (Fig. 1). These three SNPs were all in strong linkage disequilibrium and lied in intron 1 or upstream the phosphodiesterase 8B (*PDE8B*) gene: among them the SNP showing the strongest association (rs4704397) explained 2.3% of the variance of TSH levels.

To confirm this association, three additional genetically unrelated cohorts were analyzed, one of 1,858 individuals enrolled in the Progenia study but unrelated to the first group, the second (1,164 subjects) from Tuscany (Italy) enrolled in the InCHIANTI study (21) and the third of 1,136 individuals from the founder population of the Old Order Amish (22).

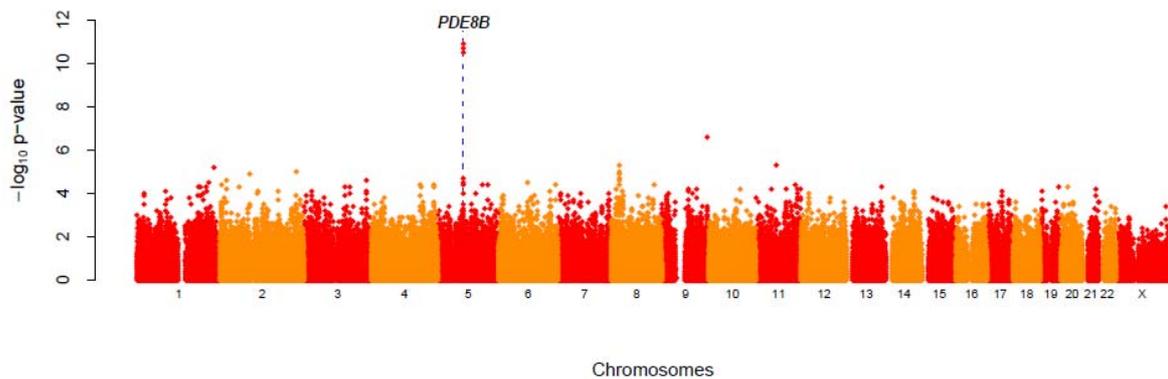


Fig. 1. Results of Genome-wide Association Scan for TSH levels. For each marker the p value of the association is plotted and the position of *PDE8B* is annotated (From (20), with Publisher's permission).

The results obtained confirmed the SNP association found in the original sample. In particular, in all populations studied, the A/A genotype of rs4704397 SNP was associated with higher median serum TSH when compared to A/G and G/G genotypes. Of particular interest the finding that this association was maintained either excluding or including subjects with evidence of associated thyroid disorders, representing a substantial proportion ($1093/8479 = 12.8\%$) of the entire population studied. Moreover, although the difference did not reach the level of statistical significance, there was a trend toward a stronger effect of the A/A allele on serum TSH of subjects with underlying thyroid disorders. Although very preliminary, this finding suggest that *PDE8B* gene could be included between the potential candidate markers for development and/or progression of thyroid dysfunctions (possibly through different expression of *PDE8B* activity in the thyroid tissue).

Although the association of *PDE8B* gene and TSH levels was very strong, this explained, as previously stated, only 2.3% of the general TSH variation, a small part of the total genetic component of this trait (~50%), which may be also explained by other genes, as briefly discussed in previous

paragraphs. We therefore selected 24 candidate genes potentially involved in TSH secretion, activity and regulation of thyroid function and tested their association with TSH levels by GWAS analysis. Evidence of association was obtained for some genes such as *THRB* and *TSHR*, whose correlation with serum TSH levels has been shown in other studies (see previous paragraphs) and other candidate genes such as *TG* (encoding thyroglobulin, involved in thyroid hormone synthesis) and *PDE4D* and *PDE7B* (encoding for other phosphodiesterases involved in the inactivation of cAMP).

Hypothetic mechanisms linking *PDE8B* to serum TSH levels.

The *PDE8B* gene encodes a high-affinity phosphodiesterase catalyzing the hydrolysis and inactivation of cAMP (Fig.2) (23).

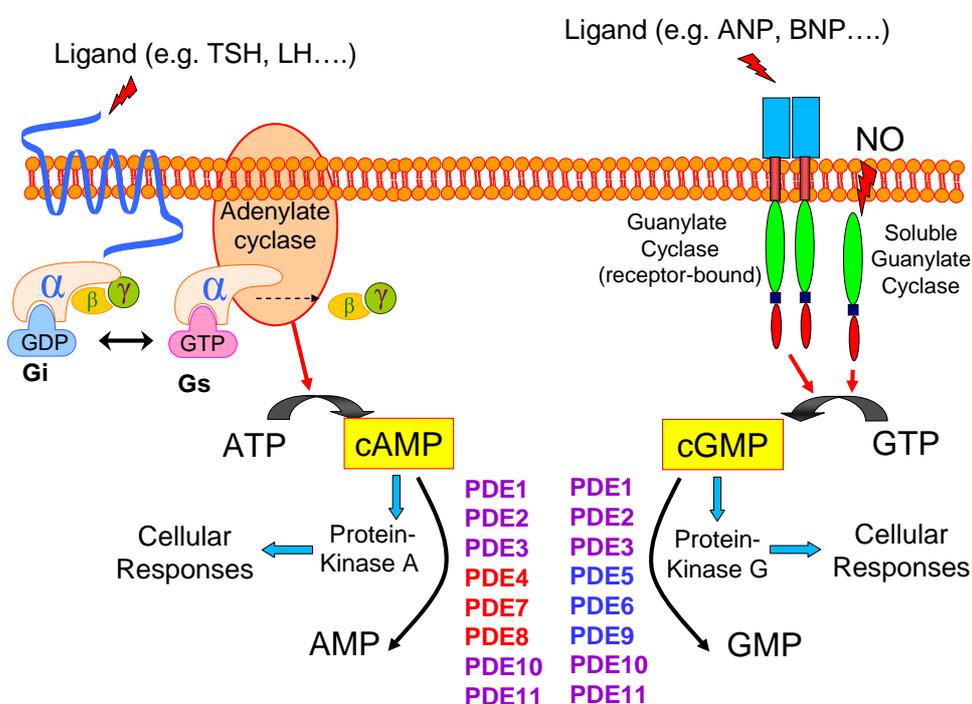


Fig. 2. Intracellular signalling pathways following activation of membrane-bound and soluble cyclases. Adenylate and guanylate cyclases generate cyclic adenosine monophosphate (cAMP) from ATP and cyclic guanosine monophosphate (cGMP) from GTP which acts as second messengers inducing cellular responses. Phosphodiesterases (PDE) in turn inactivate cAMP and cGMP in inactive AMP and GMP, respectively. To date, eleven different PDE families including several isoforms and splice variants are known. Some PDE families are specific for cAMP (PDE4, PDE7 and PDE8, in red), other are specific for cGMP (PDE5, PDE6 and PDE9, in blue) and several (PDE1, PDE2, PDE3, PDE10 and PDE11, in violet) show dual specificity.

Because *PDE8B* is undetectable in the pituitary (24), we infer that it could act primarily in the thyroid by inactivating cAMP produced after TSH stimulation. Indeed, of the 5 major isoforms of *PDE8B*, the major isoform *PDE8B1* and minor isoforms *PDE8B2* and *PDE8B3* are abundantly expressed in the thyroid (23,25,26). *PDE8B* could therefore influence serum TSH levels through its effect on TSH-dependent thyroid hormone synthesis and secretion (Fig. 3). Interestingly, in our study some evidence of association with serum TSH levels was provided for other genes such as *PDE4D*, *PDE7B* (both specific for cAMP (23)) and *PDE10A* (a phosphodiesterase with dual specificity for cyclic

nucleotides and stimulated by cAMP (23)). Further studies, however, involving differential transfection of thyroid cells with different phosphodiesterase variants, are needed to confirm their actual contribution to intracellular cAMP levels and to thyroid functional activity.

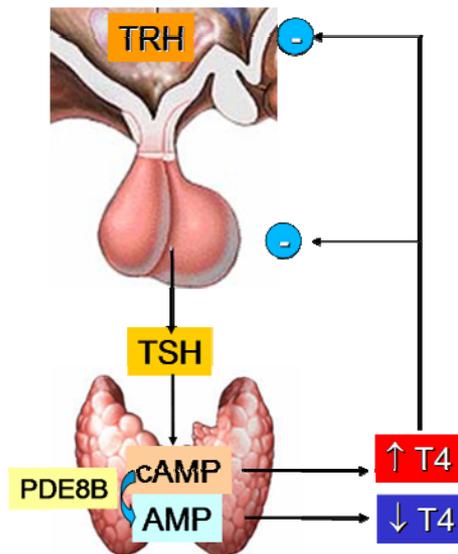


Fig. 3. Hypothetical link between PDE8B and TSH levels. PDE8B activity may affect intrathyroidal cAMP concentrations and subsequent thyroid hormone secretion which in turn, via hypothalamic-pituitary negative feedback, modulate TSH production

If the above hypothesis will provide to be correct, it should also be considered that mutations of *PDE8B* gene could be responsible of some cases of inappropriately high serum TSH in the absence of thyroid autoimmunity or loss-of-function mutations in *THRB* or *TSHR* genes.

Several studies already support the biologic relevance of PDE8B activity in human diseases. A *PDE8B* mutation leading to increased intracellular cAMP has been described in pituitary adenomas (24) and in a case of Cushing's syndrome with bilateral hyperplasia (27). Increased cAMP-degrading PDE8B activity has also been found in autonomous hyperfunctioning thyroid adenomas and interpreted as a compensatory mechanism to the constitutively activated cAMP pathway typical of these tumours (28). *PDE8B* upregulation has also been reported in Alzheimer's brain (29) and PDE8B activity has been linked to insulin secretion (30).

Finally, our finding that other cAMP-specific phosphodiesterase genes (*PDE4D* and *PDE7B*) are also significantly linked to serum TSH levels strongly supports the concept that intrathyroidal cAMP concentration represent a key point in the regulation of hypothalamic-pituitary-thyroid axis.

Other studies linking *PDE8B* to serum TSH

To our knowledge, only one additional study has been published so far on the relationship between *PDE8B* gene and serum TSH levels (31). In this paper, the *PDE8B* SNP rs4704397 (showing the strongest association in our study) was evaluated in a large cohort of 877 pregnant women at 28 week of gestation. The results obtained confirmed the involvement of this SNP in the

control of serum TSH concentration; in particular, the AA genotype was associated with the higher median serum TSH concentration (2.16) while lower levels (1.84 and 1.73) were found in the AG and GG genotypes, respectively ($p=0.004$). No association was found between rs4704397 SNP and FT4, FT3, or anti-thyroid peroxidase (anti-TPO) autoantibody, as well as with offspring birth weight or gestational age at the delivery. This study has important practical implications since the current guidelines (32) for treating subclinical hypothyroidism in pregnant women are still based on a fixed cut-off since specific serum TSH reference ranges taking into account the main genetic contributions to individual variation which is always narrower than that of the general population.

Conclusions

A recent GWAS provided compelling evidence for a role of PDE8B gene (encoding a phosphodiesterase specifically catalyzing hydrolysis of cAMP) in the control of circulating TSH concentration and this finding has been reproduced in a cohort of pregnant women. This phenomenon is probably mediated through different degradation activities of intrathyroidal cAMP, leading to different thyroid hormone secretion rates. In keeping with this concept, other cAMP-specific phosphodiesterase genes (*PDE4D* and *PDE7B*) have also been found by GWAS associated to serum TSH levels. Further studies are needed to ascertain whether and to what extent *PDE8B* may also represent a candidate gene for thyroid dysfunction and/or response to treatment.

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EXPLORING THE UTILITY OF A POPULATION-BASED REGISTRY OF CONGENITAL HYPOTHYROIDISM (CH): THE MODEL OF THE ITALIAN NATIONAL REGISTRY OF INFANTS WITH CH¹A.Olivieri, ²E. Medda, ¹C. Fazzini and The Italian Study Group for Congenital Hypothyroidism*¹Department of Cell Biology and Neurosciences, Istituto Superiore di Sanità, Roma;²National Center of Epidemiology Surveillance and Health Promotion, Istituto Superiore di Sanità, Roma, Italy.

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ABSTRACT

A population-based Registry of congenital hypothyroidism (CH) has the potential to assess temporal and geographical trends of the disease incidence, to identify possible clusters of the disease, to characterize the affected population, and to promote development of *ad hoc* investigation of suspected exposures as possible causes of the disease. Moreover, by identifying critical points in screening program procedures it can contribute to develop recommendations to improve diagnosis, treatment, and follow-up of CH babies.

In Italy the neonatal screening program for CH is a complex system including screening, diagnosis, treatment, follow-up and nationwide surveillance of the disease. This is performed by the Italian National Registry of Infants with Congenital Hypothyroidism (INRICH). All the Italian Centres in charge of screening, diagnosis and follow-up of infants with CH take part in the INRICH. The aims of the Registry are to monitor efficiency and effectiveness of neonatal screening, to provide disease surveillance and to allow identification of possible aetiological risk factors for the disease. Therefore, the INRICH represents a useful epidemiological tool for surveillance of the disease and a powerful resource of information on CH babies. In fact, the results derived from epidemiological studies performed by using the INRICH data have contributed to deepen knowledge of CH, to start identifying the most important risk factors for the disease, and to orient molecular biologists towards the identification of new genes involved in the aetiology of CH, which still represents the most frequent endocrine disease in infancy.

Key-words: congenital hypothyroidism, neonatal screening, surveillance, population-based registry.

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Introduction

Congenital hypothyroidism (CH) is the most frequent congenital endocrine disorder and the leading cause of preventable mental retardation. This represents the most dramatic long term sequel of the disease. The etiologies include thyroid dysgenesis (agenesia, ectopia, hypoplasia), thyroid dyshormonogenesis, hypothalamic-pituitary deficiency (central hypothyroidism), and transient hypothyroidism. By measuring Thyroid Stimulating Hormone (TSH) or TSH + total thyroxine (T4) in dried blood spots in all babies shortly after birth, newborn screening programs are able to identify biochemically infants who may have CH even before there are any signs or symptoms of hypothyroidism. The eradication of mental retardation, the main objective of neonatal screening, has been achieved in industrialized countries where nationwide screening programmes have been established [1-3].

In Italy the nationwide newborn screening programme for CH began in 1977 and 100% coverage of neonatal population has been achieved since the 90's thanks to an efficient network of 26 regional and inter-regional Screening and Follow-up Centres. At present blood spot detection of TSH is used as primary screening test in 15 of the 26 Italian Screening Centers, while in the remaining Centers TSH+T4 screening strategy is used. In all the Centers positive results of screening are confirmed by definitive tests of thyroid function on serum (TSH, free T4 and/or T4). Thyroid ultrasound and/or scintigraphy are generally performed to complete the CH diagnosis. Infants with confirmed primary CH are then referred to the Follow-up Center of their own region for starting replacement therapy. According to available guidelines [1, 4], when the definitive diagnosis is not established in the neonatal period and a suspicion of transient primary hypothyroidism is present, a reevaluation of diagnosis is performed at the age of 3 years after a withdrawal of the replacement therapy to ascertain the persistence of CH.

The Italian National Registry of Infants with CH (INRICH)

In Italy the neonatal screening program for CH is a complex system including screening, diagnosis, treatment, follow-up and nationwide surveillance of the disease. This is performed by the Italian National Registry of Infants with Congenital Hypothyroidism (INRICH) in which all the Italian Centres

in charge of screening, diagnosis and follow-up of infants with CH take part (www.iss.it/rnic/). The Registry was established in 1987 as a program of the Health Ministry and is coordinated by the Istituto Superiore di Sanità (Italian National Institute of Health) [5-6]. The INRICH is a population-based Registry. This implies that results obtained in the analyses conducted on the data collected in the INRICH are highly representative, can be easily used to improve the health of CH children, and provide information critical to understanding the etiology of the disease. The aims of the Registry are: 1) to monitor efficiency and effectiveness of neonatal screening, 2) to provide disease surveillance, and 3) to allow identification of aetiological risk factors for CH.

Information on new cases with CH are collected in the INRICH by means specific questionnaires filled in at diagnosis. These include anonymous data concerning screening and confirmatory laboratory tests, demographic data, details on clinical state in neonatal period (included extra-thyroidal congenital malformations), diagnostic investigations, information regarding pregnancy, birth, and family background, starting and dose of the replacement therapy. It is important to note that babies with transient hyperthyrotropinemia on the basis of spontaneous normalization of TSH between screening and diagnosis are not recorded in the INRICH. The Screening Centres are responsible for collecting the questionnaires containing information from birth clinics and follow-up centers, for accuracy of their compilation, and for sending them to the INRICH. This system allows to optimize the flow of information toward the Registry by reducing sources of data concerning one CH baby. Data are coded and stored in an informed database at the Istituto Superiore di Sanità and results of data analyses are reported in a web site (www.iss.it/rnic/), presented in national and international conferences, and published in international scientific journals (FIG.1).

The INRICH surveillance activity

In the first years of the INRICH activity a strict surveillance of the Italian screening program allowed to identify some aspects which had to be improved. During the years the active and continuous collaboration between the Registry and the Italian Screening and Follow up Centres

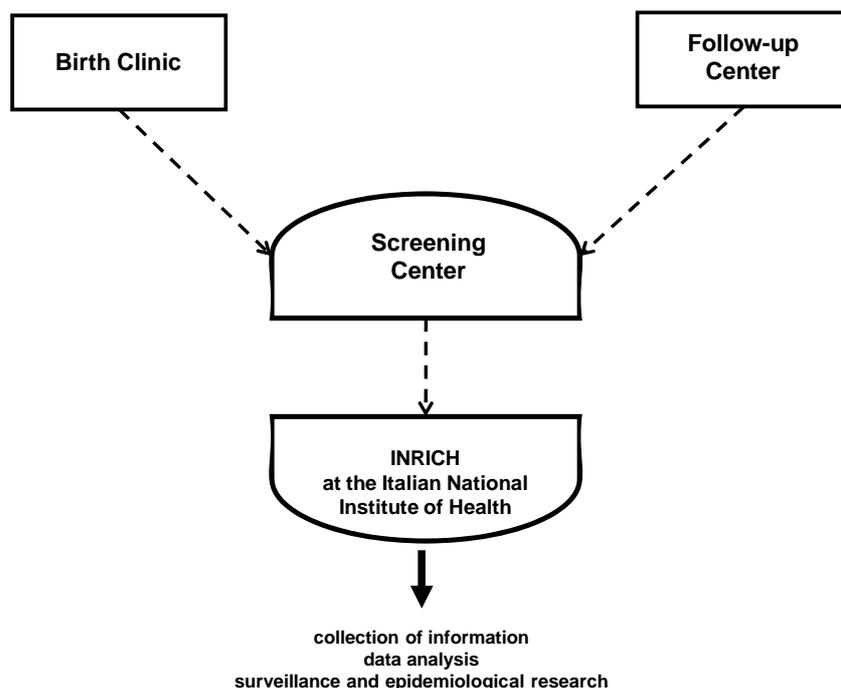


Fig.1. Flow of information on new cases with CH collected by the INRICH

has allowed to improve standardization of screening procedures with considerable improvements in terms of reduction of age at starting treatment and adequacy of dose of the replacement therapy (FIG.2). In fact, while the median value of infant's age at starting therapy was 23 days between 1987 and 1999, a reduction of this value was observed in the period 2000-2006 (19 days) although significant differences among diagnoses were observed: ectopia: 15 days; agenesis: 16 days; hypoplasia: 20 days; normal/hyperplastic thyroid: 23 days. Moreover, the INRICH data demonstrated a high efficiency of screening program also among babies with high risk of morbidity such as twins and babies with additional congenital malformations. In fact, no delay in time of starting therapy was found when twin CH babies were compared with singletons, as well as when CH babies with extra-thyroidal congenital anomalies were compared with infants with isolated CH [7-8].

According to recent studies and available guidelines [1,9], improvements have been also observed in dose of L-T4 at starting therapy. The INRICH data showed that the median value of L-T4 dose was 8.0 $\mu\text{g}/\text{Kg}/\text{day}$ between 1987 and 1999 and 9.6 $\mu\text{g}/\text{Kg}/\text{day}$ between 2000 and 2006.

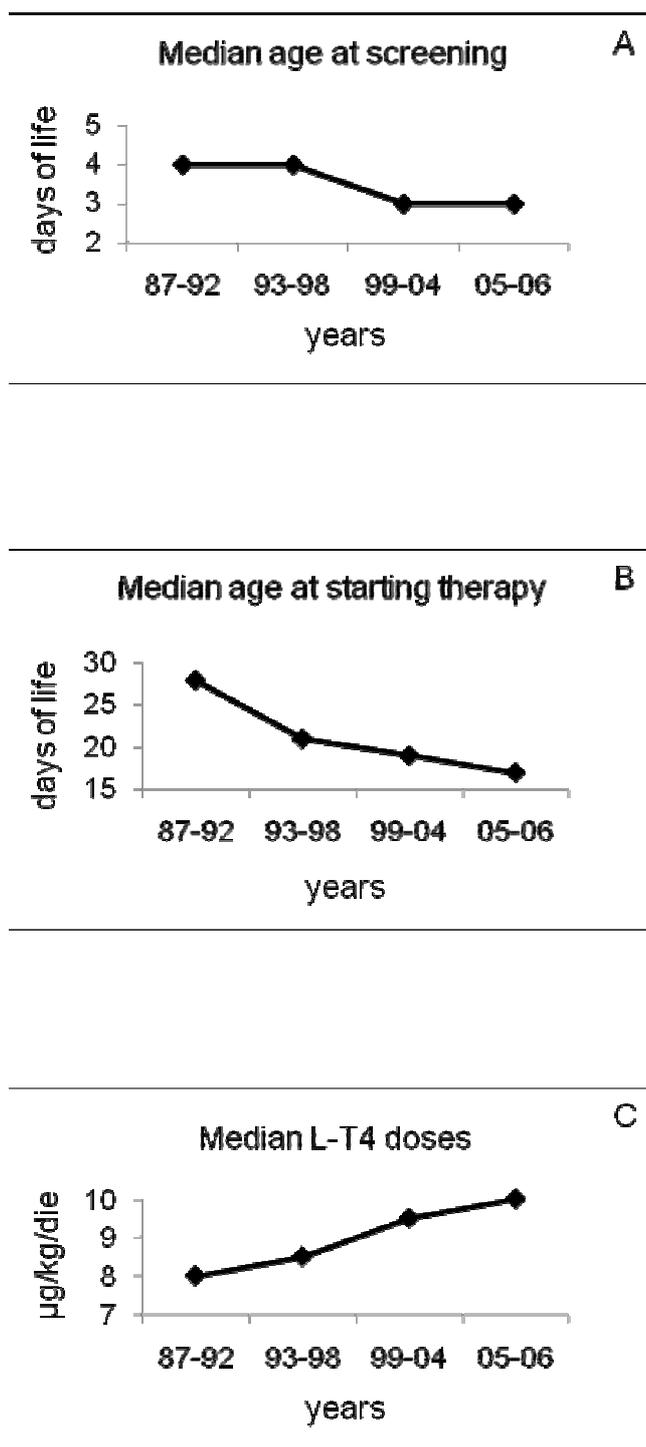


Fig.2. Graphs illustrating improvements in standardization of screening procedures obtained in Italy during twenty years of surveillance in terms of reduction of age at screening (panel A), reduction of age at starting therapy (panel B), increase of dose of replacement therapy (panel C).

Moreover, the INRICH data showed that in our country scintigraphy and/or ultrasonography was performed in 64% of CH babies before starting therapy. Among these babies 67% had thyroid dysgenesis, and 33% normal/hyperplastic thyroid (FIG.3) .

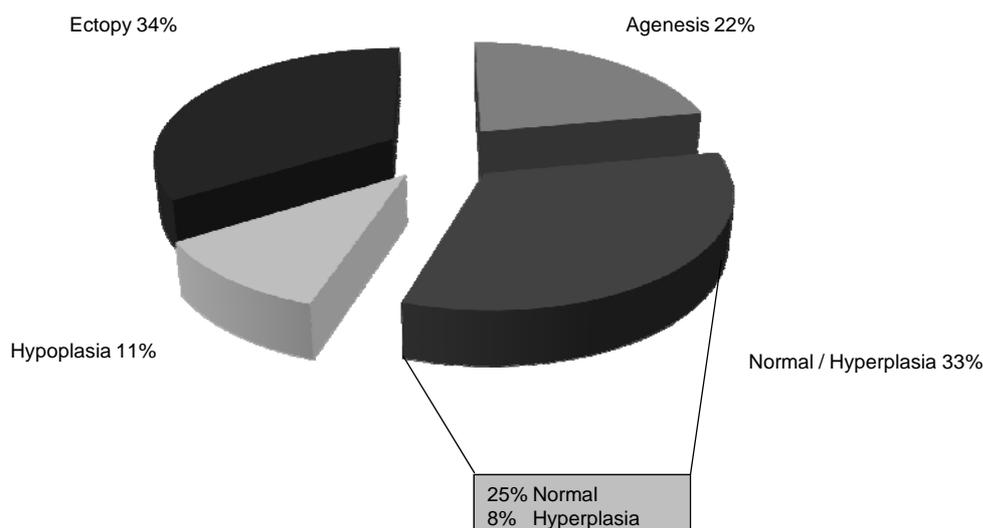


Fig.3. Thyroid scintigraphy and/or ultrasound results in the Italian population of babies with permanent CH.

As expected, the frequency of the disease is higher in female than in male babies with a F/M sex ratio = 1.7 (F/M=2.0 among babies with thyroid dysgenesis; F/M=1.0 among those with normal/hyperplastic thyroid). To ascertain the impact of CH on the Italian newborn population and to avoid the danger of drawing conclusions from an overestimation of the disease incidence, only cases with permanent forms of CH were considered while all the cases with transient hypothyroidism, ascertained by means a re-evaluation of the diagnosis after a withdrawal of the replacement therapy at 3 years of age, were not included in the incidence estimation. Indeed, to avoid the possibility of including some cases with transient hypothyroidism not re-evaluated yet, only children older than 3 years at the time of analysis were included in the evaluation. The estimated CH incidence in the period 2000-2005 was 1:2036 live borns. This value was higher than that observed previously (1987-99 = 1:2990) confirming an increasing trend of CH in our country. This is at least in part explained by a reduction of cut off value of TSH at screening, as a result of a continuous analysis of distribution of

TSH values in the screened neonatal population with a consequent more correct use of TSH threshold values [10].

The INRICH data have also shown a risk of CH occurrence 3-fold higher in twin than in single deliveries. Given the high number of CH babies recorded in the Registry, for the first time it was possible to estimate the CH incidence in multiple and single pregnancies separately. It was 10.1 per 10,000 live births in multiple deliveries and 3.2 per 10,000 live births in single deliveries [7]. Moreover, the analysis of re-evaluated infants with high suspicion of transient hypothyroidism recorded in the INRICH has also shown a twin prevalence of 1.9% among infants who resulted affected by permanent CH and 13.2% in those with final diagnosis of transient CH. Taken together these findings have demonstrated an increased risk for both permanent and transient CH in multiple than in single pregnancies. This finding has important implications in terms of public health given the high number of induced pregnancies, in Italy as well as in other Western countries, because of the increasing use of techniques of assisted reproduction and drugs inducing ovulation [11-12].

The INRICH research activity

It has been demonstrated that CH is a multigenic disease in mice [13]. Indeed, the identification of genes related to gland organogenesis or thyroid hormone biosynthesis has allowed the formulation of hypotheses on molecular mechanisms causing CH [14-15]. However, although still underestimated, the occurrence of mutations in genes known to be involved in the development of the disease have been observed only in a small proportion of the CH patients. Furthermore, the aetiological role of specific environmental risk factors has not completely elucidated yet. These considerations imply that the aetiology of CH is still largely unknown and that further efforts to identify new genetic markers and environmental (modifiable) risk factors are needed to allow an efficient primary prevention of the disease. As mentioned above, the large amount and the high quality of information collected in the INRICH during more than twenty years of activity provided a unique opportunity for research into this condition. This because data collected in the INRICH are highly representative as referred to the entire Italian population of infants with CH. One of the most important studies conducted on the basis of the INRICH data is represented by a population-based

case-control study performed with the aim to identify the most important risk factors for permanent and transient forms of CH [16]. This study showed that many risk factors contribute to the aetiology of CH suggesting a multifactorial origin in which genetic and environmental (especially iodine deficiency and maternal diabetes) risk factors play a role in the development of the disease. The multifactorial origin of CH was further supported by results obtained in the above mentioned study on CH twins recorded in the INRICH between 1989 and 2000 [7]. This study showed that, despite a low concordance rate (4.3%) for permanent CH observed among twins at birth, a higher recurrence risk for the disease was present among siblings of CH babies than in babies of the general population (35-fold higher). These findings strongly suggested the occurrence of non-inheritable post-zygotic events in the aetiology of CH and that environmental risk factors may act as a trigger on a susceptible genetic background in the aetiology of the disease.

The importance of a susceptible genetic background was also supported by another study conducted on data recorded in the INRICH. This demonstrated that not all congenital malformations but only congenital anomalies of heart, nervous system, eyes, and the occurrence of multiple congenital malformations are significantly associated to CH [8]. These results strongly suggested a very early impairment in the first stages of embryo development, probably involving genes regulating first stages of differentiation, with a consequent and simultaneous involvement of different organs and structures. These findings oriented molecular biologists to focus their investigations on genes involved in heart and thyroid development. This because cardiac anomalies represented the most frequent congenital malformations observed in CH population. It was found that mutations in NKX2.5 transcription factor, a gene specifically associated to atrial septal defects [17], can contribute to thyroid dysgenesis phenotype [18]. More recently, attention was focused on JAG1 a gene associated with the Alagille Syndrome, an autosomal multisystemic disorder characterized by variable defects of several organs (mainly liver, heart, eye, bones). A genetic variation of JAG1, already described in a patient with Alagille Syndrome, has been reported in patients with CH and heart developmental defects suggesting a potential involvement of this gene in the pathogenesis of CH [19].

CONCLUSIONS

Screening, surveillance and research are vital for the optimal management of babies with CH. The information required to improve diagnosis as well as to investigate aetiology of the disease require good clinical ascertainment with the smallest amount of selection bias. In this scenario a population-based Registry for CH represents a useful epidemiological tool for surveillance of the disease and a powerful resource of information on CH babies. This because such a Registry has the potential to assess temporal and geographical trends, to identify possible clusters of the disease, and to promote development of *ad hoc* investigation of suspected exposures. Moreover, by identifying critical points in screening programme procedures it can contribute to develop recommendations to improve diagnosis, treatment and follow-up of CH babies.

In Italy the surveillance of CH carried out by the INRICH together with the early diagnosis made by the nationwide screening programme, the prompt treatment and the appropriate clinical management of the patients performed by the Italian Follow-up Centres for CH, are elements of an integrated approach to CH which has been successfully established in our country. At the same time, the results derived from epidemiological studies performed by using the INRICH data have contributed to deepen knowledge of CH, to start identifying the most important risk factors for the disease, and to orient molecular biologists towards the identification of new genes involved in the aetiology of this disease. Finally, the potential of adding data on candidate genes involved in the CH aetiology to the INRICH database will no doubt represent a great breakthrough in the disease knowledge.

At present, further collaborative research studies based on the INRICH database are going on and our efforts in the field of CH risk factors identification are continuing with hope of making possible, in a near future, primary prevention of CH which still represents the most frequent endocrine disease in infancy.

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THYROID HORMONE RECEPTORS AND CANCER

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ABSTRACT

Thyroid hormone receptors (TRs) belong to the family of nuclear receptors and act as ligand dependent transcription factors. The ligand of TRs is 3,5,3'-triiodothyronine (T3). Recent reports showed that TRs and T3 are involved in carcinogenesis and influence processes of differentiation, proliferation, apoptosis, and metastasis. The supporting specific roles of TRs in tumorigenesis include common aberrations of TRs action in cancers and results of experiments on animal models. Although the majority of currently available data suggest the suppressive role of TRs in carcinogenesis, the fact that TRs are overexpressed in several tumor types suggests that they may also act as cancer promoting factors. This review discusses the mechanisms which are triggered by TRs to exert these two opposite roles of TRs in carcinogenesis.

Key-words: thyroid hormone receptors, mutations, cancer, tumorigenesis, metastasis, tumor suppressor, signaling pathways.

Thyroid hormone receptors: structure and function

Human thyroid hormone receptors are encoded by two genes, THRA and THRB, located in 17q11.2 and 3p24.2 chromosome regions, respectively. The primary transcripts of both genes undergo several alternative splicing events, producing multiple protein isoforms of the receptors that differ in amino-acid composition and biological properties. TRs share the common structure of the

nuclear receptors family with functional domains: A/B, C, D, E, and F (Fig.1). The C domain (or DBD, *DNA Binding Domain*) is responsible for binding of specific sites at T3 responsive genes, called Thyroid Hormone Response Elements (TREs). The E domain (LBD, *Ligand Binding Domain*) binds T3 and mediates interactions with coregulatory proteins. Both domains participate also in dimerization of the receptors. Heterodimerization with RXR (retinoid X receptor) is critical for activity of TRs.

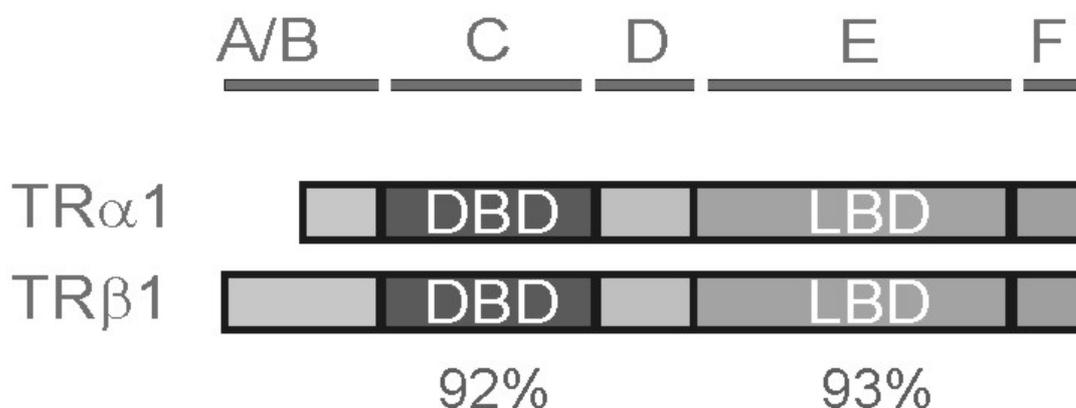


Fig. 1. Structure of thyroid hormone receptors. Two main isoforms (TR α 1 and TR β 1) are shown, coded by genes THRA and THRB. DBD: DNA binding domain; LBD: ligand binding domain. The percentage of conservation between domains of TR α 1 and TR β 1 is shown. The other TR isoforms were reviewed by Basset et al. (6).

The T3 responsive genes may be regulated in a positive or negative manner (1). The mechanism of positive regulation is mediated by positive TREs and depends on recruitment of coregulatory proteins that induce changes in chromatin structure, enabling transcriptional activation by T3-bound receptors or repression when T3 is absent. The T3-dependent repression of negatively regulated genes is less well understood. Currently several mechanisms are proposed, basing mainly on transient transfection studies. One of such mechanisms includes binding of negative TREs which act in an opposition to positive TREs. TRs may also negatively regulate the transcription without binding to DNA due to interference with other transcription factors and acting as specific traps or baits for coregulatory proteins (2).

TRs may also act in a nongenomic way (3). In this mode the TRs do not act as transcription factors but rather regulate activity of other proteins due to direct interactions. The detailed structure, function and mechanisms of TRs action have been extensively reviewed elsewhere (4 - 7).

A significant number of TRs regulated genes and proteins have been identified so far; many of them are important regulators of cellular proliferation, differentiation and apoptosis (8). Thus, it is not surprising that aberrations in functioning of TRs result in disturbances of cell physiology.

TRs and cancer

The first observations directly suggesting that TRs may be involved in carcinogenesis came from studies on v-erbA gene, encoded by the avian erythroblastosis virus and causing acute erythroleukemia and fibrosarcomas in chickens (9). TR α is the cellular homologue of v-ErbA protein, product of the viral gene (10, 11). Compared to TR α v-ErbA bears several amino-acid substitutions, partially located in DBD and LBD domains leading to loss of T3 binding and constitutive dominant negative effect to transcription activation by TRs (12). The oncogenic potential of v-erbA was proved in mice models developing hepatocellular carcinomas (13). Currently several mechanisms are proposed to explain the v-ErbA contribution to tumor formation. v-ErbA interferes with TRs due to competition for TRE or for TRs' coregulatory proteins such as RXR (14). v-ErbA may also mediate nuclear export of TRs preventing TRs mediated regulation of transcription in the nucleus (15). It was also suggested that oncogenic activity of v-ErbA may result from the ability to recognize a distinct set of target genes compared to the wild type receptors (16, 17).

The hypothesis that TRs may play a role in neoplastic transformation was supported by frequent aberrations of expression and mutations of TRs coding genes in human cancers (Table 1). Types of disturbances include aberrations in chromosomal regions of TR coding genes, epigenetic alterations, mutations, and changes in TRs expression level. The mutations are located mainly in LBD and DBD domains and result in significant disturbances of TRs action. Mutated TRs do not bind (or the binding is weakened) T3 and/or DNA; interactions with coregulators are also disturbed. Mutated TRs inhibit the activity of wild type receptors in a dominant-negative manner.

Table 1. Aberration of TRs found in human tumors. LOH: loss of heterozygosity.

Type of tumor	Type of aberration (numbers in brackets refer to number of patients with identified aberrations)	Effect of mutation on TRs function (if analyzed)	Refs.
Pituitary	TSH-oma	Mutation (2) and disturbed alternative splicing of TR β 2 (1); loss of TR α and TR β protein (1)	Loss of T3 binding 18-21
	Nonfunctioning pituitary macroadenoma	Mutations in cDNA of TR α (3); loss of TR α and TR β protein (20); lowered expression of TR β but not TR α mRNA (20)	-- 22, 23
Thyroid	Papillary thyroid cancer (PTC), follicular thyroid cancer (FTC), follicular thyroid adenoma (FTA)	Hypermethylation and LOH THRB (PTC: 10; FTC: 22; FTA: 5 FTA)	-- 24
	Papillary thyroid cancer	Disturbed expression of TR α 1 (mRNA: 21; protein: 18) and TR β 1(mRNA: 34; protein: 18) mutations in TR α 1 (11) and TR β 1 (16) cDNA	Disturbed binding of DNA 25-27
Nasopharyngeal carcinoma	Overexpression of TR α 2 mRNA (20)	--	28
Small cell lung cancer Non-small cell lung cancer	LOH of THRB (16) LOH of THRB (9)	--	29-31
Breast cancer	LOH of THRA (11) and THRB (32); amplification of THRA (12); hypermethylation of THRB (11), mutations (5) and disturbed alternative splicing (1) of TR β 1; disturbed expression of TR α 1 (mRNA: 8; protein: 15) and TR β 1 (mRNA: 6; protein: 15)	--	32-38
Hepatocellular carcinoma	Truncations (9) and mutations in TR α 1 (11) and TR β 1(13) cDNA; overexpression of TR β 1 (10); variable/increased expression of TR α 1 (16)	Impaired/loss of T3 and DNA binding; disturbed binding or release of transcriptional coregulators	39-42
Gastric cancer	Mutations in TR α 1 cDNA (19)	--	43
Colorectal carcinoma	Lowered expression of TR β 1 (mRNA: 8; protein: 36)	--	44, 45
Clear cell renal cell cancer	Mutations in TR α (3) and TR β (7) cDNAs; disturbed expression of TR α 1 (mRNA: 19; protein: 20) and TR β 1 (mRNA: 20, protein: 19)	Loss of T3 binding and/or impaired binding of DNA; disturbed binding/release of corepressors	46-48
Uveal melanoma	LOH of THRB (11)	--	49

Recent years brought significant advances in understanding the role of TRs in cancer development, tumor progression and metastasis reviewed in several excellent publications (50-54). Interestingly, it appears that TRs may act both as pro- and anti-cancerous factors. These opposite effects of TRs are discussed below.

TRs as cancer promoting factors.

Hepatocellular carcinoma is characterized by a high frequency of TRs alterations, including truncations and mutations in TR α 1 and TR β 1 cDNAs, resulting in aberrant binding of T3, DNA (39), and protein coregulators (40, 41). The mutated TRs display a target gene repertoire distinct from that of their normal TR progenitors (55). It was suggested that this “switch” in recognition of target genes could possibly be directly responsible for oncogenic potential of mutated TRs as well as v-ErbA in contrast to dominant-negative mutations of TRs found in inherited RTH syndrome (41). The other studies show, however, that not only mutations but also changes in expression of wild type TRs may contribute to tumorigenesis. For instance, elevated expression of TR β 1 protein was found in hepatoma cells, suggesting its promoting role in carcinogenesis. TR α was shown to enhance metastasis of human hepatoma due to upregulation of furin gene expression (56). Furin belongs to the family of proprotein convertases (PCs) that activate their substrates via limited proteolysis. The substrates of PCs family include several proteins involved in carcinogenesis; moreover, furin expression is associated with enhanced invasion and proliferation of several types of cancer (57). TRs positively regulate the expression of furin (56). Overexpression of TRs in hepatoma tumors leads to upregulation of furin and results in enhanced activity of matrix metalloproteinases (MMPs). MMPs catalyze degradation of extracellular matrix leading to increased tumor cell invasiveness. The effect of T3 is further enhanced by TGF- β (transforming growth factor beta), which induces signal pathway of MEK/ERK kinases. The latter enzymes catalyze the phosphorylation of TRs resulting in their stabilization (58) contributing to further stimulation of furin expression.

Another mechanism potentially contributing to pro-metastatic action of TRs in hepatoma is a negative regulation of antimetastatic gene Nm23-H1 (59). Nm23-H1 is a nucleoside diphosphate kinase (NDP) and a metastasis suppressor (60) whose lowered expression correlates with aggressive

behavior of different types of cancer. Lin et al. (59) found that expression of TR α 1 in HepG2 cell line results in repression of Nm23-H1 and leads to increased invasiveness of cells.

There is no entirely satisfying model describing the exact role of T3 and TRs in hepatoma, however. For instance, the same authors published conflicting results showing that T3 and TRs may also play antitumor role in hepatoma (61, 62). There are also other examples of antitumor activity of thyroid hormones in hepatocellular carcinoma, discussed further in detail below.

TRs as tumor suppressors.

Antitumorigenic properties of tumor suppressors are often reflected by the effects of mutations blocking their protective function. The proofs for the suppressive role of TRs in cancers include findings that mice expressing a dominant negative TR β mutant spontaneously develop thyroid and pituitary tumors (63, 64), and mouse TR knockouts (TRKO) show increased aggressiveness of skin tumors (65). The other arguments supporting the hypothesis are the loss of TR β 1 expression and multiple other alterations resulting in loss of function of TRs in cancer tissues (see Table 1).

Mouse models of TRs action in cancer.

The first *in vivo* evidence suggesting that THRB gene may act as a tumor suppressor came from knockin mutant mice harboring a PV mutation (63), originally identified in a patient with thyroid hormones resistance (RTH) (66). This mutation results in complete loss of T3 binding by TR β 1 and, in consequence, loss of ability to activate transcription by the receptor (67). Aging homozygous (TR $\beta^{PV/PV}$) mice spontaneously develop thyroid cancer (63), and TSH-secreting pituitary tumors (TSH-omas) (64), not observed in heterozygous or TRKO animals. Further studies on these mice revealed several mechanisms controlled by TR in cancer development, described in detail below.

The function of PV mutant is severely disturbed compared to the wild type receptor. These disturbances lead to activation of pro-proliferative signaling pathways and alterations in cell motility contributing to increased survival, invasive and metastatic properties of cancerous cells. These effect may be achieved via both non-genomic and genomic actions of the mutated receptor. At non-genomic level the PV receptor interacts with several cellular regulatory proteins interfering with important

signaling pathways. One of such regulators is phosphatidylinositol 3-kinase (PI3K), involved in a wide group of processes essential for survival of the cell, such as metabolism, growth, and motility (68). PI3K catalyses phosphorylation of phosphatidylinositol-4,5-bisphosphate (PIP2) to form phosphatidylinositol-3,4,5-triphosphate (PIP3), a potent activator of several protein regulators. TR β 1 binds p85 α subunit of PI3K (69); the PV mutation, however, strengthens these interactions and leads to intensified activation of PI3K downstream pathways including AKT–mTOR–p70^{S6k}. Since p70^{S6k} is known to promote cell growth, cell cycle progression and to act as an anti-apoptotic factor (70 - 72) the PV initiated activation of AKT–mTOR–p70^{S6k} may result in progression of tumorigenesis.

The PV-induced activation of PI3K results in activation of ILK (integrin-linked kinase), and its downstream target, metalloproteinase 2 (MMP-2) (69), involved in degradation of extracellular matrix and playing a pivotal role in cell invasion and metastasis (73). Since another substrate of ILK is AKT (74), the downstream pathways of PV activated PI3K may cross-talk with each other, leading to the final activation of proliferation.

Another mechanism contributing to increased metastatic potential of thyroid cancer in TR β ^{PV/PV} mice is interaction of TR β 1 with gelsolin (75), an actin binding protein involved in cytoskeleton organization (76). Compared to the wild type receptor, the PV mutant disturbs interaction between gelsolin and actin, resulting in improper properties of cytoskeleton. As a consequence, the motility of cancer cells is increased, contributing to metastatic potential of thyroid cancer (75).

The abnormalities commonly found in thyroid carcinomas include chromosomal aberrations (77-80). Ying et al. (81) found that improper action of the mutated TR β 1 may contribute to these alterations due to its interactions with PTTG1 protein. PTTG1 (pituitary tumor–transforming 1), called also securin, is a multifunctional protein involved in cell division and DNA repair mechanisms (82, 83). One of the important functions of PTTG1 is to ensure proper sister chromatid separation during mitotic cell division. During metaphase, sister chromatids are held together by a multiprotein complex called cohesin. In anaphase, protein elements of cohesin complex are cleaved by protease called separase and thus allow for cohesin dissociation from chromosomes. During other phases of the cell cycle, separase is trapped by PTTG1 that inhibits its activity. At the metaphase to anaphase transition, PTTG1 is degraded, leading to release of separase and destruction of cohesin complex

(83). Ying et al. (81) found that TR β 1 binds PTTG1 and regulates its degradation. T3 binding by TR β 1-PTTG1 complex induces interaction with steroid hormone receptor coactivator 3 (SRC-3) and binding of the latter with proteasome activator 28 γ (PA28 γ). This results in proteasome - mediated degradation of TR β 1-bound PTTG1. The PV mutant, lacking ability of binding T3, does not form complexes with SRC-3/PA28 γ and inhibits PTTG1 degradation. PTTG1 overexpression results in enhanced proliferation of hepatoma Hep3B cells and wild type TRs negatively regulate PTTG1 expression via Sp1 transcription factor (61). Concomitant observation that the expression of TRs in human hepatocellular carcinoma is reduced along with elevated Sp1 and PTTG1 suggests that TRs may play antitumorigenic role in human hepatoma. The same group, however, reported the opposite effect of TRs, suggesting their prometastatic activity (discussed above, page 5). The conflicting results of studies of the role of TRs in hepatoma are discussed on page 11.

TR β 1 regulates the stability of β -catenin, a multifunctional protein whose disturbed expression was observed in different types of cancer (84). Acting as one of the elements of Wnt signalling pathway, β -catenin functions as transcription factor controlling expression of a wide group of genes involved in processes of proliferation, migration and survival. Action of β -catenin is regulated by phosphorylation dependent ubiquitination and degradation. Inhibition of β -catenin phosphorylation leads to its cellular accumulation and activation of transcription of downstream genes. Guigon et al., (85) found that TR β 1 interacts with β -catenin in a T3 dependent manner. T3 binding by TR β 1 leads to release of β -catenin that can be directed for degradation. The PV mutant binds β -catenin, but since it does not bind T3, the dissociation is blocked. This prevents β -catenin from degradation and leads to its accumulation and constitutive activation of β -catenin signaling pathway. In consequence, the expression of β -catenin regulated genes, such as c-myc oncogene, cyclin D1, and metalloproteinases is changed in thyroid cancers of TR $\beta^{PV/PV}$ mice.

PV mutants exert their effects also at genomic level. A consequence of the PV mutation is lowered expression of PPAR γ receptor in thyroid cancers of TR $\beta^{PV/PV}$ mice (86). The PV mutant interferes also with transcriptional activity of PPAR γ receptor. Both TR β 1 and PV bind to PPRE (PPAR γ response elements) as heterodimers with RXR (retinoid X receptor) and compete with binding of PPAR γ /RXR. This competition leads to repression of transcription of PPRE regulated

reporter gene. T3 presence leads to derepression mediated by TR β 1/RXR complex. The PV mutant, however, lacks the ability of binding T3, therefore the PV/RXR constitutively represses expression of PPRE regulated genes. Since the activation of PPAR γ mediated expression is known to exert antiproliferative effect (87), loss of PPAR γ activity due to interference with mutated TR β 1 receptor may contribute to tumorigenesis of thyroid cancer in TR $\beta^{PV/PV}$ mice.

Specific effects of PV mutants are also observed in pituitary tumors secreting TSH (TSH-omas) of TR $\beta^{PV/PV}$ mice. Furumoto et al., (64) found that TR β 1 negatively regulates the expression of cyclin D1 due to interference with CREB mediated activation of transcription. CREB (CRE binding protein) is a transcription factor binding CREs (cyclic AMP response elements) located in promoters of numerous genes (88). CRE- bound CREB interacts physically with both PV and wild type TR β 1 (64, 89). In the presence of T3, TR β 1-CREB interaction is stronger, and so is the repression of transcription. PV-mediated repression of transcription is not possible, leading to constant stimulation of cyclin D1 expression. Another mechanism contributing to cyclin D1 upregulation in pituitary tumors of TR $\beta^{PV/PV}$ mice is activation of AKT with consecutive phosphorylation of GSK-3 β and inhibition of kinase activity of the latter, preventing from phosphorylation of cyclin D1 and its proteasomal degradation (90). This leads to activation of cyclin-dependent kinases (CDK4 and 6) and hyperphosphorylation of retinoblastoma protein (Rb) (64). Since TR β 1 mutations, as well as disturbances in expression of cyclin D1 are found in pituitary tumors (18-21, 91), the murine model of TR β 1 role in pituitary tumors sounds very plausible.

The studies on mutant mice raised the question whether the observed tumorigenesis results from loss-of-function or gain-of-function of PV mutation. Recent work by Zhu et al. (92) addressed this problem. They showed that the mice devoid of all known functional TRs (TR α 1 $^{-/-}$ /TR β 1 $^{-/-}$) spontaneously develop follicular thyroid cancer as they age. This study provides direct evidence that TRs in mice could function as tumor suppressors in vivo.

Anticancer effects of TRs in human cells.

Apart from mouse models, important information on the role of TRs in cancer comes from investigations on human cancer tissues and cell lines. The vast majority of studies on antitumoral

activity of TRs are focused on TR β 1 isoform. This could suggest that among the two isoforms TR β 1 is the one that controls expression of protein regulators engaged in majority of pathways contributing to protection against cancer. These different biological roles of TRs isoforms could possibly result from the ability to regulate different groups of genes. Chan and Privalsky (93) showed, however, that in fact the set of genes regulated by TR α 1 and TR β 1 overlap in HepG2 cells. Moreover, it seems that different functional properties of the two receptors result rather from isoform-specific range of T3-mediated transcriptional regulation. Thus, different biological functions of TRs result probably from subtle changes in expression of TRs regulated genes. This is in agreement with the hypothesis of procancerous effects of disturbed expression of TRs.

Aberrantly expressed TRs may lead to deregulation of proteins controlling cell cycle, such as E2F1 (94). Clear cell renal cell carcinoma is characterized by the presence of mutations and disturbances of expression of both TRs (46, 47). The mutations lead to severe impairments in TRs function, including disturbances in binding of T3, DNA and coregulatory proteins (46, 48). E2F1 is a transcription factor controlling G1 to S phase transition whose expression is negatively regulated by TRs (95). The expression of E2F1 is increased in renal cancer tissues, what is in concomitance with disturbed function of TRs. Since E2F1 is an important regulator of cellular proliferation (96, 97) its disturbed expression may potentially contribute to renal tumorigenesis. In addition, T3 differently affects proliferation of normal and cancerous kidney cells (98). While T3 treatment of cancerous cells stimulates G1 to S phase progression, its effect in normal kidney cells is opposite, leading to inhibition of proliferation. This effect is mediated by different T3-regulated expression of key cell cycle regulators (E2F4, E2F5, p107 and p130).

Yen et. al (62) found that T3 inhibits proliferation of hepatoma HepG2-TR cell line (Fig. 2A.). In these cells T3 stimulates the activity of promoter and expression of TGF- β . Upregulation of TGF- β results in repression of cell cycle regulating proteins: cdk2, cyclin E and ppRb (hyperphosphorylated retinoblastoma protein), and contributes to inhibition of cell proliferation. As discussed earlier, studies investigating the role of thyroid hormone in hepatoma generated conflicting results and the same group showed also that T3 may promote metastasis of hepatoma (56, 59). In opposition to this, Chan and Privalsky (41) found that while ectopic expression of TR α 1 in HepG2 cell line inhibits anchorage

independent growth, the mutated TR α 1 does not exert this effect. The antitumor role of T3 in liver cancers is also supported by the observation that hypothyroidism is a possible risk factor for hepatocellular carcinoma in patients with no known underlying cause of liver disease (99). This is in agreement with experiments in rats showing that although T3 treatment induces liver proliferation, it also leads to loss of hepatocellular carcinoma nodules, possibly due to redifferentiation of nodular hepatocytes (100). In both studies the specific role of TRs was not analyzed, however, therefore it is difficult to get the clear picture of their antitumor activity in these models. The conflicting results of studies in hepatoma suggest that the final role of TRs in liver cancer may depend on the molecular context within tumor cell and cooperation with other cellular regulators. It is known that tumor microenvironment may influence the activity of tumor suppressors to result in switch into their oncogenic function (101). Interestingly, one of the TR β 1 interacting partners, TGF β , exerts such a dual tumor suppressive-or oncogenic character (102). Therefore, further studies are needed focused on the influence of tumor microenvironment and molecular context on TRs function in hepatic cancer.

TR β 1 mediates inhibition of Na2- β neuroblastoma cell line proliferation and induction of morphological differentiation by an arrest in G0/G1 (103). TR expression leads also to the ras-oncogene mediated suppression of tumor formation *in vivo* in nude mice (104) (Fig. 2B.). The expression of Ha-ras^{val12} oncogene activates transcription of cyclin D1 via kinase Rsk2 mediated mechanism, leading to enhanced proliferation. T3 inhibits Rsk2 activity, expression of both TR α 1 and TR β 1 and reduces transforming ability of ras oncogene. TR β 1, however, exerts stronger antiproliferative effect and is able to inhibit ras-mediated transformation even in the absence of T3 and to totally abolish tumor formation by ras-transformed cells in nude mice.

One of the few examples showing antitumor activity of TR α 1 was published by Lee et al. (105). In this study induced expression of TR α 1 in nasopharyngeal carcinoma cells reduced proliferation, colony-formation ability in agar, and tumor formation ability in nude mice.

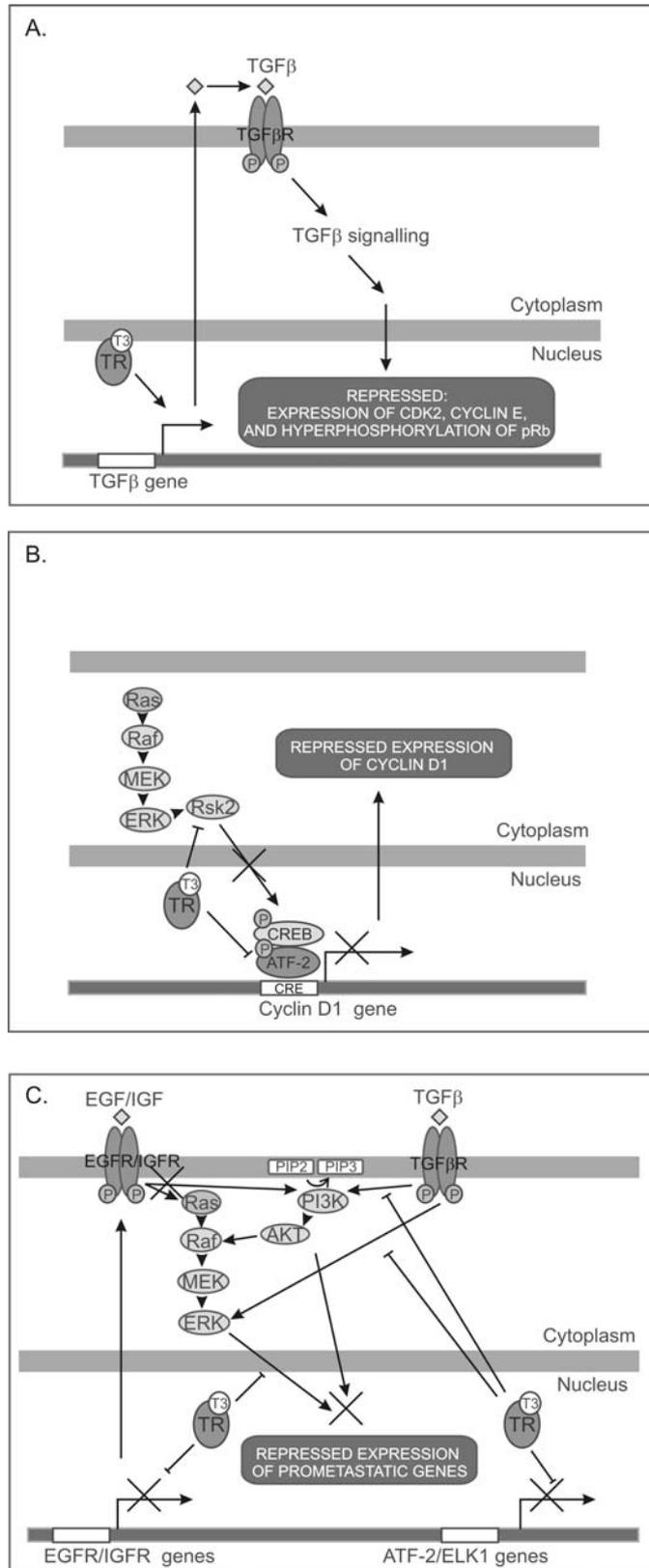


Fig. 2. TRs as anticancer factors. A. Antiproliferative effect of TR/T3 stimulated expression of TGFβ in hepatoma cell line. B. TR-mediated inhibition of ras-induced proliferation in neuroblastoma cell line. C. TR antimetastatic action in hepatocarcinoma and breast cancer cell lines. See text for details.

The ultimate evidence that TR β 1 may act as a potent tumor suppressor of tumor invasiveness and metastasis in hepatocarcinoma and breast cancer came from Martinez-Iglesias et al. (65). They showed that re-expression of TR β 1 in cells that have lost TR expression leads to tumor growth retardation, partial mesenchymal to epithelial transition and strongly suppresses invasiveness, extravasation and metastasis formation in nude mice. TR β 1 expression suppresses expression of IGFR and EGFR, the receptors of key growth factors activating signaling pathways of ERK and PI3K. TR β 1 also reduces expression of other downstream elements of these signaling pathways, and blocks TGF- β dependent activation of MAPK and PI3K. In consequence, the expression of genes involved in metastatic growth is repressed (65). Interestingly, experiments in nude mice suggest that TRs play diverse roles at different stages of tumorigenesis. TRs deficiency inhibits benign tumor formation at early stages of carcinogenesis but increases malignant transformation at later stages (65). The same group showed also that hypothyroidism in mice affects invasiveness and formation of metastasis independently of the cellular expression of TR β 1 (106). The thyroidal state has dual effect on tumorigenesis. Hypothyroidism retards growth of tumor but results in its enhanced aggressiveness, invasiveness, and metastasis formation. This is observed both in cells that express and do not express TR β 1, suggesting that the role of thyroidal status in tumorigenesis is much more substantial than direct TR β 1 mediated action on tumor cells.

Conclusions and future perspectives.

The role of TRs in cancer appears to be complex. Apart from strong evidence for antitumorigenic actions the receptors may also trigger pro-metastatic mechanisms. These effects of TRs possibly result from tissue-type and cancer-stage specific cross-talks with elements of cellular signaling cascades such as PI3K/AKT/mTOR or MAPK. Studies providing information that TRs may act as antitumor factors yield basis for hypothesis that TRs may be an interesting target for therapy. This has been recently tested by Perra et al. (107) who showed that T3 and a selective TR β agonist causes regression of hepatocellular carcinoma nodules in rats.

In conclusion, although multiple mechanisms of TRs action in cancers have been identified, future studies are needed to explain the complexity of interactions deciding on antiproliferative or prometastatic actions of TRs.

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ADIPONECTIN AND THYROID

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ABSTRACT

Available experimental data suggest that adiponectin and thyroid hormone share some biological effects and that may interact each other. Adiponectin may influence thyroid hormone production through interaction with gC1q receptor, whereas changes in the pituitary-thyroid axis may alter serum adiponectin levels through different mechanisms. Thyroid hypofunction in animals and humans did not modify serum adiponectin in most of the studies reported. However, data from experimental hyperthyroidism in animals and from clinical studies in patients with thyroid hyperfunction suggest that excess of thyroid hormone is accompanied by an elevation in circulating adiponectin. A positive association between adiponectin and thyrotropin receptor antibodies has been found in some studies, and it has been speculated that stimulation of these receptors could increase adiponectin production. Although some of these results are encouraging, the participation of adiponectin in pathogenesis of thyroid disorders is merely speculative.

Key-words: Adiponectin, thyroid hormone, hypothyroidism, hyperthyroidism, autoimmune thyroid disease

INTRODUCTION

Adipose tissue is the source of a variety of biologically active molecules, including cytokines, growth factors, complements factors, enzymes, and hormones (1). These adipocyte products, acting in autocrine, paracrine and endocrine ways, are capable of influencing not only local adipocyte physiology, but also the function of different organ systems. Among them, adiponectin is a protein specifically produced by the adipose tissue and released in high quantity into the bloodstream, accounting for up to 0.01% of total plasma protein in humans (2). As opposed to other adipocytokines, such as leptin, serum concentrations of adiponectin are decreased in states associated to insulin resistance, such as obesity and type 2 diabetes. Several lines of evidence suggest that adiponectin has a protective role against diabetes and atherosclerosis, and may behave as a cardioprotective factor.

On the other hand, thyroid hormone has profound effects of lipid metabolism and carbohydrate homeostasis (3-5). Abnormalities in serum lipids and lipoproteins are frequent findings in thyroid dysfunction, mainly in hypothyroidism. Impaired glucose tolerance and insulin resistance have been documented in patients with thyroid dysfunction. In addition, thyroid hormones are remarkable regulators of energy metabolism, being the adipose tissue the largest fuel storage compartment (6). Furthermore, thyroid hormones share some physiological actions with adiponectin, such as reduction of body fat by increasing thermogenesis and lipid oxidation (7). Hence, the interest on the relationships between adiponectin and thyroid hormones physiology (8). In this review we present an overview of participation of adiponectin in thyroid homeostasis and thyroid disease, as well as the role of thyroid hormones in adiponectin production.

ADIPONECTIN

Adiponectin, the product of the *apM1* gene, is a 244 amino acid protein, which is specifically and highly expressed in human adipose cells (9-12). This 30 kDa protein contains an N-terminal signal sequence, a short variable domain, a collagen-like domain, and a C1q-like globular domain at the C-terminal end (9-12). It belongs to the soluble defence collagen superfamily and has structural homology to collagen VIII and X and complement factor C1q. Posttranslational hydroxylation and glycosylation give rise to multiples isoforms of adiponectin (13). In plasma, adiponectin circulates in

trimeric, hexameric and high molecular weight (HMW) complexes. The globular domain of adiponectin, a proteolytic cleavage fragment, also circulates and has biological activity (14).

Serum adiponectin concentrations seem to be gender dependent being higher among women than men (15-18). Adiponectin and age are positively associated in both diabetic (19) and nondiabetic persons (16).

ADIPONECTIN RECEPTORS

Two adiponectin transmembrane receptors (AdipoR) have been cloned. These receptors contain seven transmembrane domains, but are structurally and functionally distinct from G protein-coupled receptors. AdipoR1 is expressed abundantly in skeletal muscle and has a preference for binding to globular adiponectin, while AdipoR2 is distributed mainly in liver and has intermediate affinity for both globular and full-length adiponectin (20,21). AdipoR1 acts through the AMP-dependent protein kinase (AMP kinase) signalling pathway, whereas AdipoR2 is associated with the peroxisome proliferator activator receptor (PPAR)- α pathway (22). Recent data from experimental investigation suggest that AdipoR1 and AdipoR2 deficiencies give rise to opposite effects on energy expenditure and spontaneous locomotor activity. In fact, AdipoR1^{-/-} mice showed increased adiposity associated with decreased glucose tolerance, spontaneous locomotor activity, and energy expenditure. In contrast, AdipoR2^{-/-} mice were lean and resistant to high-fat diet-induced obesity and showed improved glucose tolerance and reduced plasma cholesterol levels (23). Whether additional receptors can mediate these or other biological effects of adiponectin is still a matter of debate.

Furthermore, adiponectin may act by the expansion of subcutaneous adipose tissue with decreased levels of macrophage infiltration, similar to the action of PPAR γ agonists (24). Adiponectin is also capable to activate several other signalling pathways such as the production of nitric oxide through phosphatidylinositol-3-kinase-dependent mechanisms (25).

EFFECTS OF ADIPONECTIN

A great number of experimental investigations have shown that adiponectin increases insulin sensitivity and has noteworthy antiatherogenic and anti-inflammatory properties. Adiponectin knockout animals have shown a tendency to develop diet-induced glucose intolerance, insulin resistance (26,27), neointimal thickening, increase in vascular smooth muscle cells proliferation (26,28), and a higher expression and plasma concentrations of tumor necrosis factor- α (TNF α) (27).

In animal models, adiponectin induces the stimulation of glucose uptake in muscle, fatty acid oxidation in muscle and liver, and the inhibition of hepatic glucose production, cholesterol and triglyceride synthesis, and lipogenesis (14,29-31). Increased free fatty acid oxidation in muscle is mediated, at least in part, by increased expression of genes encoding CD36, acyl CoA oxidase, and UCP2, which enhance free fatty acid oxidation, fat combustion, and dissipation, respectively (31). Free fatty acid liver uptake also decreases and this might lead to decreased hepatic triglyceride content, which improves hepatic insulin sensitivity and reduces glucose output and blood glucose levels.

Adiponectin also inhibits monocyte adhesion to endothelial cells, macrophage transformation into foam cells, and endothelial cell activation (32,33). These actions are closely related to the inhibitory effect of adiponectin on TNF α -induced expression of several adhesion molecules on the surface of endothelial cells, including vascular cell adhesion molecule-1 (VCAM-1), E-selectin, and intercellular adhesion molecule-1, and on the effect of TNF α to induce the adhesion of monocytes cells to endothelial cells (32,34,35). Adiponectin also suppresses TNF α -induced inflammatory changes in endothelial cells by blocking inhibitory nuclear factor- κ B phosphorylation and nuclear factor- κ B activation without affecting TNF α -mediated activation of c-Jun N-terminal kinase, p38 and Akt (36). Furthermore, adiponectin also suppresses leukocyte colony formation, reduces phagocytic activity and decreases TNF α production from macrophages (34,35).

Adiponectin supplementation in mice not only leads to improved insulin sensitivity, and to inhibition of inflammatory processes above mentioned, but also exerts noteworthy antiatherogenic

effects within the vascular wall. This cytokine attenuates proliferation of vascular smooth muscle cells in response to a variety of growth factors and migration induced by heparin-binding-epidermal growth factor or platelet-derived growth factor-BB (28,37). In *in vivo* studies adiponectin has been reported to accumulate in the injured vessel wall and suppress the development of atherosclerosis in apolipoprotein E-deficient susceptible mice (38). This effect was associated with suppression of the expression of VCAM-1 and class A scavenger receptors (38). Other putative mechanism of the beneficial effect of adiponectin on the vasculature relates to the endothelial nitric oxide (NO) generation. In fact, concentrations of adiponectin similar to those found in serum have been shown to enhance NO production in cultured aortic endothelial cells (25,33). In endothelial cells treated with oxidized low density lipoproteins (LDL), adiponectin inhibited cell proliferation as well as basal and oxidized LDL-induced release of superoxide, and increased NO production by ameliorating the suppression of endothelial NO synthase (eNOS) activity by oxidized LDL (39). Furthermore, adiponectin also exerts inhibitory effects on thrombus formation and platelet aggregation in mouse models (40).

Adiponectin-induced AMP kinase activation may be a potential link between adiponectin and vascular effects of this adipokine (25,41). In fact, AMP kinase activates eNOS in endothelial cells and also ameliorates the increased apoptosis observed in endothelial cells exposed to high glucose, suggesting that this enzyme may mediate endothelial cell growth and differentiation responses (42). Other potential signalling pathways for adiponectin actions in endothelial cells include the activation of Akt, which is linked upstream to phosphatidylinositol 3'-kinase (PI-3K) signalling (25,41). Recently, a stimulating effect of adiponectin on angiogenesis by promoting cross-talk between AMP kinase and Akt signalling has been reported in endothelial cells (41).

INFLUENCE OF ADIPONECTIN IN THYROID HORMONE HOMEOSTASIS

Adiponectin might participate in the regulation of thyroid hormone production. The C-terminal globular structure of adiponectin can use the gC1q receptor, a molecule with broad tissue distribution that includes liver, smooth muscle, endothelium, immune cells and thyroid (43). Some authors have suggested that adiponectin, via this receptor found in the mitochondria of the thyroid cells, may be a regulator of thyroid hormone production (9,44).

In agreement with this hypothesis, human studies have shown that healthy subjects with high adiponectin levels had higher serum free T4 levels (45), and free T4 was found to be a predictive variable for adiponectin concentrations in humans. However, discordant results have been obtained by other investigators. The recent study by Malyszko et al. (46), performed in healthy subjects and patients with chronic kidney disease, but without a history of thyroid diseases, reported a negative correlation between adiponectin and free T3 both in healthy subjects and in patients with chronic renal failure and with kidney allograft (46). On the other hand, in hemodialysis patients adiponectin correlated negatively with free thyroxine (T4) and positively with thyrotropin (TSH). Multiple regression analysis in this study showed that, in kidney transplant recipients, adiponectin was independently related to free triiodothyronine (T3), whereas in hemodialysis patients adiponectin was independently related to free T4 (46). Lastly, in a study performed in severe obese women, TSH was inversely related with adiponectin concentrations (47).

INFLUENCE OF THYROID HORMONES IN ADIPONECTIN PRODUCTION

Rat adipose tissue has been found to express TSH receptor mRNA in amounts approaching those in the thyroid. The function of TSH receptor in rats seems to be indistinguishable from that in the thyroid and some authors have suggested that the interaction of autoantibodies with this TSH receptor plays an important role in the pathogenesis of the extrathyroidal manifestation of Graves' disease (48). Therefore, it has been suggested that, in patients with autoimmune hyperthyroidism, TSH receptor antibodies may cross-react with TSH receptor in adipose tissue to affect adiponectin production.

This finding suggests that changes in the pituitary-thyroid axis may alter serum adipocytokine levels, including adiponectin. Fujimoto et al. (49) found that, in cultures of brown adipose tissue, thyroid hormone presented a small stimulatory effect on adiponectin messenger RNA expression and on hormone secretion. However, T3 treatment did not have any effect on adiponectin gene expression in 3T3-L1 adipocytes (50).

On the other hand, thyroid hormone might stimulate adiponectin production through the PPAR pathway. It has been reported that thyroid hormone can induce the expression of PPAR γ in

hepatocytes (51), and PPAR γ stimulation increases serum adiponectin by transcriptional induction in adipose tissue because there is a functional PPAR-responsive element in human adiponectin promoter (52). It has also been shown that, in hepatocytes, thyroid hormone stimulates an increase in the mature sterol regulatory element-binding protein-1 (SREBP-1), a protein that binds the promoter regions of several lipogenic genes (53), and that the adipocyte determination- and differentiation-dependent factor 1/sterol regulatory element-binding protein 1c transcription factor (ADD1/SREBP1c) controls adiponectin gene expression in differentiated adipocytes (54). Therefore, it is likely that thyroid hormone increases transcriptional induction of adiponectin through PPAR γ or SREBP stimulation.

ADIPONECTIN AND HYPOTHYROIDISM

Hypothyroidism is accompanied by reduction in oxygen consumption, heat production and basal metabolic rate (55). A reduction in lipolysis and an increase in serum lipids are also characteristics of thyroid hormone deficiency (56). In rats with propylthiouracil-induced hypothyroidism serum adiponectin levels were significantly increased as compared to untreated rats (57). However, experimental hypothyroidism induced by methimazole treatment in rats was not accompanied by any significant change in serum adiponectin concentrations (58).

Serum adiponectin concentrations have been studied in patients with hypothyroidism before and after normalization of thyroid hormone levels with levothyroxine therapy. Most of the authors have reported that adiponectin levels remain unmodified in patients with thyroid hypofunction in comparison with euthyroid subjects (59-65). However, a few number of studies have found low adiponectin levels in hypothyroid subjects (44,66). A summary of clinical studies on adiponectin levels in hypothyroid patients is shown in Table 1.

In a group of 20 hypothyroid subjects we found that restoration of normal thyroid hormone levels after levothyroxine therapy was not accompanied by significant changes in circulating adiponectin levels (59). This finding was confirmed in a further study (61). Studies in humans have also demonstrated that the negative correlation between adiponectin and body fat mass estimated by BMI, and between adiponectin and HOMA-IR, an index of insulin resistance, is maintained in

hypothyroid patients (64). The positive association between adiponectin and high density lipoprotein (HDL)-cholesterol also persisted in hypothyroid patients (61,64).

Table 1. Serum adiponectin concentrations in patients with hypothyroidism, according to results reported in clinical studies

Number of patients	Sex (M/F)	Etiology	Control group	Serum adiponectin		Ref.
				Before	After	
20	3/17	AIT, 7; CAT, 5 RIT, 7; O, 7	Euthyroid	No change	No change	58
15	4/11	T4W	Euthyroid	No change	—	59
32	5/27	RIT	Hyperthyroid	Decreased	—	65
52	9/43	NR	Euthyroid	No change	No change (n=36)	60
22	0/22	T4W	Euthyroid	No change	—	61
23	5/18	AIT	Euthyroid	No change	—	62
67	28/39	AIT, 60; POH, 7	Euthyroid	No change	—	63
53	N.R.	N.R.	Euthyroid	Decreased	Decreased	43
27	0/27	AIT, 16; RIT, 6 T4W, 3; O, 2	Euthyroid	No change	No change	64

Abbreviations: —, no data; AIT, autoimmune thyroiditis; CAT, chronic atrophic thyroiditis; NR, not reported; O, other etiologies; POH, postoperative hypothyroidism; RIT, radioiodine therapy

ADIPONECTIN AND HYPERTHYROIDISM

Thyroid hormone excess is associated with weight loss, reduction in fat mass, depletion in lipid storage and reduction of some serum lipids (4,67). Glucose intolerance and insulin resistance are also frequent findings in patients with thyrotoxicosis (68).

Data from animal investigation showed that serum adiponectin levels in levothyroxine-treated rats were 3.2-fold higher than that of euthyroid ones (58). In rats, adiponectin concentrations correlated positively with serum T4 and T3 and negatively with TSH. Furthermore, there was a negative correlation between serum adiponectin levels and visceral white adipose mass, which was reduced in hyperthyroid animals. A positive association between serum adiponectin levels and brown adipose tissue mass was also found. (58). The elevation of serum adiponectin levels in rats with experimental hyperthyroidism was not accompanied by changes in baseline serum insulin, blood glucose concentrations or glucose tolerance, as compared with euthyroid rats.

The elevation of serum adiponectin in experimental hyperthyroidism raises the question of the putative contributing effect of this adipokine to the effects of thyroid hormone excess. On the other hand, the hyperadiponectinemia present in thyroxine-treated rats might represent a compensatory mechanism that counteracts the effects of excess thyroid hormone concentrations. Human studies evaluating circulating adiponectin in thyroid hyperfunction have shown variable results (Table 2). High adiponectin levels have been reported accompanying the elevation of thyroid hormone concentrations in hyperthyroid patients by some investigators (63,66,69,70), whereas other authors have found no significant differences in serum adiponectin between euthyroid subjects and hyperthyroid patients (44,59,60,64,65).

Table 2. Serum adiponectin concentrations in patients with hyperthyroidism, according to results reported in clinical studies

Number of patients	Sex (M/F)	Etiology	Control group	Serum adiponectin		Ref.
				Before	After	
20	4/16	GD, 17; TA, 2 TMG, 1	Euthyroid	No change	No change	58
15	4/11	GD, 10; TMG, 5	Euthyroid	No change	—	59
69	15/54	GD	Hypothyroid	Increase	—	65
32	12/20	GD	Euthyroid	Increase	Decrease	68
46	13/33	GD	Euthyroid	Increase	—	62
56	22/34	GD, 34; HT, 12 ST, 6; TMG, 4	Euthyroid	No change	—	63
39	11/28	GD	Euthyroid	No change	No change	43
120	27/93	GD	None	—	Decrease	70
76	13/63	GD	Euthyroid	Increase	—	69
24	5/19	GD, 23; TMG, 1	Euthyroid	No change	No change	64

Abbreviations: —, no data; GD, Graves' disease; HT, hashitoxicosis; ST, silent thyroiditis; TA, toxic adenoma; TMG, toxic multinodular goiter.

Treatment of thyrotoxicosis with appropriate therapy was followed by a significant decrease of serum adiponectin levels in some studies (69,71), but this decrement was not found in our study or in those of other authors (44,59,65).

The negative correlation between adiponectin and body mass index (64,65,71) and between adiponectin and insulin resistance (64) was maintained in hyperthyroid patients in some

studies, but was lost in others (69). A positive correlation between adiponectin and HDL-cholesterol has also been reported in patients with hyperthyroidism (64).

A positive correlation between adiponectin and free T4 in patients with hyperthyroidism before (44,66,69-71) and after (71) treatment has also been reported. In patients with Graves' disease treated with thionamide drugs, a weak correlation has been found between changes in adiponectin and changes in free T4 levels (71).

Discrepancies among the above mentioned studies in humans may be accounted for, at least in part, by the different etiology of the thyroid hyperfunction in the analyzed populations. Most of the studies that have found elevation of circulating adiponectin in hyperthyroid patients evaluated subjects with Graves' disease, whereas studies showing no significant changes included patients with hyperthyroidism of autoimmune and non-autoimmune etiology. It is possible that, although thyroid hormones might influence serum levels of adiponectin, some of the changes observed in patients with hyperthyroidism were also influenced by the adaptation to the changes in energy expenditure and intermediate metabolism in response to changes in thyroid hormone levels. This compensatory mechanism may be variable according to the duration of the thyroid dysfunction and may contribute to explain the discrepancies found in studies in patients with thyroid hyperfunction (62).

It has also been suggested that elevated levels of adiponectin in hyperthyroid patients might be the result of stimulating action of thyroid hormones on transcriptional induction of adiponectin through PPAR γ pathway. Thyroid hormone receptor and PPAR are members of the nuclear receptors superfamily and they both have the capacity to form heterodimers with retinoid X receptor, and it could be a crosstalk between PPAR and thyroid hormone signalling pathways (69,70).

ADIPONECTIN AND AUTOIMMUNE THYROID DISEASE

In some studies a positive association between adiponectin and TSH receptor antibodies has been found (69,70). In the study by Saito et al. (69), TSH receptor antibodies made the strongest contribution to serum adiponectin concentrations in patients with Graves' disease. It has been suggested that stimulation of TSH receptor by autoantibodies could be connected with inducing

adiponectin production (70). This is in agreement with the fact that adipokines are also secreted by immune cells contained within adipose tissue (48). However, there was no correlation between serum adiponectin and thyroid peroxidase or thyroglobulin autoantibodies (69). A recent study has shown that, in patients with Graves' hyperthyroidism, there were no differences in serum adiponectin between patients with and without thyroid associated ophthalmopathy (70), thus suggesting that adiponectin do not have a role in the autoimmune processes involved in Graves' ophthalmopathy.

On the other hand, the stimulation of TSH receptor by autoantibodies in Graves' disease could be related to the production of adiponectin. PPAR γ pathway stimulates the differentiation of preadipocytes into mature adipocytes that express TSH receptor, and also potentiates adiponectin production. High levels of adiponectin contribute to growth and proliferation of preadipocytes (72). Kumar et al. (73,74) showed that several genes were up-regulated in orbital adipose tissue from patients with Graves' ophthalmopathy compared with normal orbital adipose tissue. Among these, PPAR γ and adiponectin were found to be 44- and 25-fold overexpressed, respectively. Treatment of orbital preadipocytes with recombinant secreted frizzled-related protein-1 (sFRP-1) significantly increased adiponectin and TRH receptor RNA levels (74). Nevertheless, the possible role of this adipokine in the pathogenesis of thyroid associated ophthalmopathy is largely unknown.

Although anti-inflammatory properties of adiponectin are well known, under certain circumstances this adipokine has been involved in the regulation of the immune processes in response to T cell activation (75) and may exhibit pro-inflammatory actions (76). High levels of adiponectin have been reported in inflammatory conditions, such as rheumatoid arthritis (77), inflammatory bowel disease (78), and systemic lupus erythematosus (79). So far, now the involvement of adiponectin in the autoimmune processes in Graves' disease and other autoimmune diseases is merely speculative. As mentioned above, it is possible that elevated serum adiponectin found in patients with autoimmune conditions is a compensatory response of the adipose tissue against these pro-inflammatory states.

CONCLUSION

In summary, adiponectin, a unique adipokine with insulin sensitizing, anti-inflammatory and antiatherogenic properties, is a collagen-like protein expressed in adipose tissue that circulates in human plasma at high concentrations and in several molecular isoforms. Adiponectin and thyroid hormone share some physiological actions as reduction of body fat by increasing thermogenesis and lipid oxidation. Hence, it has been speculated that this adipokine might participate in the regulation of thyroid hormone production, through the interaction with the gC1q receptor found in the mitochondria of the thyroid cells, although a clear demonstration of the role of adiponectin in thyroid hormone biosynthesis is lacking. On the other hand, the pituitary-thyroid axis may act through several mechanisms to regulate adiponectin production. TSH receptors have been found in rat adipose tissue, and some investigators, although not all, have found that thyroid hormone are able to stimulate adiponectin expression and secretion in brown adipose tissue. The PPAR γ or SREBP pathways might also be involved in the transcriptional induction of adiponectin by thyroid hormone.

A number of clinical studies suggested that deficiency of thyroid hormone, as seen in human hypothyroidism, does not seem to be associated with significant changes in adiponectin neither before nor after control of thyroid function. However, hyperthyroidism is sometimes accompanied by an elevation in circulating levels, especially when Graves' disease is the etiology of thyroid hyperfunction. A positive association between adiponectin and TSH receptor antibodies has been found, suggesting the participation of this adipokine in autoimmune thyroid disease, although an association between adiponectin and thyroid peroxidase and thyroglobulin autoantibodies has not been found, and there is no proof for the involvement of adiponectin in Graves' ophthalmopathy. Altogether, these results suggest that changes in serum adiponectin play a modest role in thyroid dysfunction in humans. Furthermore, with present data the involvement of adiponectin in autoimmune thyroid disease is merely speculative.

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THYROTROPIN-RELEASING HORMONE:**SHEDDING NEW LIGHT ON THE HYPOTHALAMIC-PITUITARY THYROID AXIS***Leonidas H. Duntas¹, Charles Emerson²¹*Endocrine Unit, Evgenidion Hospital, University of Athens, Medical School, Greece*² *University of Massachusetts School of Medicine, Worcester, MA, USA**Reviewing Editor: Luca Persani**The authors declare no conflict of interest related to this article.***Correspondence to:**

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ABSTRACT

Thyrotropin-releasing hormone (TRH) is the dominant constituent of the hypothalamic-pituitary thyroid axis (HPT) and exerts various effects throughout the central nervous system. It has recently been reported that tanycyte pyroglutamyl peptidase II, one of the enzymes that degrade TRH, is regulated by thyroid hormones and is therefore a candidate for mediation of the feedback regulation of the HPT axis. In parallel, TRH has been found in the gastrointestinal tract, especially in the pancreas where it plays a regulatory role in the secretion of insulin, in the Leydig cells and in the prostate where it is modulated by testosterone. Of special significance are its newly identified glucoregulatory and antidiabetic effects that make the tripeptide and its analog potential targets for therapeutic intervention, while equally noteworthy are recent studies showing TRH receptor gene mutations to be the etiology of central hypothyroidism. The first family with complete resistance to TRH has also been documented of late. Forty years after its discovery, TRH continues to present us with an exceptionally wide field of research which will surely provide both exciting new challenges and enlightening perspectives in the in the coming years.

**Dedicated to all those who contributed to the discovery of TRH*

Key-words: TRH, hypothalamus, feedback regulation, TSH, pyroglutamyl peptidases

"TRF is such a simple molecule for all these years of work!" Roger Guillemin, 1969

Introduction

The isolation of Thyrotropin-Releasing Hormone (TRH), the epitome of a scientific odyssey that was passionately conducted by Roger Guillemin and Andrew Schally and their teams for almost a decade, resulted in the identification of the first hypothalamic-releasing factor and thereby the establishment of modern Neuroendocrinology (1). Subsequent to the historical pinpointing of TRH as the main physiological regulator of TSH synthesis and secretion, the description of the isolation and properties of porcine TRH was published by A. Schally on 10 August, 1969, and the structure of porcine and bovine TRH was published by J. Boler and R. Burgus on 6 and 12 November, 1969, respectively (2-4).

TRH is a tripeptide (pyroGlu-His-Pro-NH₂). It is present in a number of brain regions but of paramount importance for the hypothalamic-pituitary thyroid (HPT) axis is its secretion by the nerve terminals of the paraventricular nucleus (PVN). TRH is a tripeptide (pyroGlu-His-Pro-NH₂: the structure of TRH is presented in Figure 1) that is present in a number of brain regions.

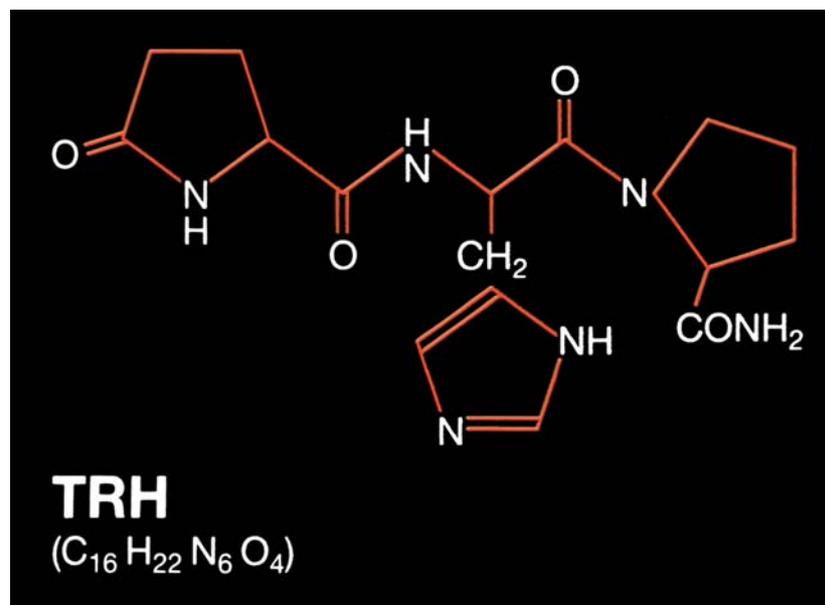


Figure 1: Chemical Structure of Thyrotropin-Releasing Hormone

However, of paramount importance for the hypothalamic-pituitary thyroid (HPT) axis is its secretion by the nerve terminals of the paraventricular nucleus (PVN). These have their cell bodies in the periventricular region known as the “thyrotropic area” which extends into the median eminence. The main action of TRH is to stimulate the synthesis and release of TSH. The description of the structure of TRH was followed by its synthesis and subsequently by its extensive use in clinical medicine. For more than two decades the TRH-test has comprised the prime diagnostic tool for detection of thyroid disease.

The aim of this review is to summarize the old and outline the many new findings with regard to TRH, a peptide which is characterized not only by its dominant role in the HPT but also by its wide range of extrahypothalamic sources and numerous actions.

Biosynthesis and metabolism

The introduction of the polymerase chain reaction and cDNA cloning methodology in the '80s made possible the elucidation of the biosynthesis of TRH. TRH is derived from the prepro-TRH peptide, which is composed of 242 amino acids in man, compared to 255 amino acids in the rat, and contains multiple copies of the TRH progenitor sequence Gln-His-Pro-Gly (5-7). Each of these sequences is flanked by paired amino acid residues that yield, via proteolytic cleavage, five copies of TRH and seven cryptic peptides, which actively participate in the intracellular routing of the precursor (8,9). For instance, the connecting peptide prepro-TRH-(160-169) (Ps4) was demonstrated to be co-released with TRH and to modulate its biological effects; Ps4 has no direct effect on TSH secretion but it potentiates TRH-mediated TSH release in a dose-dependent manner (9). However, immunochemical and immunocytochemical studies suggest that the maturation of pro-TRH, a high molecular weight immunoreactive TRH precursor form in the developing mouse hypothalamus, is a continuum starting in the endoplasmic reticulum and ending as a post-Golgi event (10). Hypothyroidism selectively increases the synthesis of pro-TRH mRNA and the prohormone in PVN. *In vitro* studies demonstrated a reduced content and increased release of pro-TRH from the median eminence (11). At least three enzymes have been observed to degrade synthetic TRH. These are a prolyl endopeptidase (post proline cleaving enzyme, TRH deamidase), which is present in brain and

lacks substrate specificity for TRH, and two pyroglutamyl aminopeptidases (PPAs). Prolyl endopeptidase, as its name indicates, converts TRH to pGlu-His-Pro, while PPAs I and II convert TRH to His-ProNH₂. PPAs II has substrate specificity for TRH and is the only one of the enzymes with TRH degrading activity that is currently listed in the International Union of Biochemistry and Molecular Biology (IUBMB) Nomenclature Database (EC 3.4.19.6) (Figure 2).

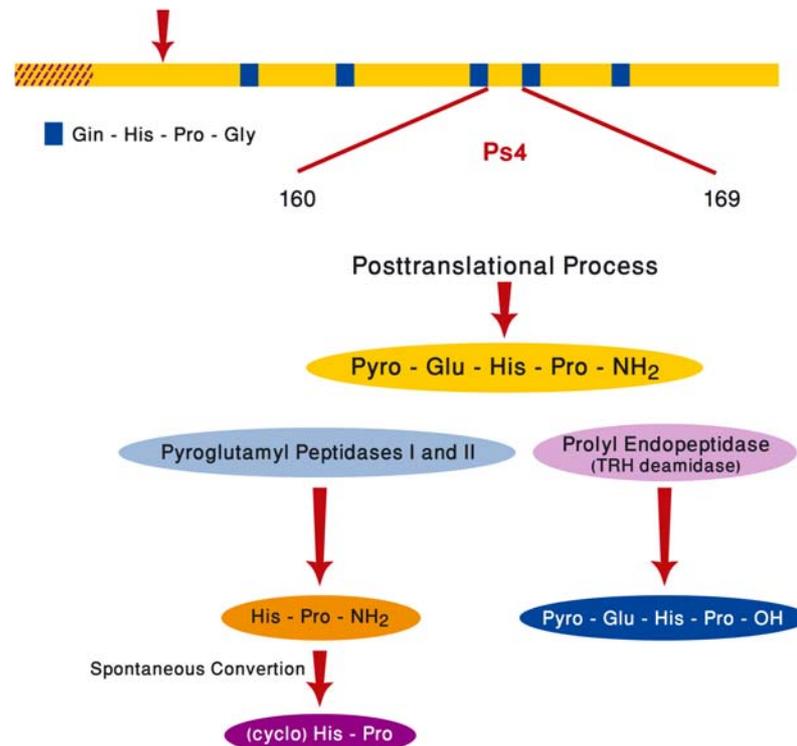


Figure 2: The metabolism of TRH. A schematic diagram of the prepro-TRH cDNA. The shaded area above left represents the signal peptide encoding region. The blue solid boxes represent the TRH coding sequences. In the posttranslational process, TRH will be at different sites by the action of pyroglutamyl-peptidases I and II degraded to its main metabolite His-Pro-NH₂, which can be spontaneously converted to cyclo (His-Pro), and by the action of prolyl endopeptidase (TRH desamidase) to desamidated pyro-Glu-His-Pro-OH.

His-ProNH₂ can spontaneously convert to cyclo His-Pro (histidyl- proline diketopiperazine). Biological activity has been reported for cyclo (His-Pro) including regulation of body temperature and, acting at variance with its pre-hormone, it has been reported to inhibit prolactin secretion *in vitro* (12) but not *in vivo* (13). Elsewhere, its co-release with glucagons by the pancreatic α -cells suggests possible exertion of modulatory actions in the insular physiology (14). Interestingly, Perry *et al.*

identified cyclo (His-Pro) in human urine in 1965, even before the elucidation of the structure of TRH, and attributed its presence to dietary sources (15).

Following its release into the portal circulation, TRH is probably rapidly degraded by serum and perhaps by tissue membrane bound PPAs activities in tanocytes (16-18). Divergent results have been reported regarding the influence of thyroid status on serum and brain PPAs activities with the ultimate aim of producing synthetic TRH (18-21). These differences are likely due to differences in species and tissues examined rather than to methodology. In the adenohypophysis, PPAs II is mainly regulated by thyroid hormone (TH), being decreased within a few days in animals rendered hypothyroid, while it is rapidly increased after 4-6h following an injection of T_3 (22). Estradiol decreases the activity of the adenohypophyseal enzyme. Ovariectomy in rats induces increase of the enzymatic activity, while treatment with estradiol benzoate leads to significant decrease (22). Thus, PPAs II exerts its influence on the adenohypophyseal function by regulating TRH catabolism in relation to hormonal status.

Two studies have recently emerged, one referring for the first time to an inactivating mutation of the TRH receptor gene causing isolated central hypothyroidism in a 9-year-old boy (23,24) and the second constituting the first report on a family with complete resistance to the action of TRH due to a nonsense mutation in the TRH receptor gene causing central hypothyroidism (25). In both cases the patients were diagnosed with isolated hypothyroidism marked by delayed growth, short stature, lethargy and fatigue. In the second case, by contrast, the pregnant sister of the proband, diagnosed with central hypothyroidism based on genetic testing of the family members, delivered two babies at term, who were subsequently both breast-fed, this indicating that TRH action is not mandatory either for female fertility and lactation nor for $TSH\beta$ and PRL genes expression. It is noteworthy that the TSH action on the thyroid was not completely absent, since withdrawal of LT_4 stimulated circulating TSH and thus resulted in increased endogenous thyroid hormone levels, this, however, insufficient to maintain euthyroidism in the absence of TRH action (25). The rhythmic pituitary function is well preserved, pointing to the fact that non-TRH signals and some other factors, such as possibly thyrostimulin, may be involved in the pituitary regulation in this case.

The setpoint and the regulation of TRH neurons

Although TRH was discovered four decades ago, it is only in the last few years that the topography of TRH-containing cells in the brain has been described, this permitting the acquisition of ever more specific data on its function and regulating activities (Figure 3).

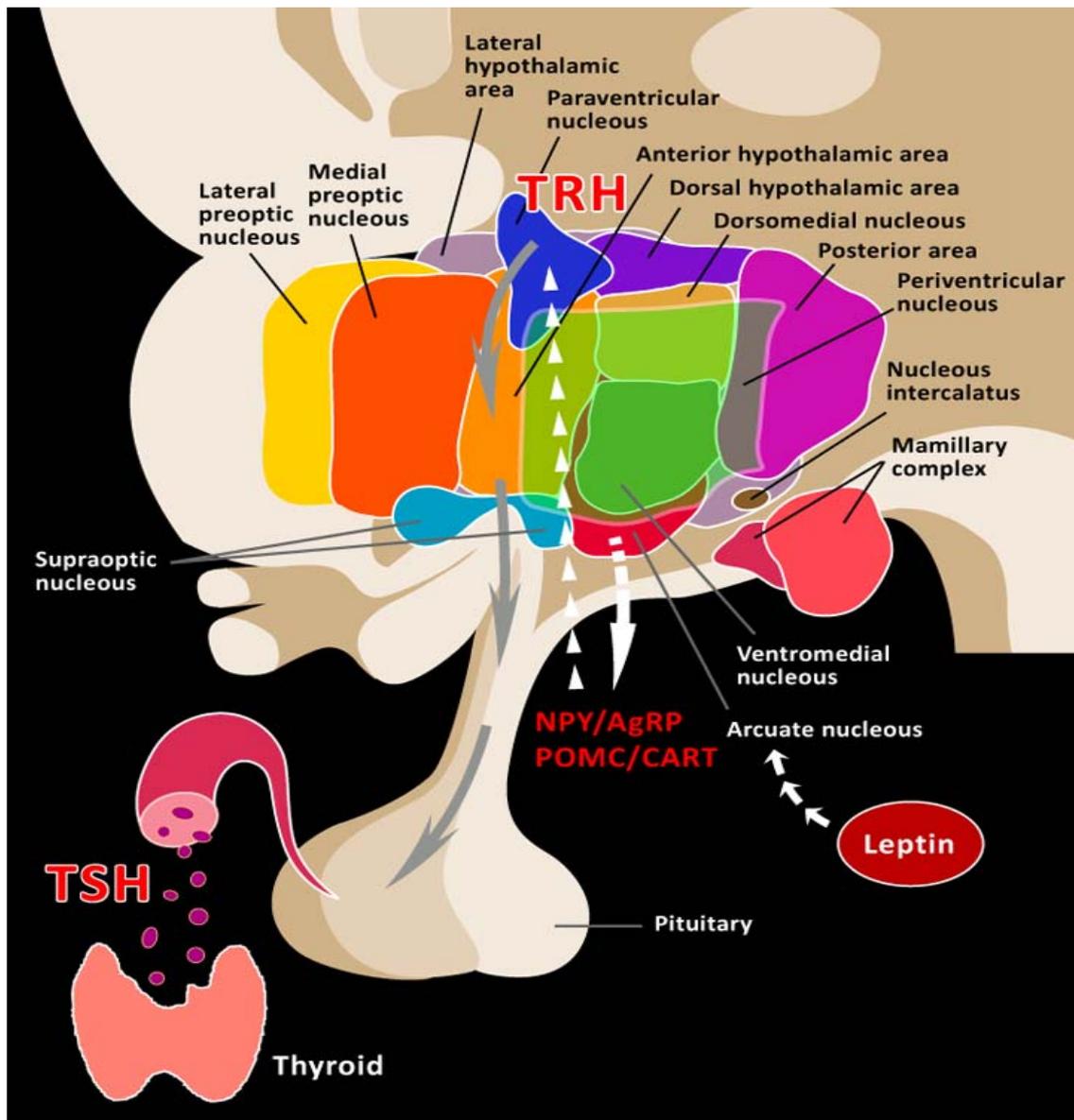


Figure 3: TRH is secreted by the hypothalamic paraventricular nucleus (PVN); it reaches the median eminence through axonal transport and is relocated via the hypothalamic portal vein to the anterior pituitary thyrotroph, where it binds to TRH receptors regulating TSH production. The arcuate nucleus (AN) is an important “relay-station” for the maintenance of energy homeostasis. Leptin signals from the periphery have an access to AN which is relayed to PVN via pre-autonomic neurons containing NPY/AgRP and POMC/CART peptides (see text).

The neurons synthesizing TRH, which are mainly located in the dorsocaudal region of the hypothalamic PVN, constitute the central regulatory unit, i.e. the setpoint, of the hypothalamic-pituitary

thyroid axis. TRH plays a major role in the posttranslational maturation of TSH oligosaccharide chains and is required for the secretion of TSH with full biological activity (26). The synthesis and release of TRH in the hypothalamus is under negative feedback regulation by TH that inhibits at the transcriptional level both TRH and TSH subunit genes (11). Hypothyroidism strongly stimulates the synthesis of pro-TRH, the release of TRH and the pro-TRH derived peptides in the hypothalamus (27). Hypothyroidism strongly stimulates the synthesis of pro-TRH as well as the release of TRH and of the pro-TRH derived peptides in the hypothalamus (27). Contrary to the general view that TH is the main regulator of the HPT-axis, recent data from transgenic animals have clearly revealed that TRH has the dominant role in the regulation of the HPT-axis and determination of the setpoint (28). Thus, central regulation of the pituitary-thyroid axis by TRH is essential for normal function. Recently it has been demonstrated that mRNA for PPAs II extends from the tanycyte cell bodies in the base of the third ventricle to the external zone of the median eminence in apposition to pro-TRH containing axon terminals (29). PPAs II activity is up-regulated by TH in the tanycyte, resulting in enhanced degradation of extracellular TRH, whereas PPAs II inhibition leads to increased TRH secretion (29). These novel and significant results indicate that tanycyte PPAs II may be an important mediator in the regulation of the HPT as far as feedback inhibition by TH is concerned.

Specific conditions, however, such as prolonged cold exposure and fasting or clinical conditions such as infection, critical illness and psychiatric disorders may alter the setpoint for negative feedback. Cold exposure induces stimulation of the HPT axis via adrenergic neurons, while fasting decreases TRH mRNA in hypothalamic neurons and enhances the sensitivity to the negative feedback by TH (30). Infection induces, via cytokines, activation of type 2 iodothyronine deiodinase (D2), which is the enzyme responsible for the conversion of T₄ to T₃ in the brain and, once stimulated, it leads to local hyperthyroidism and subsequent inhibition of hypophysiotropic TRH (31).

Recent results from neuroanatomical studies using quantitative in situ hybridization have proposed a different mechanism for thyroid setpoint regulation in pathological conditions (32). In post-mortem specimens, it was found that TRH neurons in the PVN express the thyroid transporter monocarboxylase transporter 8 (MCT8), thyroid hormones receptors and inner ring iodothyronine deiodinase 3 (D3), while outer ring iodothyronine deiodinase (D2) activity was detected in the region

of the median eminence, in the glial cells and in the tanycytes which demarcate the third ventricle (32). Following this line of evidence, it has been proposed that T4 is taken up by the hypothalamic glial cells and converted into T3, which is subsequently transported to TRH neurons to be bound to TH receptors or be metabolized into inactive iodothyronines by D3 (32). These novel findings, via the application of immunohistochemistry, mRNA in situ hybridization and enzyme activity assays, have contributed considerably to identification of the determinants of the setpoint for TH regulation of TSH secretion and have underlined the significance of the glial cells and tanycytes. In critical illness, altered metabolism of T4 due to reduced hypothalamic deiodinase activity results in decreased TRH mRNA. However, the decreased TH concentration does not raise the TSH levels, suggesting altered feedback regulation at the hypothalamic/pituitary level (32). Notably, these findings have recently been partly confirmed by experimental studies on prolonged critically ill animals showing that TRH mRNA in the hypothalamus was decreased and, although MCT8 and MCT10 were increased, TH concentration and receptors were not elevated (33). The evident conclusion is therefore that the decreased TRH gene expression and the low TSH and T3 concentrations during prolonged critical illness are not solely due to hypothalamic thyrotoxicosis but that additionally other factors are involved.

Hypothalamic and extrahypothalamic functions of TRH neuron

Besides being localized in the hypothalamus, where it represents only a small fraction of the entire brain TRH, TRH has been detected throughout the CNS in the brainstem, medulla oblongata and spinal cord (34). It is therefore conjectured to be a peptide ubiquitously distributed in the CNS where it mainly functions as neurotransmitter or neuromodulator.

The hypothalamic TRH neuron is a regulator of energy homeostasis through its impact on thyroid function, by stimulating TSH release, and on feeding behavior (35). TRH is an anorexigenic peptide suppressing, by means of central effects, food intake in normal, fasting and stressed animals. Although the precise mechanism remains unknown, the presence has been established of an interplay between TRH neurons and the leptin regulating system in the arcuate nucleus.

TRH cells in the PVN receive projections from leptin-responsive neurons that are located in the arcuate nucleus of the hypothalamus. These neurons contain the anorexigenic peptides alpha-melanocyte stimulating hormone (alpha-MSH), the cocaine and amphetamine regulated transcript (CART) and the orexigenic neuropeptide Y (NPY), and the agouti-related protein (AGRP) peptides, which promote obesity and reduce energy expenditure (36). Prolonged fasting suppresses leptin, serum T4 and TSH levels as well as proTRH mRNA in the hypothalamic PVN, presumably 1) by increasing NPY, that suppresses pro-TRH mRNA in the PVN, and 2) by decreasing prohormone convertases 1 and 2 genes, the enzymes that regulate TRH proteolysis cleavage in the PVN and median eminence (37-38). The systemic administration of leptin may entirely reverse these changes, this showing that leptin influences the setpoint for feedback inhibition of TH and that it also partially controls the biosynthesis of TRH in the PVN.

TRH exerts various effects on the central nervous system, contributing to the regulation of thermogenesis and influencing arousal and locomotor activation, while also exerting various analeptic and antidepressant effects. It controls the cephalic phase of digestion by acting, via cholinergic and dopaminergic mechanisms, on the septum and nucleus accumbens, respectively (39). Centrally administered TRH increases gastric secretion via vagal pathways, including the dorsal motor nucleus of the vagus (DMN), while it also stimulates gastric myenteric cholinergic neurons and colonic activity (40). Moreover, through the raphe pallidus neurons, medullary TRH innervates vagal preganglionic motor neurons in the dorsal vagal complex and regulates gastric functions and pancreatic insulin secretion (41).

Peripheral TRH

TRH has been localized in considerable amounts outside the CNS, as in the gastrointestinal tract, in the pancreas and in the reproductive tracts of male rats, including the rat prostate where it may have a physiological role and be modulated by testosterone (42). Further research is needed to elucidate this matter (43).

TRH is synthesized in the β -cells of the Lagherhans islets from a pro-TRH mRNA, similar to that present in the hypothalamus, and is involved as a local modulator in the pancreatic physiology

(44). Exogenous TRH increases basal glucagon secretion, suggesting a direct effect on alpha-cell secretion and a glucoregulatory role in the pancreas (45). Glucagon in contrast to arginine does not stimulate TRH release from the isolated perfused rat pancreas (46). In the neonate rat, while TRH is elevated in the pancreatic β -cells, the TRH-degrading activity is absent, appearing on day 14 and reaching adult levels on day 21 (47). A developmental role of TRH in the embryonic pancreas has been proposed by current data showing that TRH treatment of human islet-derived precursor cells for several days promotes programmed cell death, a normal pathway of human embryonic development (48). It has been proposed that TRH is a marker of insulin expressing cell, recent studies having revealed that TRH administered to rats rendered severely diabetic with streptozotocin completely reverses the hyperglycemia (49,50). In humans, following the oral glucose tolerance test, oral TRH inhibits in normal subjects the first hour increase in blood glucose, insulin and proinsulin, presumably via inhibition of gastric motility and/or paracrine actions (51). Moreover, targeted prepro-TRH gene disruption causes hypothyroidism and hyperglycemia (52), demonstrating that prepro-TRH as well as its derived peptides and their receptors are crucial not only for the functioning of various local systems but for the homeostasis of the entire organism. These results are corroborated by those of studies using mice that are lacking TRH (TRH^{-/-}). TRH^{-/-} mice, though viable and fertile, show signs of hypothyroidism with elevated serum TSH and diminished TSH bioactivity, which are reversed via TRH supplementation but not by thyroid hormone administration. Moreover, they exhibit severe hyperglycemia and impaired insulin response to glucose administration, suggesting that TRH is involved not only in thyrotroph but also in insulin regulation (53).

TRH has demonstrated a considerable inhibitory action on the gastrointestinal tract and the exocrine pancreas; an infusion of 200 μ g TRH resulted in inhibition of lipase and chymotrypsin secretion up to 44% (54).

TRH has furthermore been implicated in a range of systems whereby it exerts a wide spectrum of actions. For example, it has recently been reported that TRH and TRH receptor are expressed in the epithelium of human hair-follicles (55). TRH acts as a hair growth stimulator by promoting hair-shaft elongation, prolonging the hair cycle growth (anagen) while antagonizing its termination via TGF- β activity (55).

Finally, TRH is expressed by melanoma and its presence in nevi may be considered as a predictor for melanoma functioning as an autocrine growth factor (56). These latter findings, however, require further confirmation by means of additional research regarding TRH implication in the formation and development of melanoma.

Clinical use

The demonstration that TRH releases thyrotropin from the anterior pituitary and the subsequent synthesis of TRH led to its use as what came to be known as the TRH-test, which has been regarded for almost a quarter of century as the “Golden Test” for the study of the release of TSH and subsequently that of thyroidal status after stimulation of the thyrotroph with TRH (57-59). A suppressed TSH response to TRH indicates hyperthyroidism, while a high response evidences hypothyroidism.

In a variety of endocrine disorders, serum TSH response to TRH has been reported blunted, such as in acromegaly and hypercortisolism, or exaggerated as in Klinefelter’s syndrome and Turner’s syndrome (60). In acromegaly, TRH administration elicits a significant increase of GH in 50% of the patients; however, the increase is not dependent on basal GH and TSH levels (61,62). In Cushing’s disease and Nelson’s syndrome, TRH-testing induces a rise of plasma ACTH levels in about 30% of the patients, probably due to expression of TRH receptors on the corticotrophs (63).

TRH administration induces a 5-10 fold increase in serum prolactin (PRL), especially in women during the fertile years. However, in microprolactinomas the PRL response is rather poor, while increased response usually occurs in patients with hypothalamic or stalk lesions (64). Elevations in serum FSH, LH or even α -SU after TRH administration have been observed in patients with gonadotropinomas, and very occasionally in functionless pituitary adenomas, the rise being independent of the basal levels of the gonadotrophins (65). Increased FSH and LH response to the TRH-test was also observed in patients with acromegaly, this possibly indicating plurihormonal tumor.

The TRH-test has been performed as an adjuvant in the diagnosis and evaluation of treatment of depression (66). Human depression is characterized by an impairment of the inhibitory glucocorticoid feedback from the hippocampus to the hypothalamus, leading to increased cortisol

levels and activation of the TRH neuron. This results in increased TRH levels and down-regulation of the TRH receptor on the thyrotroph and in the typically blunted TSH response to TRH in depression (67). Administration of TRH has been reported to enhance mood and ameliorate the psychological state. This is probably due to the excitatory action of TRH throughout the neuroaxis following the blocking of various K⁺ channels linked to TRH receptors in different TRH-innervated neuroanatomical pathways and leading to increased secretory output of TRH regulatory circuits that modulate neurobehavior (68).

TRH has been therapeutically applied in patients with amyotrophic lateral sclerosis (69), ataxia cerebellaris, and experimental endotoxin shock (70), sometimes but not always with favorable results. Due to its short-half life, which amounts to 6.5 min following intravenous administration (71,72), TRH probably needs to be evaluated by administering it at a constant rate for a prolonged period. Another approach would be to develop analogs of TRH, ideally have the same neurotrophic and/or glucoregulatory properties as TRH but without hypophysiotropic activity.

Epicrisis

Four decades have passed since the ground-breaking discovery of TRH, a period marked by our constantly growing understanding of its broad spectrum of vital actions, including control of thyroid function and energy homeostasis and its influence on behavior. Its wide range of application is hence unsurprising and certainly justifies the continuing interest of the scientific community in this truly impressive molecule. Meanwhile, the constantly accumulating experience of its clinical and neurobiological effects warrants our sustained endeavor for the development of TRH agonistic drugs that can alleviate numerous diseases and symptoms. Forty years after its discovery TRH remains a unique and extraordinary peptide.

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CASE REPORT**THYROTOXICOSIS FACTITIA: A DIDACTIC EXAMPLE OF WRONG INTERPRETATION OF RESULTS LEADING TO SEVERAL UNNECESSARY PROCEDURES**

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ABSTRACT

Objective: to present an unusual and didactic case of thyrotoxicosis factitia (TF).

Case report: we present a patient suffering from TF who received unnecessary large doses of antithyroid drugs and steroids, and underwent two useless surgical procedures, i.e. total thyroidectomy and thymectomy in an attempt to treat her thyrotoxic state. We underscore that in such cases it is important to perform radioiodine uptake and serum thyroglobulin (Tg) measurements, which can be of help to establish the diagnosis. In addition, the estimation of urinary iodine, which in such cases is very high, and color-flow Doppler sonography may also be helpful for the correct diagnosis. Fecal thyroxine is also an important estimation which, however, has gradually being abandoned.

Conclusions: this case illustrates that in most - if not all - suspected cases of TF, serum Tg and urinary iodide will be sufficiently informative as additional investigations. The case report further illustrates that magnetic resonance imaging (MRI) and rhTSH stimulated radioiodine uptake are not useful and may even lead to unnecessary therapeutic (surgery, 131I treatment) procedures because of radioiodine uptake in the thymus.

Key words

thyrotoxicosis, thymus enlargement, radioiodine uptake

Introduction

The term Thyrotoxicosis Factitia (TF) is used to describe thyrotoxicosis produced by ingestion of excessive amounts of exogenous thyroid hormone (1). It is also known by a variety of other names, including alimentary thyrotoxicosis, exogenous thyrotoxicosis, occult factitial thyrotoxicosis or thyrotoxicosis medicamentosa (2).

It is most frequently iatrogenic, when thyroxine is prescribed to suppress tumor growth in thyroid cancer, to decrease goiter size or for therapy of hypothyroidism.

TF can also be caused by unintentional ingestion of thyroid hormone (accidental overdose in children, "hamburger thyrotoxicosis", cosmetic creams containing iodine and thyroid hormones, veterinary T₄ preparations).

Finally, TF is the result of surreptitious use of thyroid hormones by psychiatrically disturbed patients or for unconventional indications such as obesity, depression, menstrual disorders and infertility (3). In the first cases it is hard to make the diagnosis because patients usually do not admit the misuse of thyroid hormones (4).

In the present communication we report a patient who was unsuccessfully treated for thyrotoxicosis during 3 years with various therapies [antithyroid drugs, radioactive iodine (RAI) and two surgical procedures], and the diagnosis of TF was indicated by different endocrine tests, although the patient strongly denied exogenous thyroid hormone ingestion.

Case Report

A 29-year old woman presented initially to the outpatient endocrine clinic of Panagia Hospital, Thessaloniki in May 2008 suffering from persistent thyrotoxicosis for the last 30 months.

She reported that her symptoms started in January 2006, six months after the birth of her second child, consisting of weight loss (from 150kg to 100kg), tachycardia, tremor of the extremities and irritability. Prior to the onset of symptoms the patient was extremely obese [body mass index (BMI)=52]. Regarding her social history, no occupational factors were discovered that might have predisposed her to take excess thyroid hormones or be willing to undergo inappropriate surgery. It is of note, that the patient financially is fully dependent from her mother.

Initial investigations (January 2006) were compatible with thyrotoxicosis [free thyroxine (FT₄) = 21.85 ng/dl (NR = 0.8-2.0), thyrotropin (TSH) < 0.005 μ IU/ml (NR=0.3-4.0), thyroid receptor antibodies (TRAb) = 0.8 IU/ml, (NR < 1.8)]. Thyroid ultrasonography revealed normal thyroid volume with homogeneous pattern and a solid, hyperechoic nodule of 10.5 mm diameter in the right lobe. No isotopic scanning of the thyroid gland was performed at that time.

She was treated with antithyroid drugs for one year but euthyroidism was never achieved. Due to apparent failure of drug treatment and concern about her continuing thyrotoxicosis, a total thyroidectomy was performed on February 2007 in a hospital in Athens. Histology showed a normal thyroid gland with a single hyperplastic 6 mm nodule in the right lobe.

Immediately after thyroidectomy the patient commenced replacement therapy with L-thyroxine. About a month later she reported symptoms of thyrotoxicosis, which persisted despite the suggested discontinuation of thyroxine therapy. Neck ultrasonography and computer tomography (CT) were performed to exclude residual thyroid tissue, and both showed no evidence of thyroid remnant. A few enlarged cervical lymph nodes were noted. A fine needle aspiration of one cervical lymph node revealed reactive changes only, without any evidence of malignancy.

Three months after total thyroidectomy (May 2007), the patient presented to another hospital in Athens with severe thyrotoxic symptoms including tachyarrhythmia non responsive to medication, hypertension, irritability, hyperhidrosis, and tremor of the extremities. Her body weight was 61 kg with BMI 22. Endocrine investigation showed TSH < 0.005 μ IU/ml, free triiodothyronine (FT₃) = 16.7 pg/ml (NR=2.0-4.4), FT₄ = 25.85 ng/dl. Her TRAb were normal = 0.9 IU/ml (NR < 1.8), while her Tg levels were 7.5 ng/ml (NR = 0.0-5.0 after thyroidectomy). Moreover, thyroid peroxidase (TPO) antibodies and Tg antibodies were 37.0 IU/ml and 21.0 IU/ml, respectively (NR < 50 and < 60, respectively). Her symptoms remained unchanged, despite the administration of high doses of propylthiuracil and corticosteroids for a week. She was advised to continue taking antithyroid drugs.

She was investigated for the first time in our clinic in May 2008. After confirming that she was thyrotoxic [FT₄ = 32 pg/ml (NR=7-18), FT₃ = 7.2 pg/ml (NR=2.2-5.5), TSH=0.05 μ IU/ml (NR=0.3-4.0)], with normal TRAb and low Tg levels (< 0.1 ng/ml), a chest MRI was performed, to exclude ectopic hyperfunctional thyroid tissue, which however revealed a 4x2 cm soft tissue mass in the anterior

mediastinum. An ultrasound of her uterus and ovaries was normal. ^{131}I whole body scan after stimulation with rhTSH [(Thyrogen[®], Genzyme, USA) (2 amp x 0.9 mg)] demonstrated significant radioiodine uptake in the mediastinum. A therapeutic dose of 40mCi ^{131}I after stimulation with rhTSH (2 amp x 0.9 mg) was administered, but without any clinical improvement, at least in the first two months after treatment. No post-therapy scan was performed after the 40 mCi ^{131}I dose. Three months after ^{131}I treatment, and while the patient was waiting for her follow-up appointment in our clinic, she underwent surgical excision of the anterior mediastinal mass in a private hospital in Athens despite being severely thyrotoxic. On histological examination the mass was proved to be a hyperplastic thymus without signs of malignancy or presence of thyroid tissue.

The patient presented to our department again in December 2008, four months after her last surgery, complaining of tachycardia, diarrhea, hyperhidrosis, irritability and menstrual disorders. She denied taking any drug or supplements for the last 6 months. Her body weight was 71 kg and her BMI 24.5.

On physical examination she had a fine tremor, hyperhidrosis and lid lag. She had tachycardia 110 beats per minute and her blood pressure was 150/90 mmHg. Respiratory, gastrointestinal and urinary systems were unremarkable. Laboratory tests revealed: elevated FT₄ (6.1 ng/dl, NR = 0.8-2.0), elevated FT₃ (2.2ng/ml, NR = 0.7-2.0) and suppressed TSH (0.065 $\mu\text{IU/ml}$). A whole-body scan with ^{131}I after stimulation with rhTSH (2 amp x 0.9 mg) was performed and no pathological uptake was found. In addition, serum Tg was low (0.2 ng/ml), and did not rise after stimulation with rhTSH. Anti Tg, anti TPO and TRAb antibodies were within normal range. Finally, very high levels of urine iodine (1150 $\mu\text{g}/24\text{h}$, NR<150) were found. All the above tests were indicative of a case of excess thyroid hormone ingestion, which the patient strongly denied.

Discussion

The term of TF has been used in several ways. One is any situation where thyrotoxicosis is induced by self administration of thyroid hormones, even in cases where the patient is unaware of it (i.e. hamburger thyrotoxicosis) and the other is when the patient is surreptitiously ingesting enough thyroid hormone to make himself thyrotoxic (5). The diagnosis of TF should be considered in any

patient with apparent hyperthyroidism, but lacking any thyroid enlargement. It becomes more likely if the patient has a low thyroid radioiodine or pertechnetate uptake and a low serum Tg concentration (6). Usually, patients on thyroid hormone for the unconventional indications such as obesity, depression, menstrual disorders and infertility are as likely to admit the use of thyroxine as those receiving the same medication for hypothyroidism.

In TF exogenous thyroid hormone suppresses TSH secretion by negative feedback on the pituitary thyrotroph cells and this leads to suppression of endogenous thyroid function. As a result, the thyroid radioiodine or pertechnetate uptake is very low to undetectable (4). Serum Tg measurement can distinguish TF from other causes of thyrotoxicosis with low thyroid radioiodine uptake, since serum Tg levels are low or undetectable in cases of excess thyroid hormone ingestion, but normal or elevated in most other cases of thyrotoxicosis. In order to increase the uptake of eventual thyroid remnant, rhTSH was administered in our case on an empirical basis. It is of note, that the presence of anti-Tg antibodies in serum, can interfere with the serum Tg assay, yielding in most cases false negative results (7-9). In our case, Tg antibodies were elevated in May 2007 and low in May 2008.

In our patient, the diagnosis was further complicated by the pathological radioiodine uptake in the anterior mediastinum on whole body iodine scanning, leading to the erroneous diagnosis of ectopic thyroid tissue, which proved to be thymus on the histology. Radioiodine uptake by the thymus is a well-recognized phenomenon. Wilson et al. described uptake in the thymus in 10 of 38 patients undergoing post radioiodine therapy scans (10). Also, Veronikis et al. confirmed the same phenomenon (11) with reported frequencies between of 3.5-25% (10, 12). Radioiodine uptake in the thymus can occur in the absence of ectopic thyroid tissue or thyroid metastases (13) and reflects expression of human sodium-iodide symporter (14). Serum Tg determination should be performed in such cases to differentiate between ectopic or metastatic thyroid tissue and physiological uptake in thymus. It is noteworthy that rhTSH stimulation test is not useful in the diagnosis of TF per se and is not recommended in the investigation of such cases. In our case, it was used to evaluate if the uptake of the anterior mediastinum mass could be increased and in a positive case to administer a therapeutic dose of RAI. After demonstrating significant uptake of the mass, we proceeded to the administration of 40 mCi RAI after rhTSH administration. In our case rhTSH was used on an empirical

basis before the administration of the diagnostic dose of RAI to check if the mass in the anterior mediastinum was trapping radioiodine. After demonstrating significant uptake of the mass, rhTSH stimulation was also used immediately before the administration of the therapeutic dose of 40 mCi ^{131}I , to augment the uptake and to achieve the best therapeutic results. However, the whole procedure was proved unsuccessful the reason being that the mass was not an ectopic thyroid but an enlarged thymus.

Another helpful diagnostic tool in the evaluation of our patient was the measurement of urine iodine, which was extremely raised. Since most of the dietary iodine is excreted in the urine, a 24-hour urinary iodine concentration is an accurate index of dietary intake (15). Our patient, who was thyrotoxic, had a very low serum Tg concentration, had no evidence of thyroid tissue on imaging and had not received radiographic contrast. On these grounds iodine induced thyrotoxicosis was excluded. The only remaining diagnosis consistent with thyrotoxicosis associated with low serum Tg and high urine iodine is exogenous thyroxine ingestion. One reliable measurement, which is very useful to establish the diagnosis of the latter, is the measurement of thyroxine in the feces. Fecal T_4 measurements are approximately 1 nmol/g in normal healthy subjects, are mildly increased in Graves' disease (about 2 nmol/g), and are markedly elevated in individuals with Tg (over 12 nmol/g) (16). However, this diagnostic procedure has progressively been abandoned in such cases. Finally, color-flow Doppler sonography has been used to distinguish Graves' disease or toxic nodular goiter, in which the thyroid is hypervascular, from TF, in which thyroid hypervascularity is absent (17). In our case, this procedure could not be used as the patient had previously undergone total thyroidectomy. We believe that the incentive for the patient may have been to address her morbid obesity. Also, as the T_4/T_3 ratio was not as high as it would have expected if she was taking thyroxine, the combination of T_4 and T_3 or thyroid extract should be the most possible thyroid compound. However, no specific data on this issue can be provided. Finally, it should be mentioned that according to the law in Greece, thyroxine should be provided by the chemists only by official prescription. However, it is not unusual in some cases to be supplied over the counter.

In conclusion, we present a patient suffering from TF who received large doses of antithyroid drugs and steroids, and underwent two major surgical procedures in an attempt to treat her

thyrotoxicosis, all of which failed. We suggest that in such cases it is important to perform radioiodine uptake and serum Tg measurements, which will help to establish the diagnosis. In addition, an important investigation in suspected TF, is the estimation of urinary iodine, which in such cases is very high. Fecal thyroxine, which is also a reliable method to establish the diagnosis, is being gradually abandoned. Usually, the patient denies the exogenous use of thyroid hormones and confirmation of the diagnosis is often very difficult.

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Case Report**Euthyroid Graves' Ophthalmopathy patients remaining stable on more than 20 months follow up – a report of 9 patients.**

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ABSTRACT

Thyroid Associated Ophthalmopathy (TAO) is generally associated with altered thyroid function. Patients who are euthyroid, generally become hyperthyroid within 6-12 months of diagnosis. We report a group of patients who have remained euthyroid for more than 20 months following diagnosis of TAO. The diagnosis of TAO was supported by the presence of TSH receptor antibody and graded clinically by the 'NO SPECS' system of the American Thyroid Association. Free T3, Free T4 and TSH were measured every 4 months using a chemiluminescence technique. Out of 78 patients with TAO, six patients were euthyroid and were followed for more than 20 months and remained euthyroid. Additionally, 3 patients seen earlier, all of whom had visual loss secondary to TAO, remained euthyroid for more than 39 months. In the present study, a subset of patients were identified who did not develop hyperthyroidism by 20 months. These patients are unlikely to be referred to an endocrinologist, are at risk of visual loss and warrant further study.

Key-words: Graves' Disease; Graves' ophthalmopathy; TSH receptor antibody

INTRODUCTION

Thyroid-associated orbitopathy (TAO), is an organ-specific autoimmune process that is strongly associated with dysthyroidism. In the medical literature, TAO was formally described by Graves in 1835 and by von Basedow in 1840. Twenty percent of patients indicate that the ocular morbidity of TAO is more troublesome than the thyroid problems. It may result in eyelid retraction, proptosis, chemosis, periorbital edema, and altered ocular motility with significant functional, social, and cosmetic consequences. Although most cases of TAO can be managed medically and without visual loss, it may result in vision-threatening exposure keratopathy, troublesome diplopia, and compressive optic neuropathy. TAO may precede, but more frequently coincide or follow the onset of dysthyroidism (1).

Many patients with TAO are hyperthyroid, but the following also are associated with TAO: Hashimoto thyroiditis, thyroid carcinoma, neck irradiation and hypothyroidism (2). In patients who are hyperthyroid, the eye signs of TAO usually develop within 18 months of dysthyroidism; very often, they develop concurrently (3). We describe here 9 cases of TAO, who did not develop hyper-thyroidism on more than 20 months of follow up.

METHODS

Patients

The study was conducted at the Endocrine clinic of Ram Krishna Mission Seva Pratisthan Hospital. All patients with TAO attending the clinic of between Jan 2006 to Dec 2007 were included into the study if they satisfied the following criteria.

Inclusion criteria:

TAO with normal thyroid function for more than 18 months. All were TRAb positive except 3 patients who were seen 4 to 6 years earlier, as the test was not available to us at that time. The patients were diagnosed and TAO was graded following the "NO SPECS" classification (4) (see Table 1 and photos in the appendix)

Exclusion criteria:

1. Thyroid associated orbitopathy with thyrotoxicosis
2. Anti-TSH receptor antibody negative
3. Development of hyperthyroidism within 18 months of TAO.

Photographs of patients have been included in an appendix at the end of the report with their written consent. Patient No. 7 has died and consent was received from her son. This patient had enucleation of her right eye with fitment of prosthesis. Permission was given by Patient No. 8 to include his MRI scan.

Methods

TSH receptor antibodies were measured by a competitive binding radioreceptor assay manufactured by Immunotech, SA, Marseille, France. Free T3, Free T4 and TSH were measured by sensitive chemiluminescence microplate assays (Monobind Inc, Lake Forest CA, USA). A thyroid scan with 99m Technetium pertechnetate (99Tc scan) was performed on all patients. All patients also had CT or MRI scans of their orbits

RESULTS

We reviewed all retrievable charts of patients seen at our institution for Graves' ophthalmopathy between January 2006-December 2007. TAO was diagnosed by a combination of clinical symptoms and signs and CT scans. CT scans of the orbits were performed on all patients, primarily to exclude other pathology. All showed enlargement of the external ocular muscles in varying degrees. The 99Tc scans were normal in all patients. Most patients with TAO had some form of thyroid dysfunction (dysthyroid group), as evidenced by a history of abnormal thyroid function tests, previous diagnosis of thyroid dysfunction by an outside physician, or current or past use of thyroid medications. Nine patients (9/78 total patients; 11.5%) were found to be euthyroid for over a minimum of 18-months of follow-up., based on consistently normal thyroid function tests, lack of hyperthyroid clinical features, absence of medical or surgical treatment for thyroid dysfunction, and a negative history of any thyroid associated abnormalities (see Table 1). These patients were seen every four months in our clinic and their thyroid function tests were continuously monitored. Patients who

subsequently developed thyroid dysfunction were excluded from our follow up. However, we cannot exclude the possibility that some of these patients had an undetected, transient episode of asymptomatic hyperthyroidism. In 3 patients (Pts no. 7,8,9), TRAbs could not be done as this test was not available in our centre at that time.

Table 1. Clinical and biochemical data of the 9 patients.

Pts	TAO manifestations and NO-SPECS class ¹	Baseline data						Follow-up data		Follow-up duration months
		FT4 ng/dl <i>0.9-2.4</i> ²	TSH mU/L <i>0.3-6.2</i>	TRAb U/L <1	⁹⁹ Tc Scan	anti-TPO kU/L <40	anti-Tg kU/L <125	FT4 ng/dl <i>0.9-2.4</i>	TSH mU/L <i>0.3-6.2</i>	
1	Bilateral exophthalmous Class 3	1.86	2.48	3.1	N ³	-	-	1	0.4	21
2	Monolateral left exophthalmous Class 3	1.26	3.24	1.2	N	18	165	1.36	2.2	20
3	Bilateral exophthalmous Class 3	1.48	0.6	1.2	N	10	260	0.86	1.75	30
4	Right ophthalmopathy with redness, swelling & watering Class 3	2.1	0.4	7.3	N	2.5	53	2.2	0.47	24
5	Bilateral exophthalmous and diplopia Class 4	2.0	0.6	6.7	N	20.6	58	1.60	1.0	24
6	Bilateral exophthalmous with congestion & watering Class 3	2.2	0.6	21.5	N	1.6	55	1.2	13.50	36
7	Bilateral exophthalmous, right visual loss Class 6	1.86	1.44	nd ⁴	N	8.2	45	1.22	2.24	41
8	Bilateral exophthalmous Class 3	1.96	1.28	nd	N	33	78	1.76	3.8	39
9	Bilateral exophthalmous with congestion & watering Class 3	1.66	1.04	nd	N	12	685	1.96	5.2	67

¹ see also the photos in the appendix.

² normal ranges are reported in italic

³ normal

⁴ not determined

DISCUSSION

The diagnosis of TAO was based on the presence of ophthalmopathy and confirmed in 6 out of 9 cases by positive serum TSH receptor antibodies, both specific indicators of Graves' disease. In one of the largest series reported in the literature (3), eye disease was associated with hyperthyroidism in more than 90 % of patients with active Graves' ophthalmopathy. Another recent review (5) reported that 10% of Graves' ophthalmopathy patients are euthyroid, but made no

comment as to the natural history of the euthyroid state. In the present series, ophthalmopathy and was not accompanied by thyroid hyperfunction (euthyroid Graves' disease) in 10% of the patients and did not evolve toward thyroid dysfunction during a follow-up of >20 months in 9 patients.

TRAbs detected with the Dynotest TRAK human have the highest diagnostic power to differentiate Graves disease from other thyrotoxic conditions and should be obtained for all patients with non-typical Graves disease (6). However, euthyroid Graves' ophthalmopathy with negative autoantibodies (including TRAb) has also been described (7,8). Graves' ophthalmopathy as a rule develops at a time when thyroid autoimmunity also exists. Hyperthyroidism precedes the eye disease or occurs simultaneously in most of the cases and in only minority of cases it develops after the eye disease and usually within one year after the onset of eye disease (9).

In our study, 9 patients with euthyroid TAO were identified who did not develop thyrotoxicosis when followed for more than 20 months. However at least one patient (no. 6) developed hypothyroidism. In comparison to a series of patients with dysthyroid ophthalmopathy (10), most of our patients were males (6 out of 9), they were younger (mean age \pm SD: 44.6 \pm 15.9 vs 56 \pm 13.5 years), had less severe ophthalmopathy and a similar body mass index (mean BMI \pm SD: 26.5 \pm 2.1 vs 26 \pm 0.8).

CONCLUSION

A large body of evidence suggests that when TAO occurs in euthyroid Graves' disease patients, hyperthyroidism supervenes within 12 months of diagnosis. In the present study, a subset of patients were identified who did not develop hyperthyroidism by more than 20 months. These patients were referred fairly late to our clinic, after having ineffective treatment for glaucoma and various eye problems. It is important to be aware of the connection of TAO to thyroid disease and early endocrinology referral should be mandatory.

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APPENDIX: Photographs of the 9 patients and MRI scan of patient 8.

Patient 1, female, 42 years



Patient 2, male, 28 years



Patient 3, male, 49 years



Patient 4, female, 31 years



Patient 5, male, 18 years



Patient 6, male, 60 years



Patient 7, female, 58 years



Patient 8, male, 52 years



Patient 9, male, 64 years



WHY THYROID HORMONE TRANSPORTERS ARE IMPORTANT

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ABSTRACT

Thyroid hormone (TH) plays an essential role in the proper development of the brain and peripheral tissues. Lack of sufficient TH results in abnormal development, including mental retardation. It has become clear that TH transporters are necessary for proper TH metabolism and action inside the cell. Different specific TH transporters are known to date including MCT8, MCT10 and OATP1C1. MCT8 and MCT10 are widely expressed throughout the body, whereas expression of OATP1C1 is rather restricted to specific areas in the brain and testis. The clinical importance of TH transporters is dramatically shown in patients with mutations in *MCT8*, suffering from severe psychomotor retardation in combination with disturbed TH levels, especially high serum T₃ levels. No patients have been identified yet with mutations in *MCT10* or *OATP1C1*. *Mct8* deficient mice show no overt neurological deficits but have the same marked disturbed TH serum levels. Apparently mice have a different subset of TH transporters important for TH transport into the brain. It is expected that more TH transporters will be identified to explain the cell-specific subsets of TH transporters in normal tissue and brain. (*Hot Thyroidol. 2009: e14*).

Key-words: thyroid hormone; transport; MCT8; MCT10; OATP1C1; brain; development.

Introduction

Thyroid hormone (TH) is important for the foetal growth and development of different tissues, especially the brain, and for the regulation of the basal metabolic rate throughout life. Disturbances in TH supply due to, for example, maternal iodine deficiency during foetal development cause severe neurological abnormalities in the neonate (1). However, also in the postnatal period TH is essential for further development of the brain, and in many countries neonatal screening programs have been instituted to detect congenital hypothyroidism and to prevent mental retardation by early supplementation of TH (2).

The biological effects of TH are mediated by binding of the active form T_3 to its nuclear receptor, resulting in a change of interaction of the receptor with T_3 -responsive elements in regulatory regions of the target genes (3). The thyroid itself produces predominantly T_4 , which is converted to T_3 through outer ring deiodination by the deiodinase D1 or D2. T_4 is also metabolized to receptor-inactive rT_3 or T_3 is inactivated to $3,3'$ - T_2 through inner ring deiodination by D3. TH availability is regulated by these three different deiodinases (4). D1 is expressed in liver, kidney and thyroid and is assumed to contribute to the production of serum T_3 and clearance of serum rT_3 . D2 is important for local production of T_3 in the central nervous system but may also contribute to the production of serum T_3 . D3 is expressed in adult brain and skin, and at high levels in multiple foetal tissues as well as in the placenta and the uterus during pregnancy. D3 is only capable of degrading TH and is thus important for the negative control of both tissue and serum T_3 levels.

The biological activity of TH is determined by the intracellular T_3 concentration available for binding to its nuclear receptor, and this depends on a) the circulating concentrations of T_4 and T_3 , b) the activities of the different deiodinases catalyzing the production or degradation of T_3 and c) the presence of transporters regulating TH specific uptake and/or efflux. Although it has been thought for a long time that the lipophilic THs are capable of crossing the plasma membrane by simple diffusion, it has become increasingly clear that this is impossible without transporters (5).

Existence of TH transporters

Studies already published in the 1970s by Krenning *et al* (6) and Rao *et al* (7), have shown saturable and energy-dependent transport of T₃ and T₄ into rat hepatocytes. Since then different research groups have reported studies confirming carrier-mediated, mostly energy- and Na⁺-dependent transport of TH into a variety of cells from different species.

Fifteen years ago, we decided to use the *Xenopus laevis* oocytes expression system, at that time the most successful method known for cloning and characterization of plasma membrane transporters, to find the long-sought Na⁺-dependent hepatic TH transporter (8). For this purpose, *X. laevis* oocytes were injected with rat liver mRNA and analyzed for TH transport. Only a modest increase in TH uptake was found in oocytes injected with different mRNA size fractions, but we were not successful in cloning a single TH transporter. The investigations were shifted towards functional screening of already known transporters for homologous ligands still using the oocytes expression system. Using this approach, we were successful this time as we identified different candidates as potential TH transporters within the organic anion, fatty acid and amino acid transporter families (9).

In the last decade, different groups have identified transporters that are capable of transporting TH (9, 10). One specific transporter is the Na⁺-taurocholate cotransporting polypeptide (NTCP) (11). Although NTCP is Na⁺-dependent and exclusively expressed in liver it appears not to be the long-sought hepatic TH transporter as it has a low affinity for TH. Also different members of the Na⁺-independent organic anion transporting polypeptide (OATP) family were characterized as TH transporters although with low affinity (12). Most of the OATP family members are widely expressed and multi-specific accepting a wide variety of ligands. A notable exception is OATP1C1, which is almost exclusively expressed in brain, in particular in capillary endothelial cells and in the choroid plexus, and shows high specificity for only T₄ and rT₃ (13-15). Recently, van der Deure *et al* reported that also T₄ sulphate (T₄S) is transported by OATP1C1 (16). This transporter is thought to be very important for the transport of T₄ across the blood-brain-barrier (BBB) into the brain. The heterodimeric L-type amino acid transporters, LAT1 and LAT2 are capable as well to facilitate Na⁺-independent cellular entry and efflux of TH, but show restricted tissue distributions (17). We also found that fatty

acid translocase (FAT) expression in oocytes induces TH uptake (18). However, FAT (also known as CD36) is not a true transporter but may facilitate TH transport by forming a complex with neighbouring transporters.

Identification of MCT8 as TH transporter

Due to the structural relation between aromatic amino acids and THs, the group of Blondeau and Francon (19, 20) had already suggested in the 1990s the involvement of a T-type amino acid transporter in the uptake of TH. Such a transporter has been cloned and characterized by Kim *et al* in 2001 (rat, (21)) and 2002 (human, (22)), termed T-type amino acid transporter 1 (TAT1), later referred to MCT10 (SLC16A10). This transporter is a member of a larger family of monocarboxylate transporters (23, 24). The family name is derived from the preference of the first 4 members (MCT1-4) for the substrates lactate and pyruvate. Kim *et al* have clearly shown the transport of L-DOPA and the aromatic amino acids phenylalanine (Phe), tyrosine (Tyr) and tryptophan (Trp) (21). However, they could not find any transport of THs by MCT10. Within the MCT family, MCT10 and MCT8 share the highest level of amino acid sequence identity (49%). So, we hypothesized that MCT8 could be the T-type amino acid transporter that also accepts THs.

In close collaboration with Prof Andrew Halestrap we studied rat MCT8 in our former *X. laevis* oocyte expression system and we found an incredible 10-fold induction in TH transport (25). This was by far the best TH transporter we had studied up till now. Substrates studies revealed that MCT8 showed ligand specificity exclusively towards iodothyronines; sulphated THs, lactate, Leu and the aromatic amino acids Phe, Tyr and Trp were not transported.

The gene coding for human *MCT8* is located on the X-chromosome (Xq13.2) and consist of 6 exons (26). The putative structure of MCT8 consists of 12 transmembrane domains and both the N- and the C-terminus are located intracellular (see Fig 1). In contrast to most species, including the mouse and rat, the human *MCT8* gene contains two possible translation start sites (TLSs). Depending on which of these TLSs is used, proteins are generated of 613 (hMCT8L) or 539 (hMCT8S) amino acids *in vitro*. Preliminary studies using human liver revealed the presence of mRNA species containing both TLSs. The function of the additional N-terminal sequence in hMCT8L

is still under investigation. The N-terminal domain in both hMCT8 isoforms is enriched in Pro (P), Glu (E), Ser (S) and Thr (T), also known as PEST domain (27). PEST domains are often associated with rapid protein degradation, but the function of this domain in MCT8 is yet unknown.

In the meantime we changed our expression system from *X. laevis* oocytes to mammalian cells like COS1 (Green monkey kidney cells) or JEG3 (human choriocarcinoma cells).

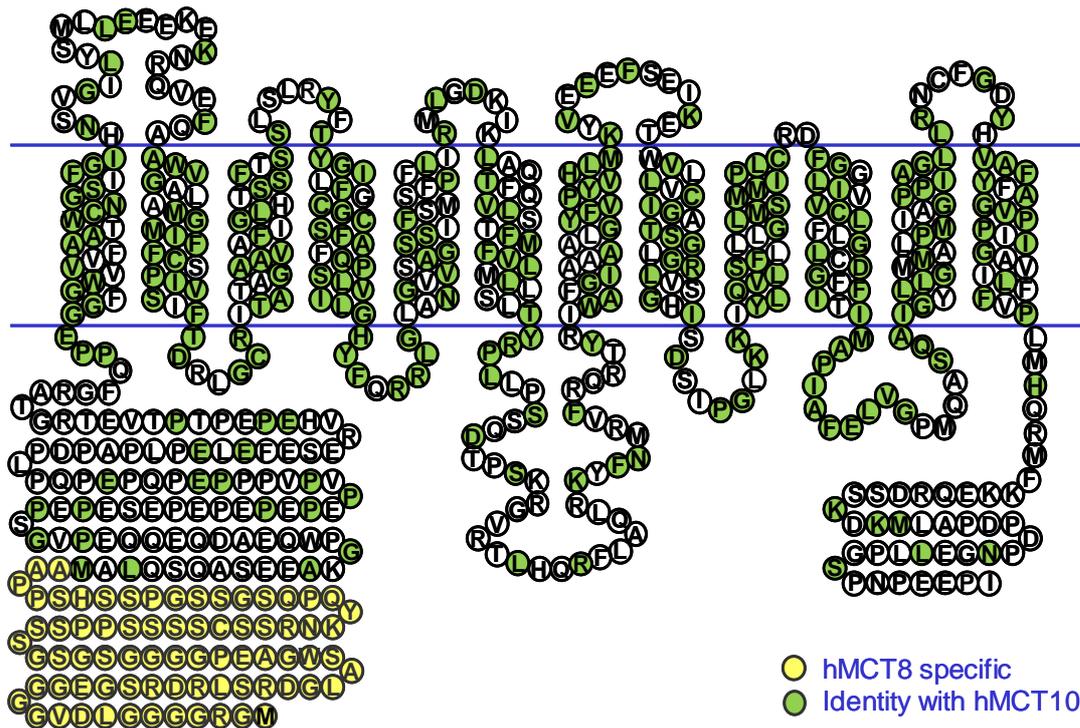


Figure 1. Putative structure of human MCT8. In yellow, the hMCT8 specific structure is indicated; in green, amino acid identity between hMCT8 and hMCT10 is indicated. Both the N- and C-terminus are located intracellular.

Transfection with hMCT8S results in a significant increase in TH transport but the fold induction was much lower than observed in oocytes (28). Further studies revealed the capacity of hMCT8S to mediate efflux even faster than the uptake of TH. To prevent the rapid efflux of TH in our transport studies we co-transfected our cells with mu-crystallin, a cytosolic TH binding protein. When doing so, we increased the uptake signal to the same level as what we had found earlier in our *X. laevis* oocyte expression system. To demonstrate that MCT8 increases the intracellular availability of TH for metabolism by the different deiodinases, cells were co-transfected with MCT8 and for instance D3 to

measure T_3 metabolism. The results showed that expression of a TH transporter markedly increased intracellular TH metabolism.

MCT8 is widely expressed in liver, heart, intestine, placenta, kidney and brain (29, 30). Detailed studies of the mouse brain by the groups of Heuer *et al* (31) and Roberts *et al* (32) revealed high MCT8 expression in neuronal populations of the cerebral and cerebellar cortex, hippocampus, striatum and hypothalamus. This suggests that MCT8 is involved in neuronal TH transport. MCT8 is also expressed in the choroid plexus and in large capillaries indicating its involvement in the transport of TH across the BBB and/or blood-cerebrospinal fluid (CSF) barrier. Studies from Alkemade *et al* have shown that at the interface of the human hypothalamus and the peripheral circulation, MCT8 protein is present particularly in neurons of the paraventricular and infundibular nuclei (33). Also strong MCT8 expression has been observed in tanycytes that are located in the central lining of the third ventricle. These cells are in close contact with the CSF and the hypothalamus and median eminence. As OATP1C1 and D2 are expressed in these tanycytes, conversion of T_4 to T_3 in these cells plays an important role in the negative feedback of TH at the hypothalamus (31).

Pathophysiology of human MCT8

In 2001, the group of Grueters in Berlin and our group in Rotterdam investigated two apparently identical severely mentally retarded male patients with abnormally high serum T_3 levels. Since no mutations were found in the genes coding for the T_3 receptors or the different deiodinases, we raised the hypothesis that this syndrome of TH resistance was caused by a defect in cellular TH uptake. Therefore, we screened the *MCT8* gene for mutations. In the first patient we could not amplify the first exon of the *MCT8* gene and later results showed the exact deletion of 24 kb, comprising part of the 5'-UTR, entire exon 1 and also a part of intron 1. Family investigation revealed that the mother was carrier of this deletion and that one of her other sons was also affected with this deleterious mutation in *MCT8*. In the *MCT8* gene of the second patient we found a missense mutation in the second exon resulting in an Ala224Val substitution (34).

Since 2004, more than 40 families have been described with hemizygous affected males carrying mutations in the *MCT8* gene (34-43). Recently, a sporadic case of a female carrying a *de*

novo translocation that disrupted the *MCT8* gene in combination with unfavorable nonrandom X-inactivation has been reported (44). The reported mutations range from large deletions, resulting in the loss of one or more exons, smaller frame-shift deletions, triplet (1-amino acid) deletions or insertions, nonsense mutations resulting in a premature truncation of the MCT8 protein, and missense mutations resulting in 1-amino acid substitutions. All patients share the severe neurological deficits and markedly elevated serum T₃ and low T₄ and rT₃ levels. The neurological phenotype includes in most patients central hypotonia, with poor head control; initially peripheral hypotonia, which evolves into spastic quadriplegia; inability to sit, stand or walk independently; severe mental retardation; and absence of speech (45). This severe form of X-linked psychomotor retardation had already been described in 1944 by Allan, Herndon and Dudley (46), since then referred as AHD syndrome (OMIM 300523).

We have tested a variety of the mutations found in patients with AHDS, especially the 1-amino acid deletions, insertions and substitutions as for these mutations it is not clear what the effect will be on the proper function of MCT8 (37, 43, 47). To test the effect of the mutation, we compared the function of the mutated with the wild-type hMCT8S using transiently transfected mammalian cell lines. TH transport was measured in cells transfected with the different variants of MCT8 alone, and metabolism of T₃ was measured in cells co-transfected with the different transporter variants and D3. The results from both tests showed that most mutations resulted in a complete loss of hMCT8S transport function, but significant residual activity was observed with a few MCT8 mutations, associated with a somewhat milder clinical phenotype. Thus, with the functional analysis of MCT8 mutations found in patients with AHDS we found impaired uptake and subsequently impaired metabolism of TH *in vitro*. These results predict that mutations in a TH transporter result in an impaired tissue TH supply especially into the brain and represent a novel mechanism for TH resistance.

***Mct8* deficient mice**

To study the pathophysiology of MCT8 deficiency Dumitrescu *et al* (48) and Trajkovic *et al* (49) have generated independently two different *Mct8* knockout mouse strains. Unexpectedly, these

mouse mutants do not show any overt neurological deficits, but they exhibit the same marked increase in serum T_3 and decrease in serum T_4 and rT_3 as found in the AHDS patients with mutations in *MCT8*. Analysis of the mutant mouse liver showed an increased activity of D1 as well as an increased T_3 content. This indicates that the liver is in a hyperthyroid state. In contrast, the T_4 and T_3 content in the brain was diminished and associated with an increase in D2 activity and a decrease in D3 activity, reflecting a hypothyroid state of this tissue. In the mutant mouse brain T_4 entry was not affected as T_3 uptake was almost completely diminished. Recently, comprehensive studies on the *Mct8*-deficient mice revealed several behavioral abnormalities (50) as decreased anxiety-related behavior reported in hyperthyroid mice and, in contrast, reduced grooming and increased latency of grooming reported in hypothyroid rats. This indicates that also certain brain areas may be hyperthyroid, whereas other areas remain hypothyroid in *Mct8* knockout mice.

Apparently, *Oatp1c1* is involved in the specific transport of T_4 via the BBB or blood-CSF barrier into the mouse brain (15, 32). The local conversion of T_4 to T_3 in the mouse brain is sufficient to provide neuronal cells as cerebellar Purkinje cells with enough TH to prevent serious neurological damage, as they show normal dendritic outgrowth and responded normally to T_3 treatment *in vitro*. Studies by Ceballos *et al* (51) reported that the brains of *Mct8* mutant mice do not respond to a low dose of T_3 due to the critical restriction of T_3 transport into the brain via the BBB rather than at the plasma membrane of a neuronal cell. The fact that *Mct8* mutant mice do not show any overt neurological deficits could be explained by a different subset of TH transporters in the mouse brain when compared to the human brain leading to a less severe TH deficiency in mouse brain. Wirth *et al* (50) speculate that the L-type amino acid transporter *Lat2* might compensate in the mouse, but not in the human brain for the lack of *Mct8* as only a low *LAT2* expression was found in developing neurons in the human brain. Also, the rat brain expresses the TH transporter variants *Oatp1a4* and *Oatp1a5* (52), but both transporters have to our knowledge no ortholog in the human brain.

Recently, Di Cosmo *et al* (53) reported the use of the ligand 3,5-diiodothyropropionic acid (DITPA) as available analogue of TH bypassing the involvement of *MCT8* to be transported into different target tissues. Using the *Mct8* mutant mouse they found that DITPA is relatively *MCT8* independent for entry into the hypothyroid brain, and normalizes the thyrotoxic state of the liver,

resulting in the achievement of an overall euthyroid state. According to the authors, the clinical use of DITPA in MCT8 patients needs further studies.

Identification of human MCT10

The human *MCT10* gene is located on chromosome 6q21-q22 and has the same structure as the human *MCT8* gene. The mRNA contains one single TLS and codes for a protein of 515 amino acids, containing 12 transmembrane domains (see Fig 1). Also the MCT10 protein contains a PEST domain as described for MCT8. Based on its expression pattern human MCT10 may be important for TH transport specifically in intestine, kidney, liver, skeletal muscle and placenta (21, 22, 30). Ramadan *et al* (54) reported on the function of mouse MCT10 as a net efflux pathway for aromatic amino acids and showed localization of the protein to the basolateral membrane of small intestine and proximal kidney tubule cells.

In view of the involvement of a T-type amino acid transporter in uptake of TH and due to the homology between MCT8 and MCT10 (see Fig 2), we decided to reinvestigate the possible transport of TH by MCT10.

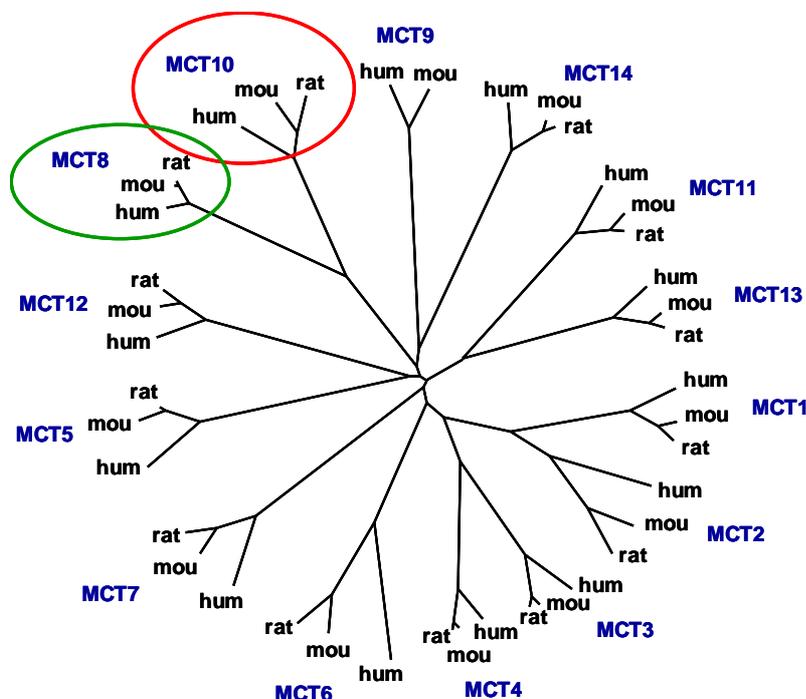


Figure 2. Phylogenetic tree of the monocarboxylate transporter (MCT) family. In the green cycle are the MCT8 members located and in the red cycle the closely related MCT10 members.

Our studies showed clear TH transport capacity by MCT10 showing preference of T_3 over T_4 (55). Like MCT8 also MCT10 facilitates TH uptake as well as efflux. As with MCT8, cells co-transfected with MCT10 and one of the deiodinases largely stimulated the intracellular deiodination of TH. Uptake of T_3 in cells transfected with MCT10 was significantly inhibited by the aromatic amino acids. Also the uptake of T_3 was more inhibited by an excess of different iodothyronines in cells transfected with MCT10 than MCT8 (personal observation).

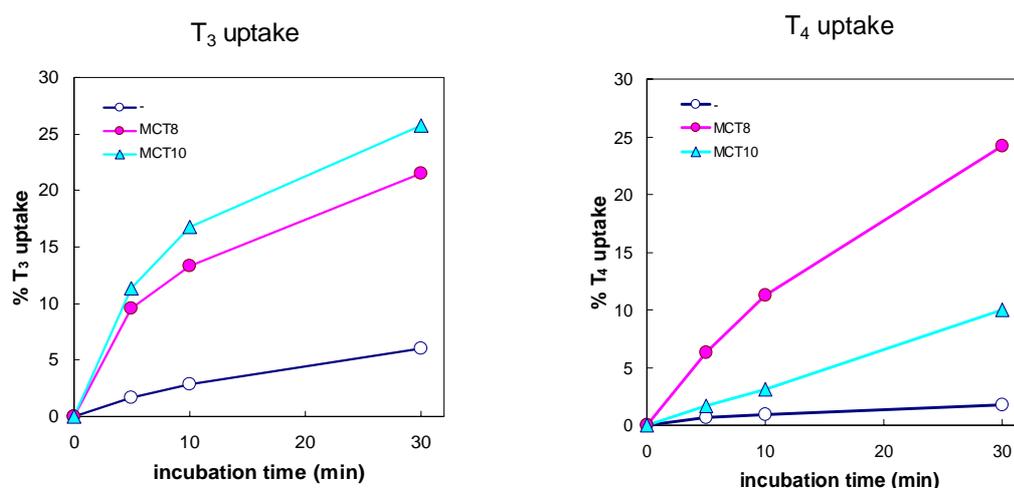


Figure 3. Transport of T_3 or T_4 into cells transfected with hMCT8 or hMCT10. Uptake of TH was measured in the presence of mu-crystallin, a cytosolic TH binding protein (55).

Together with its high homology with MCT8, it is very likely that MCT10 could be an important transporter in human physiology by regulating local and tissue TH levels. But so far, no patients with mutations in MCT10 have been identified.

Concluding Remarks

With the discovery of patients with mutations in MCT8 the physiological importance of transporters for the metabolism and action of TH has been generally recognized. Next to MCT8, also MCT10 and OATP1C1 are characterized as specific TH transporters (see Table 1). It is clear that more TH transporters will be identified in the near future, because the Na^+ -dependent, high affinity TH transporter expressed in liver has not yet been discovered. From mouse and human studies it is also

confirmed that different subsets of TH transporters are present in the two species, especially in specific areas of the brain.

Table 1. Important candidates for specific TH transport.

	hMCT8	hMCT10	OATP1C1
Function	Uptake/efflux T ₃ + T ₄	Uptake/efflux T ₃ >T ₄ Export of aromatic amino acids	Uptake T ₄ + rT ₃ > T ₄ Sulphate
Chromosome	X	6	12
Physiological relevance	AHDS: mental retardation + elevated serum T ₃ levels	Unknown	Unknown
Tissue expression	Liver, kidney, heart, skeletal muscle, brain, thyroid, placenta	Intestine, liver, kidney, skeletal muscle, placenta	Blood-brain-barrier brain capillaries, testis

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CASE REPORT**GRAVES' HYPERTHYROIDISM IN A PATIENT WITH PENDRED'S DYSHORMONOGENESIS**IM Ibrahim,¹ DO McDonald,² CJ Owen,^{1,2} P Kendall-Taylor,^{1,2} SHS Pearce^{1,2}¹Endocrine Unit, Royal Victoria Infirmary, Newcastle upon Tyne, UK and ²Institute of Human Genetics, Newcastle University, UK.

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ABSTRACT

The clinical details of a young woman with Pendred's syndrome who developed autoimmune hyperthyroidism are presented. Although the dysmorphonogenesis of Pendred's syndrome is associated with a defect in iodide organification, her hyperthyroidism was successfully treated with ¹³¹I radioiodine. The nature of the thyroid hormonogenic defect in Pendred's syndrome and the relative functional importance of the apical iodide transporting mechanisms are illustrated. In addition, the hyperthyroidism, and its subsequent treatment, clearly demonstrate that Pendred's syndrome results only from a partial block to thyroid hormone synthesis. Radioiodine, perhaps administered after recombinant TSH thyroid stimulation, may be an alternative treatment for goitre in Pendred's syndrome. (*Hot Thyroidol. 2009: e13*).

Key-words: dysmorphonogenesis; iodide transport; hyperthyroidism; Graves' disease; Pendred syndrome

Introduction

Pendred's syndrome (PDS) is an autosomal recessive disorder characterized by congenital sensorineural hearing loss and progressive goitre (1). Other features of PDS are enlargement of the vestibular aqueduct and "Mondini" malformation of the cochlea, which are typically present on imaging studies. In about 50% of patients, the circulating thyroid hormone levels are normal, while the remainder develop overt hypothyroidism due to defective iodide transport, which results in thyroid dysmorphogenesis (2). Typically, affected subjects demonstrate avid thyroidal iodide uptake but impaired iodide organification, as determined by an exaggerated release of uncoupled radioiodine tracer from the thyroid following perchlorate administration (a positive perchlorate discharge test) (3). PDS was assigned to chromosome 7q31 by linkage analysis (4,5), and mutations in the gene encoding an anion transporter, *SLC26A4*, also termed "Pendrin" were found in affected patients (6). There are now more than 50 such independent *SLC26A4* gene mutations that have been characterized as causing PDS (6-12). The mature *SLC26A4* transporter is located on the apical membrane of thyrocyte (13,14), where it is responsible for the transport of inorganic iodide into the colloid space. Iodide is then available for organification and subsequent incorporation into iodotyrosine compounds, the precursors of thyroid hormones. Pendrin acts as an anion exchanger, allowing iodide flux out of the thyrocyte with a reciprocal influx of chloride. In the presence of a loss of function in the *SLC26A4* transporter, apical iodide transport is defective, leading to impaired organification of iodide and the hypothyroidism of Pendred's syndrome. However, as many patients with PDS remain euthyroid, organification is only partially deficient, suggesting that there may be either functional heterogeneity in the activity of the abnormal transporters in PDS or that other mechanisms exist that can compensate for lack of the *SLC26A4* protein activity (15). In this paper we report a rare association of autoimmune hyperthyroidism in a patient with PDS, and its treatment.

Case report

The patient is the daughter of unrelated parents who were both known to have Pendred's syndrome (PDS). There was no family history of autoimmune disorders. At the age of 2 years, she was found to have sensorineural deafness and clinical examination showed her to have a large goitre. Because of her family background, she was thought likely to be an obligate carrier of two *SLC26A4* gene mutations, and compound heterozygosity for a 1101+1G>A and 2015G>A base changes within the *SLC26A4* gene were demonstrated (9). These DNA changes predict a donor splice-site mutation of exon 8 and a non-conservative G672E missense mutation in exon 17 of *SLC26A4*, respectively. Both mutations are likely to result in a non-functional protein, encoding a transporter either with a missing domain, or one that is unable to reach the cell surface due to misfolding, respectively (16).

At the age of eleven years, she was first found to be biochemically hypothyroid with a serum TSH of 6.4mU/l; she was treated with thyroxine 150µg daily, with the aim to keep the serum TSH towards the lower limit of normal to suppress the growth of her goitre. She attended clinic sporadically, when her

mother and a sign-language interpreter were available to accompany her. However, at the age of 18 she lost 17 Kg in weight, associated with tremor, heat intolerance and palpitations. There were no eye signs or thyroid dermopathy. Her repeat thyroid function tests showed a raised free T4 of 64 pmol/l (normal range 11-23), free T3 of 13.7 pmol/l (3.5-6.5), and an undetectable TSH. Thyrotropin-binding inhibitory immunoglobulins (TBII) were raised at 36 U (normal <10) and thyroid peroxidase antibodies were positive at 89 kU/l (normal <60). Her serum thyroglobulin measured by RIA was 63.4 µg/l. Full details of the course of her thyroid function tests are shown in Table 1. A ^{99m}Tc thyroid uptake scan showed a patchy but widespread increase in uptake (Figure 1).

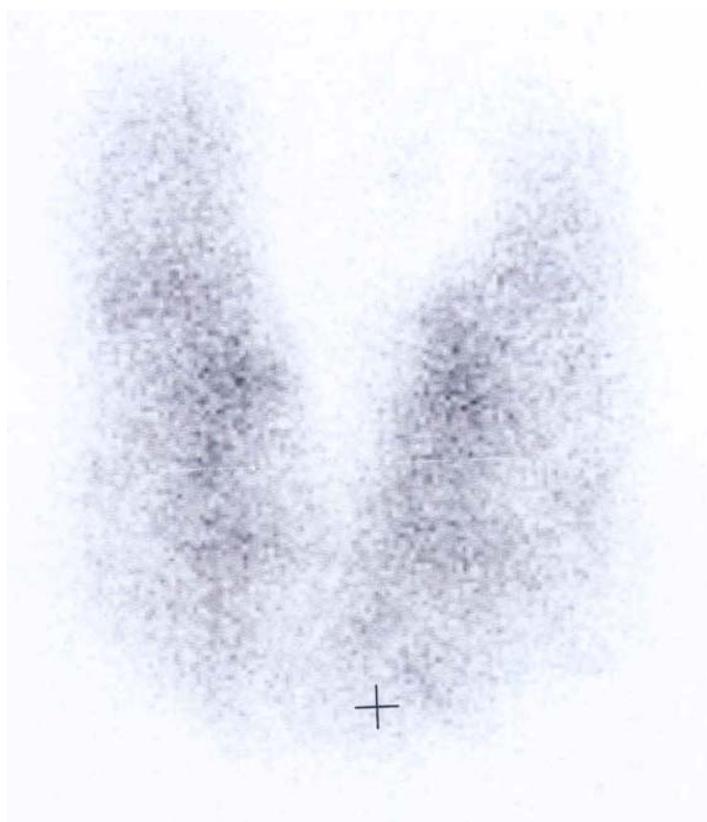


Figure 1. ^{99m}Tc (pertechnetate) thyroid uptake scan. The 20 minute thyroid uptake was 10% of tracer (normal <3.5%).

After repeat testing, her thyroxine medication was stopped, but she remained mildly hyperthyroid despite this. After some discussion about the uncertain outcome, she agreed to radioiodine as therapy for her hyperthyroidism and was treated with 400MBq (11mCi) ^{131}I . Her thyroid function tests became normal 7 weeks after the treatment and she was restarted on thyroxine and remains well at last follow up (Table 1). There was substantial reduction in her goitre noted over the 6 months following her radioiodine therapy.

Table 1. Thyroid function testing and weight

Date	TSH	Free T4	Free T3	Thyroxine dose	Weight
<i>Normal ranges</i>	<i>0.3-4.7 mU/l</i>	<i>11-23 pmol/l</i>	<i>3.5-6.5 pmol/l</i>	<i>µg per day</i>	<i>Kg</i>
09/04/98	2.82	15	-	150	82
06/07/00	3.54	14	-	175	104
09/12/02	0.55	17	-	200	115
03/02/03	0.15	31	6.0	200	118
28/02/05	<0.05	64	13.7	200	100
16/05/05	<0.05	67	13.4	Discontinued	100
13/06/05	<0.05	26	7.6	-	101
14/07/05	Radioiodine 400 MBq (11mCi) administered				
22/08/05	1.13	12	-	150 Re-started	104
20/03/06	0.06	21	-	150	105
12/06/06	0.28	20	4.8	150	104

Methods

Mutational analysis.

The genetic analysis of this patient (III-1) and her family have been previously reported, with her paternal and maternal relatives being designated as families 14 and 13, respectively (9).

Biochemical assays.

Serum thyrotropin (TSH) was analyzed using the Bayer ADVIA Centaur analyzer (Bayer Diagnostics Division, Newbury Berkshire, UK) by 2 site sandwich immunoassay using direct chemiluminescence. Serum free T4, free T3 and anti-thyroid peroxidase antibodies were measured by competitive immunoassay using direct chemiluminescent technology (Bayer centaur). Thyrotropin-binding inhibitory immunoglobulins (TBII) assay (RSR Ltd, Pentwyn, Cardiff, UK), measure the ability of TBII in the patient sample to inhibit the binding of ¹²⁵I-Labelled TSH to recombinant thyroid receptors on coated polystyrene tubes. The % inhibition is calculated as an index.

Thyroglobulin (Tg) was measured using coated tube 2 site immunoradiometric sandwich assay, using 4 monoclonal anti (Tg) antibodies to specific sites on (Tg) molecule on coated tubes (CIS Bio international). The intra-assay coefficients of variation for each measurement (at the given concentration) were: TSH 3.8% (5.3mU/l), FT4 7.9% (13.5pmol/l), FT3 6.3% (4.5pmol/l), thyroid peroxidase antibodies 3.7% (522kU/l), TBII 8% (37U), Tg 8.4% (17µg/l).

Discussion

Our investigations show that this young woman who had Pendred's syndrome, a state of thyroid dyshormonogenesis, developed hyperthyroidism owing to Graves' disease. Although at first, the diagnosis of thyrotoxicosis 'factitia' due to excessive thyroxine ingestion was suspected, several factors suggested true autoimmune hyperthyroidism as the cause of the thyrotoxicosis. These include a progressive rise in circulating thyroid hormone levels during treatment with a stable dose of L-thyroxine, detectable serum thyroglobulin at the time of thyrotoxicosis, positive thyroid peroxidase and thyrotropin-binding inhibitory autoantibodies, and a low serum thyroxine to triiodothyronine ratio once thyroxine was discontinued. Her thyroid uptake scan is particularly difficult to interpret, since a rapid uptake of pertechnetate tracer has been reported in untreated Pendred's syndrome, probably driven by a high-normal or high TSH. However, in the context of an undetectable TSH, the high pertechnetate uptake (10% at 20 mins; normal <3.5%) is caused, at least in part, by her endogenous hyperthyroidism due to thyroid stimulation with TSH-receptor antibodies (Figure 1). In a person with no underlying thyroid disease, low isotope uptake would be expected in the presence of an undetectable TSH during exogenous thyroxine administration. The improvement in thyrotoxicosis following the radioiodine and the subsequent reduction in goitre size are also in keeping with the diagnosis of Graves' disease.

Radioiodine was selected as treatment for this patient because she was keen to avoid thyroid surgery, which both her mother and her maternal uncle (who was also affected with PDS) had undergone, and there was the additional possibility of shrinkage of her goitre with the treatment. We were unsure whether radioiodine would be efficacious in this circumstance: there are no previous reports of radioiodine use in PDS. Nevertheless, as we were confident she had endogenous hyperthyroidism, it was clear she could trap and organify enough iodide to become thyrotoxic. Therefore, it seemed reasonable to assume there would be a therapeutic effect from radioiodine. Since she had a reduction in goitre size with the radioiodine, it is possible that this may be a useful therapeutic option for goitre reduction in other patients with PDS, perhaps administered following recombinant TSH stimulation, rather than with the endogenous TSH-receptor stimulation from autoantibodies as in this case. This is an area of practice where there is little published experience.

Thyroid autoantibodies, including TSH receptor stimulating antibodies, have been previously reported in Pendred's syndrome (17), which suggests that there may be a predisposition to thyroid autoimmunity in PDS patients. The large size of the thyroid gland in PDS, together with increased expression or turnover of the proteins involved in thyroid hormone biosynthesis, which are also the targets of the autoimmune attack, could explain an association of thyroid autoimmunity and this form of dyshormonogenesis. In particular, there is evidence of upregulation of thyroid peroxidase activity in PDS thyroid tissue (18, 19). Although, one study has shown genetic linkage of a large family with autoimmune thyroid disease to the chromosomal interval containing the PDS locus (20), there is currently no formal evidence to support the idea that genomic variation in the *SLC26A4* gene itself is

responsible for autoimmunity. Interestingly, autoantibodies recognizing Pendrin protein have recently been described in Hashimoto's thyroiditis and, to a lesser extent in Graves' disease, using immunoblotting with patient sera (21).

During thyroid hormone synthesis, the initial transport of iodide from the circulation (extracellular fluid) occurs at the basolateral surface of the thyrocyte through the sodium-iodide symporter (NIS). This has the ability to transport iodide up a 20-fold concentration gradient to allow its accumulation in the thyrocyte. Inorganic iodide is then transported across the apical membrane of the thyrocyte, to bring it into close proximity to thyroid peroxidase, which is anchored to the apical membrane but with its catalytic "head" in the colloid space. Iodide crossing the apical membrane via SLC26A4 is available to TPO to organify, whence it is rapidly incorporated into the tyrosyl residues of thyroglobulin to form the iodotyrosine thyroid hormone precursor molecules. Thus, one might predict that it would be difficult or impossible for an individual with the defective apical iodide transport found in PDS to become hyperthyroid. However, it is clear from our clinical findings in this case, that under certain conditions SLC26A4-mediated iodide transport is not a significantly rate-limiting step in thyroid-hormone synthesis. Since many patients with PDS remain euthyroid for many years, iodide organification is only partially deficient in many individuals with this condition, suggesting that other mechanisms of iodine transport exist that can compensate for lack of the SLC26A4 protein (15). Some studies (22, 23) have suggested that iodide efflux can also be mediated through a TSH-induced iodide porter via either a cAMP or a Ca^{2+} PIP₂ pathway. Golstein *et al.*, (24) used a membrane vesicle preparation of bovine thyroid to characterize two iodide channels with distinct biophysical properties. A second iodide transport mechanism has recently been proposed, termed the human apical iodide transporter (hAIT or SLC5A8), with substantial structural homology to the basolateral NIS co-transporter, but with an apical distribution in thyrocytes (25). This report goes some way to confirming the physiological relevance of an additional apical iodide channel(s) in man, that can compensate for defective transport mediated by SLC26A4.

To summarize, we report the probably unique case of a young woman with PDS who developed hyperthyroidism owing to Graves' disease. The clinical findings are used to illustrate the normal physiology of thyroid hormone synthesis, the mechanisms of apical iodide transport in the thyrocyte and the pathophysiology of dyhormonogenesis. The patient was successfully treated with a conventional dose of radioiodine, and this treatment may be worthy of further investigation in this condition.

Acknowledgements

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The Thyroid and Pregnancy: Historical & Scientific Vignette on The Brussels' Studies

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ABSTRACT

The first part of this manuscript is an overview of two successive prospective cohort studies, carried out in Brussels (1988-1998), aiming at evaluating the main changes in thyroid function and the clinical epidemiology of thyroid diseases associated with the pregnant state. Main results were to show the inadequate adaptation of thyroid function in pregnant women with a restricted iodine intake, leading to excessive thyroid stimulation, relative hypothyroxinemia and goiter formation in both mother and fetus. Another finding was the demonstration that CG is a maternal thyroid regulator (especially in 1st trimester), with 2% of the women at risk of developing gestational transient thyrotoxicosis when CG levels remain abnormally elevated during a prolonged period. The second study showed an overall 6.5% prevalence rate of positive thyroid autoantibodies, and women with autoimmune thyroid disorder (AITD) had a significantly increased risk of spontaneous miscarriage. Furthermore in women with AITD whose gestation progressed to term, frequent development of hypothyroidism was evidenced. These results led us to propose that thyroid function screening should be part of the routine management of pregnant women.

The second part outlines the landmarks that allowed to extend our views from the clinical epidemiology of thyroid disorders to their management, in particular iodine deficiency prevention during pregnancy, algorithms for systematic screening of thyroid dysfunction, and the establishment of consensus guidelines for the treatment of thyroid diseases during pregnancy and postpartum.

The third part is a short discussion on unresolved issues, in an attempt to help define some perspectives for future research. (*Hot Thyroidol. 2009: e12*).

Key-words: pregnancy, thyroid disorders, goitrogenesis, fetal development, iodine deficiency, thyroid autoimmunity, screening, consensus guidelines.

Part 1: Overview of the pioneering observational studies

Two successive prospective studies, carried out in Brussels over the span of a decade (1988-1998), allowed to delineate the main aspects of the clinical epidemiology of thyroid function and disorders associated with pregnancy and present a comprehensive view on how thyroid function is regulated in the pregnant state, in an attempt to delineate the pathways of thyroidal adaptation from physiology to pathology (1).

A cohort of 726 unselected consecutive apparently healthy pregnant women was investigated first in 1988-1989. Among them, 606 women had no known or detectable thyroid abnormality. Thyroid function was evaluated using a double cross-sectional and sequential study design, between first visit at our prenatal clinic and delivery (2). The results showed that iodine deficiency (ID) – considered mild to moderate in our country – was aggravated during pregnancy. As a result of more severe ID, in a context where the thyroid machinery must increase physiologically its hormone production to maintain the homeostasis of thyroid economy, one third of pregnant women presented relative hypothyroxinemia with preferential T₃ secretion and increased serum TG levels, as well as a doubling of serum TSH during the second half of gestation (although TSH remained within the normal range).

This pattern of changes in thyroid function was compatible with the concept of *excessive and prolonged thyroidal stimulation* including, as its most visible hallmark, development of gestational goiter in 9% of normal pregnant women. Goiter formation did not affect only a small fraction of pregnant women. It was shown that goitrogenesis was a general phenomenon and affected 75% of the women, with a increase in thyroid volume (TV) between early gestation and delivery (3). Furthermore, goiter formation affected also the fetus. This finding provided the first demonstration that goitrogenesis takes already place *in utero* in conditions where normal pregnant women have a restricted daily iodine intake (4). Thus, pregnancy constitutes a stimulus for both the maternal and newborn thyroid glands in conditions with ID. The importance of this notion is that it showed that, although maternal and fetal thyroid economies were regulated independently, the link between them was the iodine nutritional status of the mother. Maternal goitrogenesis was directly correlated to the biochemical indices of excessive thyroidal stimulation due to ID. In a subsequent study, we showed that a gestational goiter may persist one year after delivery, thus allowing us to propose the novel concept of a “*ladder phenomenon*” (Figure 1), whereby each subsequent pregnancy carried an added risk to aggravate goiter formation (5). At the same time already, we recommended that the iodine intake should be fortified as early as possible during pregnancy in our country to reach 150-250 µg/day, in order to avoid such pathologic sequence of events.

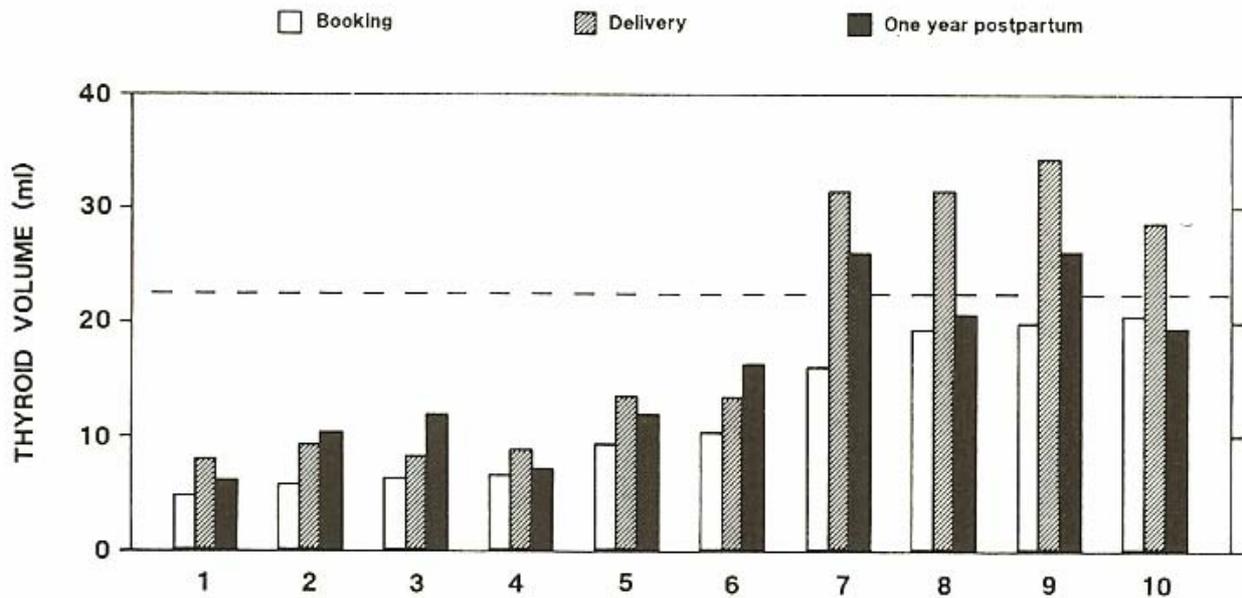


Figure 1. Thyroid volume (TV) was determined by ultrasonography in 10 women in 1st trimester, at delivery, and 1 year postpartum. Women selected for the study had TVs covering the entire normal range (from 5 to 22 mL) at the beginning of their pregnancy and TVs that increased by >25% of the initial size during gestation. Twelve months after delivery, TVs, which had increased by 54% on the average during gestation, had not reverted to initial individual TV sizes. Moreover, a goiter was still evident in 2 of the 4 women in whom a gestational goiter had developed (cases N° 7 & 9).

Another important finding was the blunting of serum TSH due to the thyrotropic action on the thyroid gland of elevated serum concentrations of human chorionic gonadotropin (hCG) near the end of 1st trimester, with a mirror image between peak hCG values and a nadir of serum TSH values. Turning our efforts to evaluate more systematically the role of elevated hCG levels on the pituitary-thyroid axis, a study showed that ~20% of pregnant women underwent transiently partial or total suppression of serum TSH levels. In 10% of the latter (i.e., 2% of the female pregnant population), TSH suppression was associated with supranormal free T₄ levels, hence leading to a state of transient biochemical hyperthyroidism of non autoimmune origin, that was coined “GTT” (gestational transient thyrotoxicosis) (6). Normal TSH values were progressively restored during the 2nd trimester. In a later study where twin and singleton pregnancies were monitored sequentially during the first weeks of gestation, we showed that it was the both the amplitude and duration of peak hCG values that geared the changes in thyroid function (7). Specifically, peak hCG levels were much higher in twin compared with singleton pregnancy (mean of 170.000 IU/L vs. 65.500 IU/L) and significantly prolonged (~6 weeks vs. <1 week). Thus, GTT results from the abnormal stimulation of the thyroid gland when hCG levels exceed 75.000-100.000 IU/L and when enough time is given for such functional abnormality to develop (Figure 2). In summary, these studies showed that hCG is a maternal thyroid regulator, especially during the first trimester of gestation and also that GTT is the

most frequent cause of (non autoimmune) thyrotoxicosis in the pregnant state, as part of the Hyperemesis Gravidarum syndrome (1, 8).

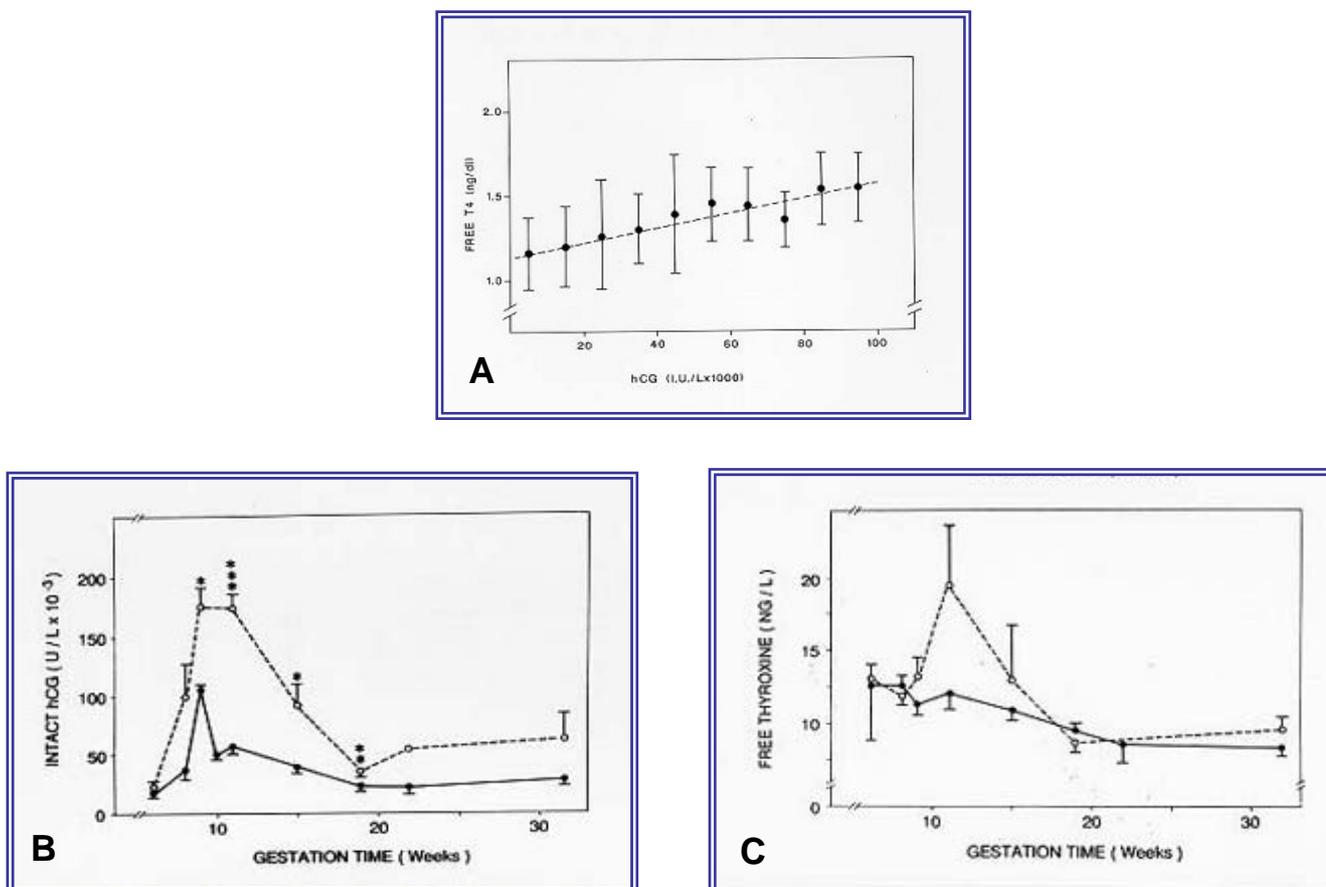


Figure 2. A. Direct correlation between increments in peak hCG values (by 10.000 IU/L) and progressively increasing free T₄ levels in normal women with singleton pregnancy near the end of 1st trimester. B. Comparison of hCG values between singleton (solid line) and twin (dotted line) pregnancy, showing the marked difference in both the amplitude and duration of hCG peaks. C. Comparison of serum free T₄ concentrations between singleton (solid line) and twin (dotted line) pregnancy, showing the transient burst in serum free T₄ values associated with higher and more prolonged hCG values in twin pregnancy.

After the study of normal pregnant women, we started additional investigations on the outcome of pregnancy in women with preexisting thyroid abnormalities. This group was part of our first cohort and encompassed 120 pregnancies, i.e. 17% of the initial cohort. These women were shown to present subtle, underlying – and hitherto undisclosed – thyroid anomalies: past history of thyroid disease, goiter, nodules, and thyroid autoantibodies (Th-Abs). It was shown that both size and number of these nodules increased during pregnancy. Another finding was that women with positive Th-Abs have a 3-fold increased risk of early spontaneous miscarriage. Finally, the study showed that euthyroid women with Th-Abs frequently develop subclinical hypothyroidism (SCH) as gestation progresses, giving us the first clear indication that asymptomatic autoimmune thyroid disorder (AITD) is a major cause of an impaired thyroid functional reserve, which is revealed by pregnancy (9). To evaluate further the risk of hypothyroidism in euthyroid women with AITD, we initiated a second cohort investigation encompassing 1.660 new consecutive pregnant women. Main results were that

the prevalence of positive Th-Abs (TG-Ab and/or TPO-Ab) reached 6.5% of the population. Despite an overall 50% decrease in Th-Abs titers during gestation, many women developed SCH. The risk of presenting SCH during late gestation was predictable in first trimester already, on the basis of Th-Abs titers and the serum TSH shift toward higher normal values (10). Published in 1994, these data gave us the opportunity to propose for the first time that pregnant women should be screened systematically for thyroid disorders, if such thyroid function abnormalities (both frequent and most usually unknown) were to be diagnosed (11). An overview of the various aspects of the clinical epidemiology of thyroid function abnormalities and diseases associated with pregnancy is illustrated in Figure 3.

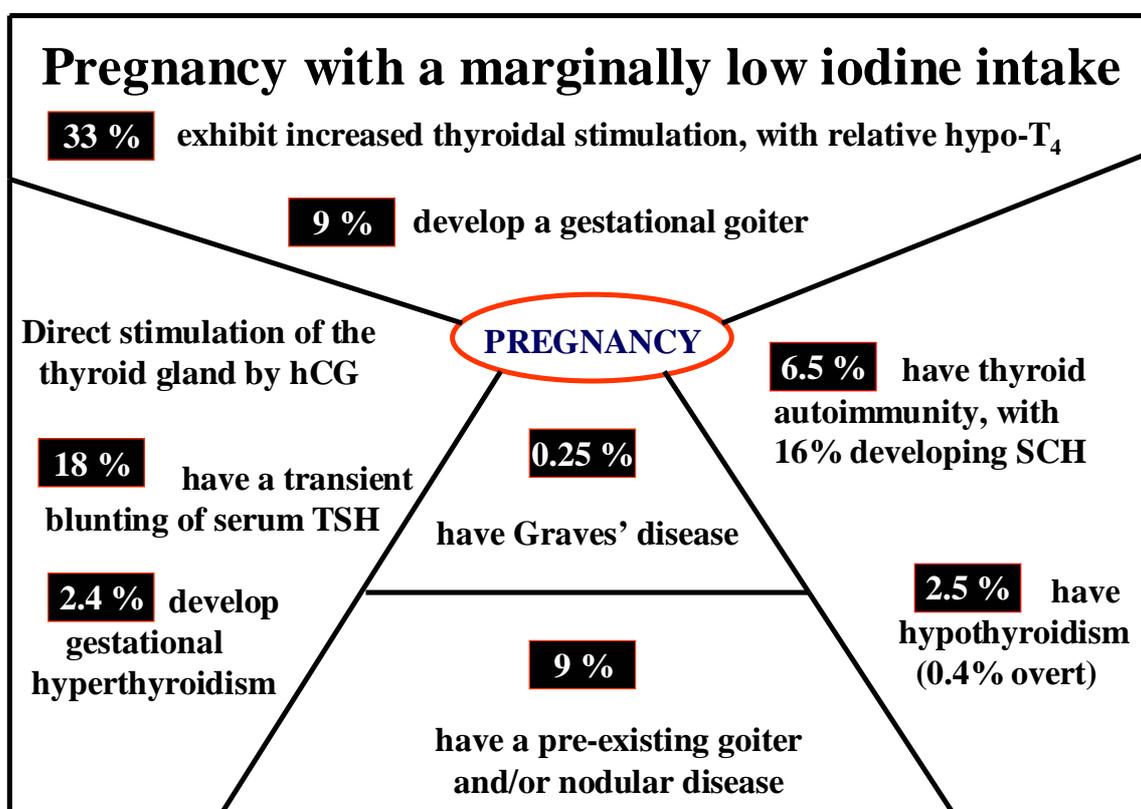


Figure 3. Overview of the clinical epidemiology of the most prevalent thyroid function abnormalities and thyroid diseases associated with pregnancy, summarizing data obtained in two successive population studies carried out in Brussels between 1988 and 1994.

Part 2: From the observational studies to the consensus guidelines

In this section, we outline a number of landmarks that have allowed us – and many other investigators – to extend our views from the clinical epidemiology of thyroid disorders associated with pregnancy to their management.

In 1995, we presented the results of the first prospective double-blind randomized clinical trial for the prevention of ID during pregnancy (12). Women, selected to present biochemical indices of excessive thyroid stimulation in early pregnancy, were subdivided into three groups and treated with either placebo, daily iodine supplementation with KI, or the combination of L-T4 + KI. Main results were that dietary iodine fortification allowed to improve markedly the pattern of thyroid function tests, with a decrease in serum TSH and TG levels, an increase in urinary iodine excretion, and a marked reduction in the risk of maternal goiter formation as well as a complete eradication of neonatal goiter (Figure 4). The study showed also that there was an inevitable lag period of approximately one trimester before the benefits of iodine fortification on thyroid function could be observed, a finding that prompted us to recommend that the iodine supplementation should ideally start before conception and, when this was not feasible, as soon as possible after the onset of pregnancy.

In 1998, we proposed an algorithm for the systematic screening of thyroid disorders during pregnancy, using a 2-step scheme to detect AITD, SCH and OH. With a procedure derived from the screening scheme proposed for the detection of thyroid underfunction, the algorithm could be easily extended to the screening of hyperthyroidism (13).

In 1999, Haddow *et al.* reported the first study showing that school-age children, born to mothers with thyroid insufficiency during pregnancy, presented a risk of impairment in neuro-psychological development (14). Because the design of the study was prospective for the offspring's evaluation, but retrospective for the part that concerned maternal thyroid function, these hypothyroid women had either remained undiagnosed during pregnancy or had been diagnosed before conception and already treated with L-T4, but hormone replacement therapy not correctly adapted, hence leading to more severe thyroid insufficiency during gestation. One of the consequences of this study was to reinforce the proposal to systematically screen pregnant women for thyroid disorders, and particularly for hypothyroidism that constitutes, by far, the most prevalent thyroid disease in this age range (15). Another major consequence of this pioneering study was the launching of several population-based studies thereafter in the U.S. (some of these are still in progress today), especially after the joint meeting organized by CDC and ATA in 2004 and entitled "*The impact of maternal thyroid diseases on the developing fetus: implications for diagnosis, treatment, and screening*" (16).

In 2005, WHO (Geneva headquarters) organized a "Technical Consultation" of world experts to revise the existing programs for iodine supplementation in pregnant and lactating women. This meeting led to the recommendation that the daily iodine intake should be increased to 200-300 µg/day (average: 250 µg/day) during pregnancy and iodine fortification pursued during breastfeeding

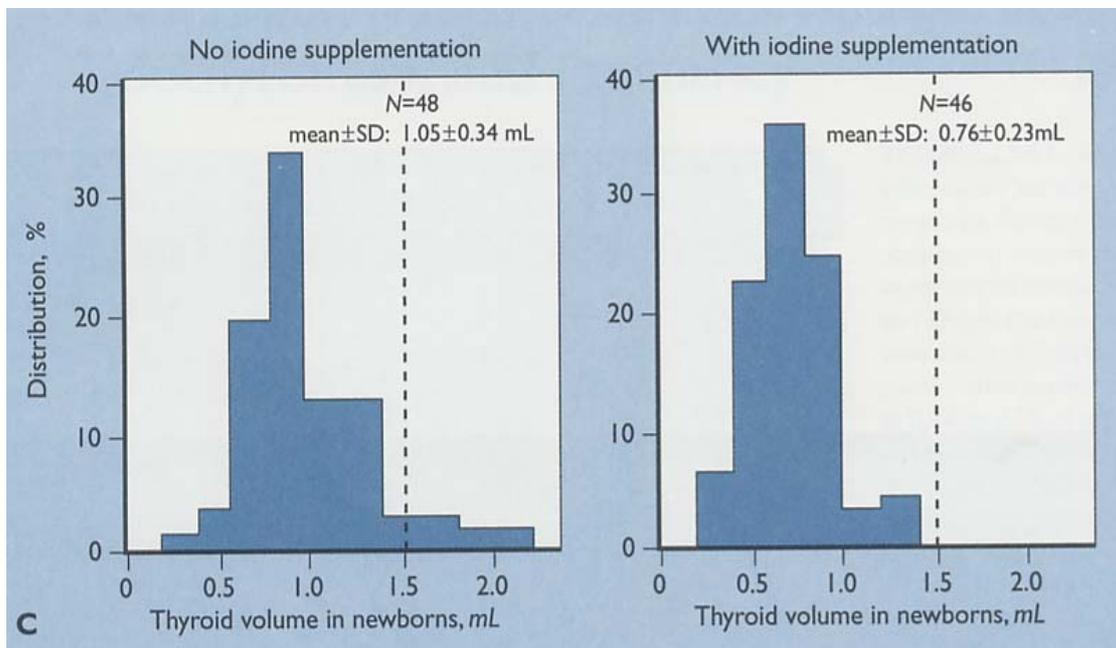
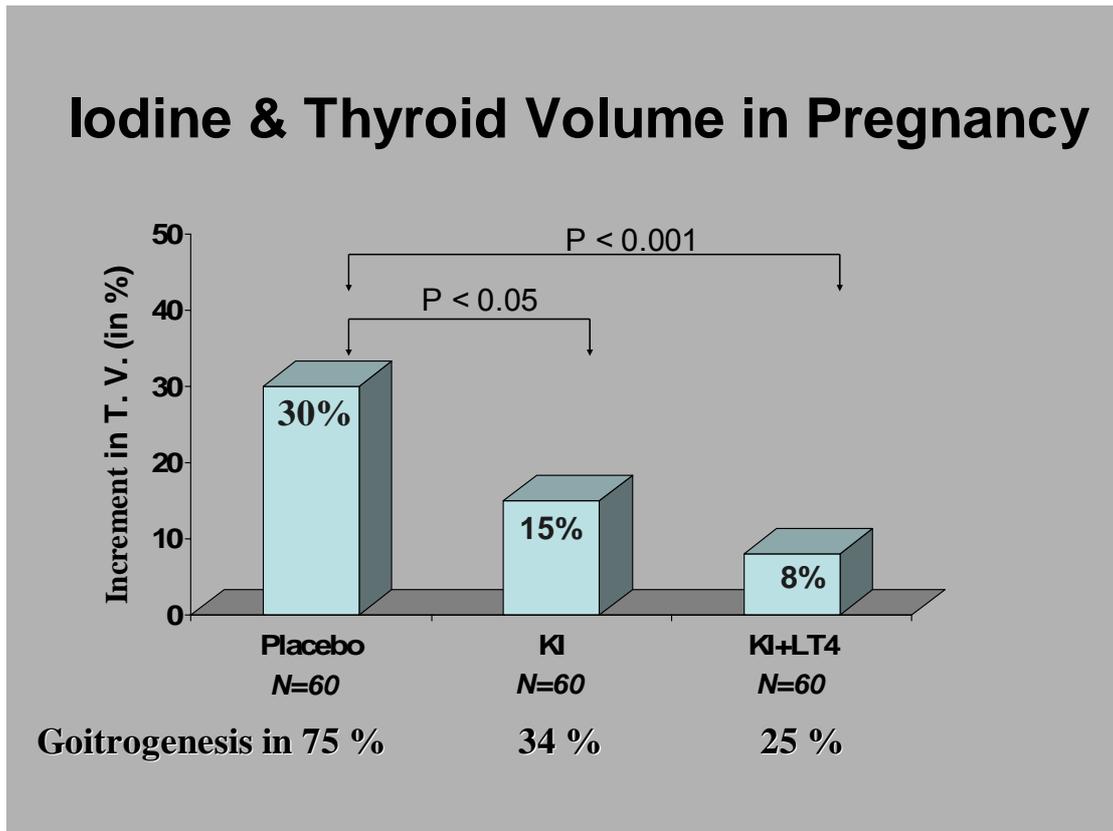


Figure 4. Upper panel shows that in pregnant women who were given daily supplements of potassium iodide (KI), thyroid volume increments were reduced from 30% in placebo-treated women to 15% in KI-treated women, and TV increases affected only 34% of the latter women compared with 75% in placebo-treated women. With the combination of L-T4 and KI, the beneficial effects were even better.

Lower panel shows that in the offspring of pregnant women, administration of iodine supplements to mothers eradicated entirely the risk of neonatal goiter and resulted in an overall 30% reduction in neonatal thyroid volume.

(Figure 5) (17). Another important result of this meeting was to differentiate three geographical situations for the implementation of iodine fortification, in an attempt to tailor strategies to actual iodine intake levels as well as to the practical possibilities in a given population. First, for those countries considered to have reached iodine sufficiency or with a well-established universal salt iodisation (USI) program (Ex: USA), the recommendation was that there was no need for global public health measures, although individual counselling was still advocated. Second, for those countries without USI program or with a USI program known to have only partial coverage (Ex: several countries in Europe), the recommendation was to provide women with multivitamin pills containing the amount of iodine required to reach the recommended nutritional intake. Third and finally, for those remote areas with no accessible USI program and frequent difficult socio-economic conditions (Ex: several countries in Africa and Asia), the recommendation was to administer orally, as an emergency measure, iodized oil (Lipiodol) in early pregnancy.

WHO – Geneva 2005
Revision of Recommendations
(Published in Public Health Nutrition, Dec 2007)

RNI: 200 – 300 µg/day

Population Group	Median Urinary Iodine Conc. (UIC)	Category of Iodine intake
Pregnant women	< 150 µg/L	Insufficient
	150 – 249 µg/L	Adequate
	250 – 499 µg/L	More than adequate
	> 500 µg/L	Excessive
Lactating women	< 100 µg/L	Insufficient
	> 100 µg/L	Adequate

Figure 5. Median values or ranges in urinary iodine concentrations (UIC) used to categorize the adequacy of iodine intake in pregnant and breastfeeding women (RNI: Recommended Nutritional Intake).

Beginning of 2005, an international *ad hoc* task force was established under the auspices of the American Endocrine Society (TES) to prepare consensus guidelines for the management of thyroid disorders during pregnancy and postpartum. After two years of hard work and thorough discussions within this committee, clinical practice guidelines were published in 2007 (18). These guidelines have been endorsed by TES (The Endocrine Society), AACE (American Association of Clinical Endocrinologists) as well as by the four world regional thyroid associations (ATA, ETA, LATS, & AOTA). Note that ACOG (American College of Obstetricians and Gynecologists) did not endorse the recommendations, essentially because they opposed the screening of pregnant women with the

argument that *“there just isn’t any data to support the routine screening of millions of pregnant women every year because the long term effects are not certain and there is no evidence that any treatment would make a difference in the long run”*.

Finally, and to buckle my personal research buckle, I am pleased that my younger collaborator in Brussels, Kris Poppe, has taken over the challenge to continue working in this field, which he has already nicely prolonged and extended during recent years on specific issues related to thyroid autoimmunity and dysfunction in the context of infertility and assisted reproduction (19).

Part 3: The future issues: “Where do we go now, with the presently accepted consensus guidelines?”.

In this section, we discuss shortly some personal views on unresolved issues in an attempt to define perspectives for future research activities in this field.

A first problem concerns the validity and normal reference limits of serum TSH and free T₄ measurements in pregnancy (20, 21). While most TSH assays are intrinsically sturdy and pose no actual validity problems, this is not the case for free T₄ determinations whose intrinsic validity has recently been questioned. Both reference ranges are modified during pregnancy. For serum TSH, there is a downward shift of the entire reference range, which is maximal in first trimester but prolonged during later gestational stages (Figure 6). For serum free T₄, there is a narrowing and clustering of serum free T₄ estimates near – or just below – the lower normal limit of non pregnant healthy individuals (Figure 6). This difficulty raises an important question, namely that of the correct interpretation of an isolated serum free T₄ lowering, i.e. isolated maternal hypothyroxinemia (hypo-T₄). It is possible that, in many instances, hypo-T₄ (in the absence of a concomitant TSH rise and absence of detectable thyroid autoantibodies) may primarily reflect dosage interferences. Thus in summary, it will be important to delineate better in the near future a more univocal approach of gestation-specific, trimester-specific, and perhaps also assay-specific reference values for serum free T₄ and TSH determinations in pregnancy.

A second question concerns gestational hypothyroxinemia, with or without concomitant serum TSH elevation. It remains unclear today what degree of maternal T₄ lowering must be reached – and during how long? – for thyroid function abnormalities to be associated – beyond doubt – to detrimental effects on the neuro-psychological development in the offspring. Answering such questions is crucial to help us define the best possible strategies for the detection (by early screening, etc.) and management of these disorders.

A third unresolved issue concerns subclinical thyroid disorders associated with pregnancy (both hypo- “SCH” and hyperthyroidism “SCHR”). There is some evidence that SCHR – which is primarily related to GTT – has no detrimental effect on the pregnancy outcome (22). This is not the case for SCH, as various arguments (more or less direct or indirect) exist to suggest a possible

relationship between mild thyroid insufficiency and a poorer pregnancy outcome (23). If this is so,

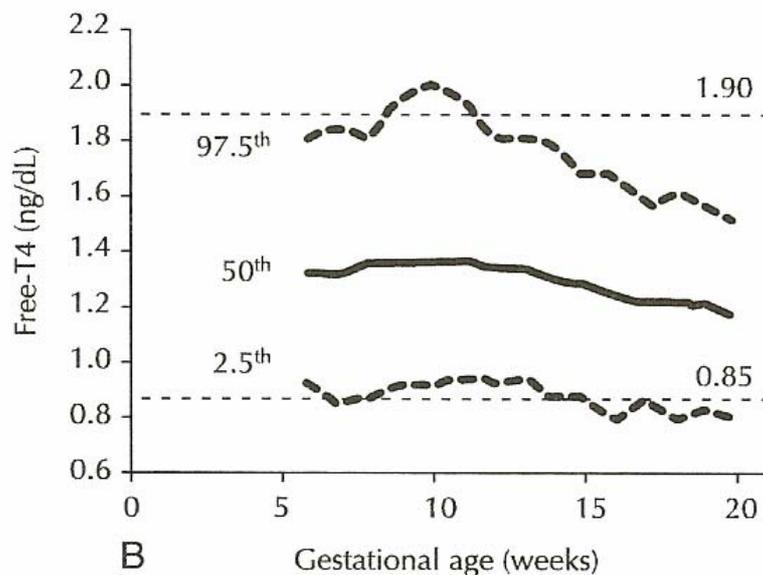
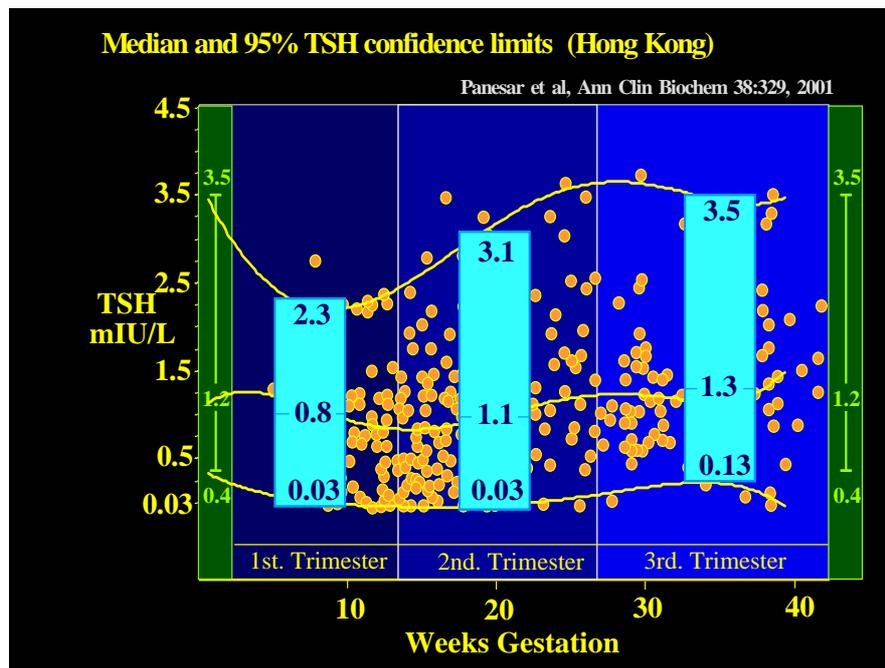


Figure 6. Upper panel shows the changes in serum TSH reference range in the 3 trimesters of gestation (adapted from Panesar et al., Ref. N° 20). Lower panel shows the gestational-age specific nomogram for upper and lower serum free T4 limits during first half of gestation (adapted from Casey et al., Ref. N° 21).

then systematic screening is required since, in most cases, the diagnosis has not been made before the onset of a pregnancy. A controversy persists today between endocrinologists and obstetricians on whether thyroid screening should be performed in all pregnant women. In the consensus guidelines endorsed in 2007 by the four world Thyroid Associations, a middle-way consensual view was taken in

favour of targeted screening in high-risk groups (18). These included women with a personal or family history of thyroid disease, symptoms of thyroid dysfunction, history of other autoimmune diseases, infertility, type I diabetes, history of head and neck irradiation, and positive thyroid antibodies. Our personal view is that this approach is unrealistic, somewhat hypocritical and will, in any event, prove to be insufficient to solve all the remaining questions. Other colleagues have argued, however, that it was better to reach a consensus (albeit unsatisfactory) than to remain in a quandary with total absence of any consensus.

The last question concerns the future implementation of consensus guidelines. The role of guidelines is to lead the way for the best possible management of patients and their diseases, based on evidence-based medicine combined with common sense. Unfortunately, the evidence is not always available and there is an evident lack of good randomized clinical trials to help us decide on the best attitude. Thus for the moment, the guidelines should essentially be viewed as a guide to help the multiple care providers who intervene in the management of pregnant women. Finally, it is worth mentioning that the existence of guidelines raises the issue of liability and only the future will show how this difficult question may be tackled by both endocrinologists and obstetricians.

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Nonthyroidal Illness Syndrome

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ABSTRACT

Nonthyroidal Illness Syndrome (NTIS), also called Sick Euthyroid Syndrome or Low 3,5,3'-tri-iodothyronine (T3) syndrome, refers to a thyroid phenotype consisting of low T3, normal-high reverse T3 (rT3), normal-low thyroxine (T4), and inappropriately normal or low serum thyroid stimulating hormone (TSH) observed during illness and starvation. The pathophysiology of NTIS involves a central hypothyroidism together with changes in thyroid hormone production, transport, cellular uptake, and metabolism, and a decrease in thyroid hormone receptor levels. These changes reduce the bioavailability and probably also the actions of thyroid hormones, thus creating a clinical situation of hypothyroidism. Whether such changes are directed to protect the organism from the actions of thyroid hormones in catabolic situations, or whether the changes are themselves harmful and therefore indicate thyroid hormone replacement treatment in patients with NTIS, is a question of debate (*Hot Thyroidol. 2009: e11*).

Key-words: Nonthyroidal Illness Syndrome; deiodinase activity; cytokines; thyroid hormone action; thyroid hormone receptors.

Introduction

The term Nonthyroidal Illness Syndrome (NTIS) refers to characteristic changes in thyroid hormone levels during illness and starvation (1). These changes are low T3 and normal-high rT3 in serum and tissues, normal or low serum T4 and inappropriately normal or low serum TSH relative to serum levels of T4 and T3 (2) (Figure 1).

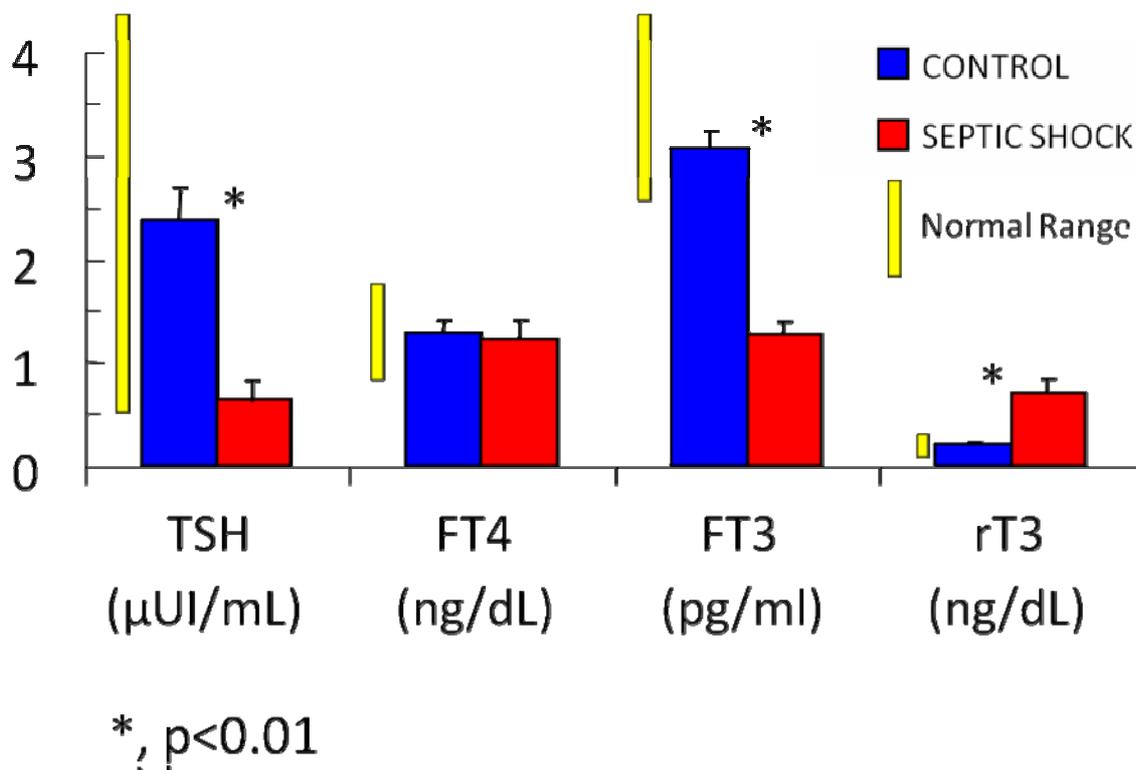


Figure 1. Serum thyroid hormone levels in a group of patients with NTIS related to septic shock, and controls. Patients with NTIS have lower serum TSH and free T3, higher reverse of T3, and no differences were observed in serum free T4 levels, relative to controls. Yellow bars indicate hormonal normal range values.

NTIS can be considered as a part of the neuroendocrine response to severe stress that also includes an increase in serum glucocorticoid levels and a decrease in serum gonadotropins. It remains unclear whether NTIS is a beneficial adaptive response to reduce energy consumption, or a form of secondary hypothyroidism that requires thyroid hormone treatment (3-6).

Patients with NTIS and low serum T4 have an increased probability of death (7-10), probably because the duration and magnitude of NTIS are dependent on the severity of the underlying disease. However there is no clinical evidence that under these circumstances thyroid hormone treatment is advantageous, nor indeed disadvantageous (6). In the few prospective studies conducted to assess the response to thyroid hormone therapy of patients with severe NTIS no reduction in mortality was observed (11, 12); an increase in mortality in the treated group was reported in one study (13), and no changes in other clinical outcomes were observed (12, 14). Nevertheless some authors advocate T3 therapy in cardiac transplantation recipients since T3 seems to lead to a rapid restoration of energy stores, together with an improvement in myocardial function, a reduction in the incidence of inadequate heart function early after transplantation, and an increase in graft survival (15). T3 administration to humans is associated with improved hemodynamics, reduced peripheral vascular resistance, and increased cardiac output. T3 administration to adults during coronary artery surgery neither changes the requirement for inotropic drugs nor increases the incidence of arrhythmia (16). Studies in experimental animals have shown that T3 decreases during a period of regional myocardial ischemia and that T3 administration is associated with an improved left ventricular ejection fraction, an important indicator of outcome after acute myocardial infarction (15,17). Taken together, these studies suggest that T3 administration to patients with cardiac-related NTIS is safe, well tolerated, and potentially of benefit to these patients.

A recent study in children that underwent cardiac bypass surgery for congenital heart lesions showed that all the children presented NTIS within the first day postoperatively. NTIS changes correlated to prolonged hospital stay and T3 levels within 6-14 h from surgery predicted the patients outcome (18). However the beneficial role of thyroid hormone replacement in children with NTIS remains unknown.

Hypothalamic-pituitary function in NTIS

The inappropriately normal or low serum TSH levels in NTIS patients suggest an impairment of hypothalamic-pituitary function. Low TRH mRNA expression levels have been found in the periventricular nuclei of the hypothalamus in NTIS patients (19). A decrease in pulsatile TSH

secretion as well as an increase in basal but not in pulsatile TSH secretion after TRH infusion have been found in prolonged critically ill patients (19). The finding that thyroid axis alterations are partially reversed by the combined infusion of TRH and GH secretagogues (20) provides further evidence of the importance of the role of central hypothyroidism in NTIS. Increased levels of proinflammatory cytokines, endogenous glucocorticoids and glucagon typically seen in critically ill patients, together with administration of glucocorticoids and dopaminergic drugs, could directly suppress TRH secretion, the pituitary response to TRH, and TSH secretion (19-21). In humans, administration of tumor necrosis factor- α (TNF- α) (22), interleukin-6 (IL6) (23) and interferon alpha (24) causes a decrease in the plasma concentration of TSH and T3 and an increase in rT3, resembling the biochemical changes in NTIS. In rats, interleukin-1 α (IL1- α) decrease hypothalamic pro-TRH, pituitary TSH beta gene expression and plasma TSH levels, while IL6 reduce plasma TSH without decrease hypothalamic pro-TRH and pituitary TSH beta expression, indicating that IL1- α affects the synthesis and release of TRH and TSH, and that IL6 affects TSH release (25). Fasting decreases TSH secretion in humans (26), while starvation decreases thyroid function in rats due to a reduction in the synthesis and release of TRH and TSH (27); an increase in adrenal glucocorticoid secretion is probably responsible for TSH suppression during fasting and also for the reduced TSH levels seen in NTIS (26).

The molecular mechanisms of the impairment of hypothalamic–pituitary function in NTIS are not well characterized. Bacterial lipopolysaccharide (LPS) administration to rats induces type-2 iodothyronine deiodinase (DIO2) activity in tanocytes located in the mediobasal hypothalamus, and it has been hypothesized that the increase in DIO2 activity could increase the conversion of T4 into T3, creating a local hyperthyroidism that prevents an increase in TRH and/or TSH secretion in response to low T3 (28). Although a decrease in TRH and an increase in DIO2 mRNA expression in the hypothalamus were found in a model of prolonged critically ill rabbits, the hypothalamic concentration of T4 was low and of T3 was low-normal, with no changes observed in thyroid hormone receptor beta (THRB) or alpha (THRA) mRNA expression (29). Moreover, in autopsy samples from humans with severe NTIS a decrease in T3 concentration was found at the levels of the hypothalamus and the pituitary (30).

These studies indicate that in NTIS there is a relatively hypothyroid state at the hypothalamus, arguing against an increase in hypothalamic conversion of T4 to T3 as a cause of low TRH mRNA expression in NTIS.

Thyroid hormone production and thyroid gland function in NTIS

Although T4 production is decreased in NTIS patients, thyroid gland function in NTIS has not been systematically studied. As already mentioned, NTIS thyroid axis alterations are partially reversed by the combined infusion of TRH and GH secretagogues (20), indicating that the thyroid gland is able to release thyroid hormones if appropriately stimulated by TSH. These observations do not, however, rule out a role of the thyroid gland in the pathophysiology of NTIS, especially in severe cases with low T4 levels. Patients with NTIS have altered TSH glycosylation which is associated with reduced biological activity (31), and lethal NTIS is associated with major morphological changes of the thyroid gland including loss of colloid and reductions in follicular size and thyroid weight (32). Finally, cytokines affect thyroid cell function in several ways (21,26), including a decrease in basal and TSH-stimulated iodide uptake by IL1 and TNF, inhibition of thyroglobulin synthesis by IL1, TNF- α and interferon gamma (IFN- γ), a decrease in thyroperoxidase expression by IL1, IL6 and IFN- γ , and a decrease in T3 secretion by IL1, TNF- α and IFN- γ .

Low serum T4 levels in patients with NTIS are due not only to a decreased daily T4 production but also to a reduction of T4 binding to carrier proteins (33) which is mainly due to a decrease in serum concentration and binding affinity of thyroid binding globulin (TBG). In NTIS there is an increased cleavage of TBG by elastases and an increase in the levels of non-esterified fatty acids (NEFA) which compete and displace T4 for binding to TBG.

Deiodinase expression and activity

In humans, approximately 80% of T3 is produced by extra-thyroidal enzymatic deiodination of T4, mainly in the liver and kidney by type-1 iodothyronine deiodinase (DIO1), and in skeletal muscle by DIO2 (34). In post-mortem analyses of patients with NTIS, low T3 and high serum and tissue rT3

have been found to be related to decreased activities of liver DIO1 and skeletal muscle DIO2, and to increased activities of type-3 iodothyronine deiodinase (DIO3) in the liver and in skeletal muscle (2,35). Similar findings have been reported in an animal model of prolonged critical illness (36). In patients with septic shock and NTIS there is a decrease in skeletal muscle DIO2 mRNA expression but not in DIO2 activity, and an increase in DIO3 activity. However, no changes in DIO1 or DIO3 activity were observed at the level of subcutaneous adipose tissue, suggesting that the changes in deiodinase activity occurring in patients with septic shock and NTIS are tissue specific (37). The changes in deiodinase activity in NTIS patients could be attributable to increases in serum glucocorticoids and proinflammatory cytokines (38-41), and/or to a decrease in thyroid hormone bioavailability within particular tissues. TNF- α , IL1- α and IL6 inhibit DIO1 activity in rodents (21) and in human hepatocarcinoma cell lines (40). It has been proposed that one of the causes of a decrease in DIO1 activity could be due to competition between the promoters of *DIO1* and the multiple genes regulated by those cytokines for the few cellular coactivators available, such as SCR-1 (42), or for transcription factors such as NF-kB (43). Activated NF-kB, a transcription factor that plays a pivotal role in immune and inflammatory responses, is a potential molecular factor at the root of NTIS in patients with increased cytokines (44,45); in vitro studies using HepG2 cells have shown that NF-kB activation attenuates the induction of DIO1 by T3 (44) and that the decrease in DIO1 mRNA expression by IL1- β is mediated by the simultaneous activation of NF-kB and the activator protein-1 (AP-1) pathway (43).

Deiodinases are selenoproteins, while selenium deficiency is frequently observed in sick patients and selenium deficiency decreases DIO1 activity (45). Mutations in SECIS binding protein 2 (SBP2), a protein that incorporates selenocysteine into the catalytic center of deiodinases, also cause a decrease both in deiodinase activity and in serum T3 (46). However, selenium administration to critically ill patients has not been found to alter serum thyroid hormone levels (47), nor have changes been found in SBP2 mRNA expression levels in skeletal muscle or subcutaneous adipose tissue samples from patients with septic shock NTIS, relative to controls (37). These results suggest that selenium does not play a major role in the pathophysiology of NTIS.

Although changes in peripheral deiodination may be necessary, it seems that they are not sufficient to cause NTIS. Mice deficient in deiodinase activity have normal serum T3 levels and an increased serum T4 concentration (48). Thus decreased DIO1 gene expression and low DIO1 activity observed in NTIS patients could be an effect rather than a cause of low T3. Recent studies have questioned the physiological relevance of DIO2 activity in human skeletal muscle due to its normally low activity (49), while DIO2 activity in skeletal muscle biopsies from patients with septic shock NTIS is not significantly different from controls (37). Continuous infusion of TRH and GHRP2 has been found to increase T4 and T3 but not rT3 serum levels, suggesting that central hypothyroidism is a major component of NTIS syndrome (20).

Thyroid hormone tissue uptake and actions

Patients with fatal NTIS show decreased T4 and T3 in most tissues (30), caused in part by reduced uptake (50). Several factors, including NEFA and bilirubin, have been identified as inhibitors of cellular T4 transport, although their mechanisms of action remain unknown (51). Also NTIS observed in patients with anorexia nervosa or in obese patients under caloric restriction seems to be related to carbohydrate restriction, free unsaturated fatty acid increase and low tissular ATP levels that could decrease thyroid hormone uptake and metabolism (52). However, the administration of T4 plus T3 to NTIS patients previous to death increased iodothyronine levels in liver and skeletal muscle (2) showing that thyroid hormone bioavailability is not limited by reduced tissue uptake if appropriate replacement therapy is given. The thyroid hormone transporters monocarboxylate transported 8 (MCT8, SLC16A2) and MCT10 (TATA1, SLC16A10) do not seem to play a role in controlling thyroid hormone uptake in skeletal muscle, liver or kidney (37,53), although MCT8 might play a role in thyroid hormone uptake at the level of adipose tissue (37).

Thyroid hormone action depends on the tissue distribution and expression levels of thyroid hormone receptors (TRs), ligand regulated transcription factors that bind to the thyroid hormone response elements (TREs) of target genes. TRs are encoded by the *THRA* and *THRB* genes that by alternative splicing give rise to different TR isoforms (54). TRs bind to TREs predominantly as heterodimers with

the retinoid X receptor (RXR) and together they also bind to other regulatory proteins that act as co-repressors and co-activators modulating TR transcription function. Nuclear receptor co-repressor, NCOR1, and silencing mediator of retinoid and TR, SMRT, recruit histone deacetylases and inhibit the basal transcription machinery (55). Steroid receptor co-activator, SRC1, has intrinsic histone acetyl transferase activity and mediates the chromatin remodeling that allows transcription (56). TR expression is reduced in skeletal muscle and subcutaneous adipose tissue of patients with septic shock NTIS (37), and similar findings have been obtained in mice after LPS administration leading to an acute decrease in TR α 1, TR α 2 and TR β proteins and mRNA expression in white fat and the liver and heart (57,58). Interestingly, a previous study in humans found an increase in TR α and TR β mRNA concentrations in peripheral mononuclear cells from patients with NTIS due to liver disease or chronic renal failure, but no differences were observed in cells from patients with severe acute illness recruited within 48 h of admission to ICU (59). A recent study of liver biopsies from critically ill patients who died in ICU reported that the ratio of mRNA expression of the two THRA isoforms TR α 1 and TR α 2 was higher in patients with more severe disease (60); interestingly, however, in six patients who died after a very short stay in ICU, the ratio was not different from that seen in controls. Together, these data show that *THR* gene expression decreases during the acute phase of severe illness. Skeletal muscle and subcutaneous adipose tissue express the three RXR isoforms, *RXRA*, *RXRB* and *RXRG*, and patients with septic shock NTIS show a decrease in *RXRG* mRNA expression in skeletal muscle and adipose tissue, and an increase in *RXRA* expression in skeletal muscle, but no changes in *RXRB* expression in either skeletal muscle or adipose tissue (37). RXRs have an important metabolic role in skeletal muscle, increasing the uptake and oxidation of saturated fatty acids, and the ratio of unsaturated to saturated fatty acids. Moreover, *RXRG*-deficient mice have increased skeletal muscle lipoprotein lipase activity and lower triglyceride levels (61). The RXR isoform expression changes seen in skeletal muscle and subcutaneous adipose tissue samples from septic shock NTIS patients may be directed at decreasing insulin resistance and increasing ATP production from the fat within skeletal muscle during septic shock.

In conclusion, NTIS forms a part of the neuroendocrine response to moderate-severe illness and starvation, caused by impairment of hypothalamic-pituitary function and of the physiological mechanisms producing T3 in peripheral tissues: NTIS is a systemic inflammatory reaction triggered by a variety of deleterious agents and by a reduction in energy intake. Depending on the severity of the noxious agent, extent of damage and time taken to recover, several molecular changes affecting transport, tissue uptake, metabolism and action of thyroid hormones may be observed (Figure 2). Whether such changes are beneficial and directed towards protecting the cell from thyroid hormone action under catabolic conditions, or whether they are harmful and thyroid hormone supplementation should be given to NTIS patients, is still a question of debate.

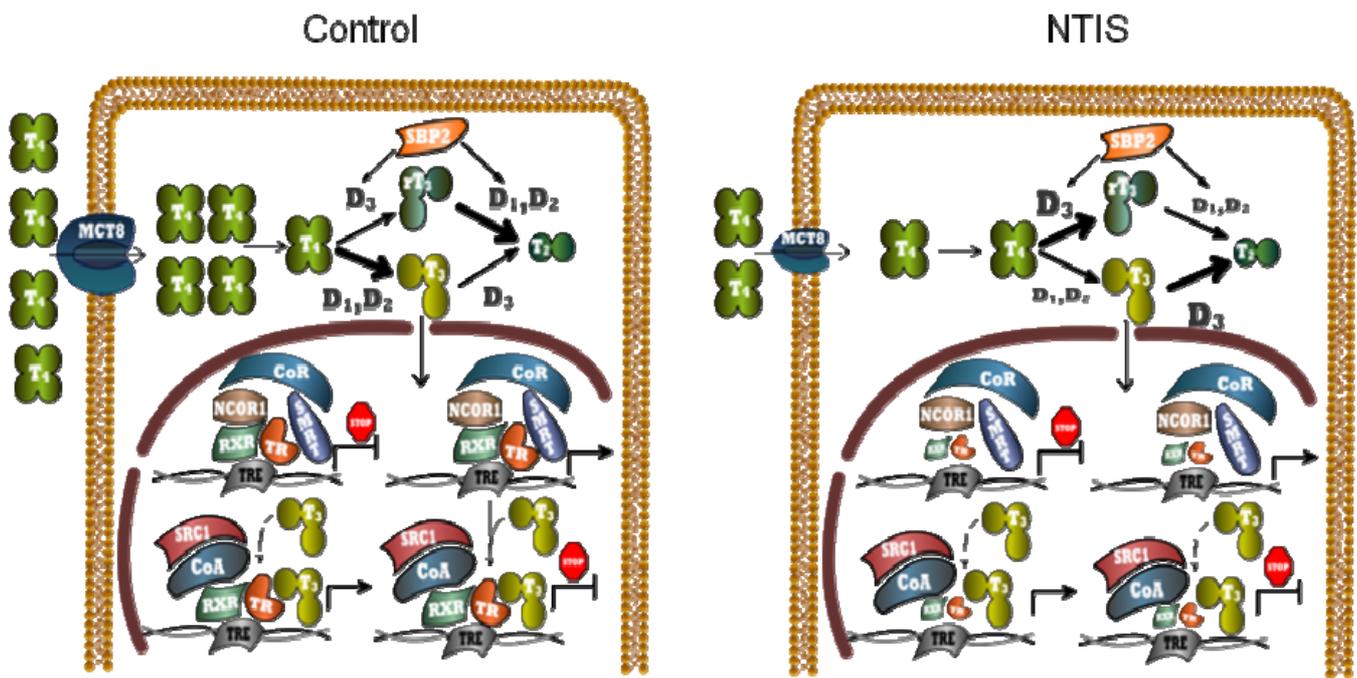


Figure 2. Thyroid hormone metabolism and action in normal (A) and NTIS (B) subjects. A decrease in serum thyroid hormone levels, cellular uptake and tissue levels of thyroid hormones, expression of MCT8, DIO1, DIO2, thyroid hormone receptors THRA and THRB, and RXRG, together with an increase in activity of DIO3, have been described in tissue samples from patients with NTIS.

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CRIBRIFORM-MORULAR VARIANT OF PAPILLARY THYROID CARCINOMA. A PROTOTYPE OF CLINICAL, PATHOLOGICAL AND GENETIC CORRELATION.

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ABSTRACT

The cribriform-morular variant of papillary thyroid carcinoma is an unusual neoplasm developing in patients with familial adenomatous polyposis but also occurring as a sporadic tumor. Histologically this variant is characterized by a combination of cribriform, follicular, papillary, trabecular, solid and spindle cell growth patterns with morular areas. Its peculiar morphological features are related with the permanent activation of the WNT pathway, with nuclear and cytoplasmic accumulation of beta-catenin. In addition to the genetic alterations in the APC/beta-catenin pathway, *RET/PTC-1* and *RET-PTC-3* rearrangements have been found in this variant, supporting this tumor as being a subtype of papillary thyroid carcinoma. Because this follicular cell derived carcinoma can develop before familial adenomatous polyposis becomes clinically manifest, the recognition of its particular histological features should alert to the possibility of FAP whenever such a tumor is found. In this article we describe the main clinical, pathological, immunohistochemical and molecular characteristics of the cribriform-morular variant of papillary thyroid carcinoma, including the genotype-phenotype correlations.

Key-words: cribriform-morular variant; papillary carcinoma; thyroid cancer; WNT pathway; APC; beta-catenin; familial adenomatous polyposis.

While pathological findings remain as the “gold standard” for tumor diagnosis, discoveries made in the field of molecular pathology have served to support the great value of the histological evaluation as well as opening possibilities for new therapies. Thyroid cancer is particularly interesting for pathologists given that each histological tumor type (and subtype) strongly correlates with its own particular molecular alterations, biological behavior (e. g. routes of metastatization), and clinical aggressiveness (1, 2). The genotype-phenotype correlation in medullary carcinoma of the thyroid is well recognized (1). In this paper, upon reviewing the principal features of the cribriform-morular variant (CMV) of papillary thyroid carcinoma (PTC), we portray a clear example of association between morphological, immunohistochemical, molecular and clinical findings.

The CMV of PTC was first recognized in 1994 by Harach et al (3) as a peculiar form of thyroid carcinoma developing in patients with familial adenomatous polyposis (FAP). Five years later, Cameselle-Teijeiro and Chan (4) reported the sporadic counterpart of this tumor as a peculiar subtype of papillary carcinoma. This is a rare tumor representing approximately 0.1%-0.2% of all PTC (4), with a prevalence of up to 12% in patients with FAP who have about 160 times higher risk of developing thyroid carcinoma than healthy people (5). The presentation of this tumor was over 10 times more frequent than that expected for sporadic papillary thyroid microcarcinoma (6). The CMV of PTC has a striking female predominance (female/male ratio \approx 17:1); one still ignores the genetic and/or epigenetic and/or environmental reason(s) for this gender difference. The mean age at diagnosis was about 28 years (range 12 to 53 yrs), sometimes preceding the diagnosis of FAP (7). The term *cribriform-morular variant* is now generally used to describe this tumor type when it occurs as a sporadic tumor (often solitary) as well as in the setting of FAP (often multicentric).

The CMV has a very unusual histology, characterized by a combination of cribriform, follicular, papillary, trabecular, solid and spindle cell growth patterns with morular (squamoid) areas (3, 4, 8, 9) (Figure 1). Characteristically, the luminal spaces are devoid of colloid, and morules with peculiar nuclear clearing caused by biotin accumulation are scattered in the tumor. The neoplastic cells are columnar with chromatin rich nuclei usually showing nuclear grooves, sometimes a clear appearance and, very occasionally, with intranuclear cytoplasmic pseudoinclusions. Immunohistochemically, most tumor cells are positive for the thyroid transcription factor-1, cytokeratins 7 and 19, vimentin, estrogen

and progesterone receptors, bcl-2, E-cadherin and galectin-3. Thyroglobulin may be positive focally or totally negative and there is no immunoreaction for calcitonin or cytokeratin 20 (3, 4, 8-10). Strong cytoplasmic and nuclear immunoreactivity for β -catenin is very characteristic (7) (Figure 2). A single case that was partially positive for chromogranin and synaptophysin but negative for thyroglobulin and calcitonin has been reported (7). The CMV of PTC could be diagnosed preoperatively after cytology examination because its tall columnar cells, fascicular spindle cells, and cribriform and morular patterns can be found in the fine-needle aspirates (10). Since this neoplasm can develop before FAP becomes clinically manifest, the recognition of the peculiar cytological and/or histological features of the CMV of PTC should alert to the possibility of FAP whenever such a tumor is found, especially in young patients (3, 4, 6-10).

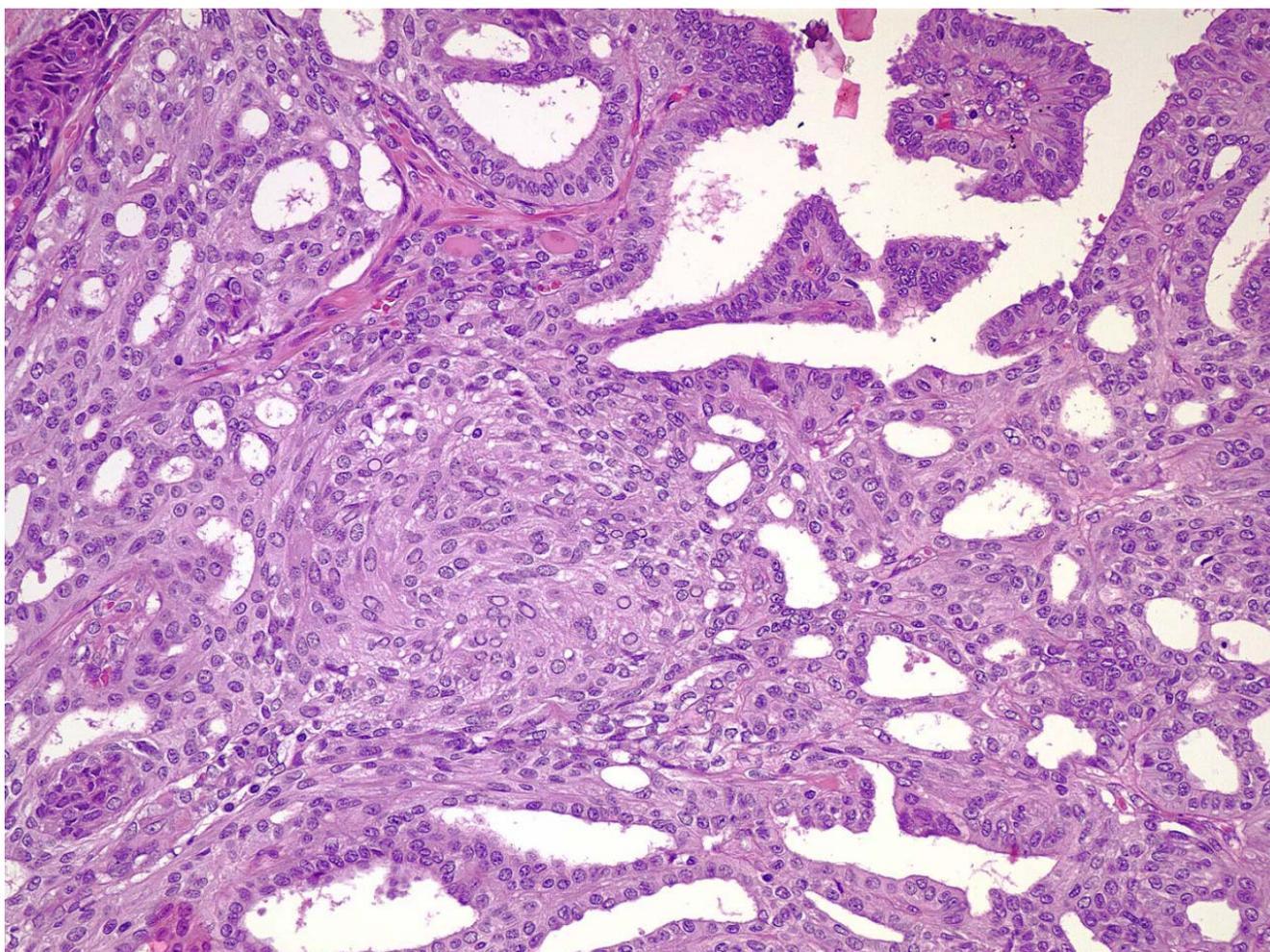


Figure 1. Microscopic appearance of the cribriform-morular variant of papillary thyroid carcinoma (CMV of PTC). A combination of cribriform, follicular, papillary, solid and spindle cell patterns with morules can be seen (x200).

FAP is an autosomal dominantly inherited cancer-predisposition syndrome characterized by the progressive development of multiple colorectal adenomatous polyps and an increased incidence of colorectal carcinoma. It is often accompanied by various benign and malignant manifestations, including epidermoid cysts, dental abnormalities, gastric and duodenal tumors, osteomas, hepatoblastomas, desmoid tumors, osseous tumors, congenital hypertrophy of the retinal pigmented epithelium (CHRPE), adrenocortical neoplasms, brain tumors, and, of course, thyroid tumors (11).

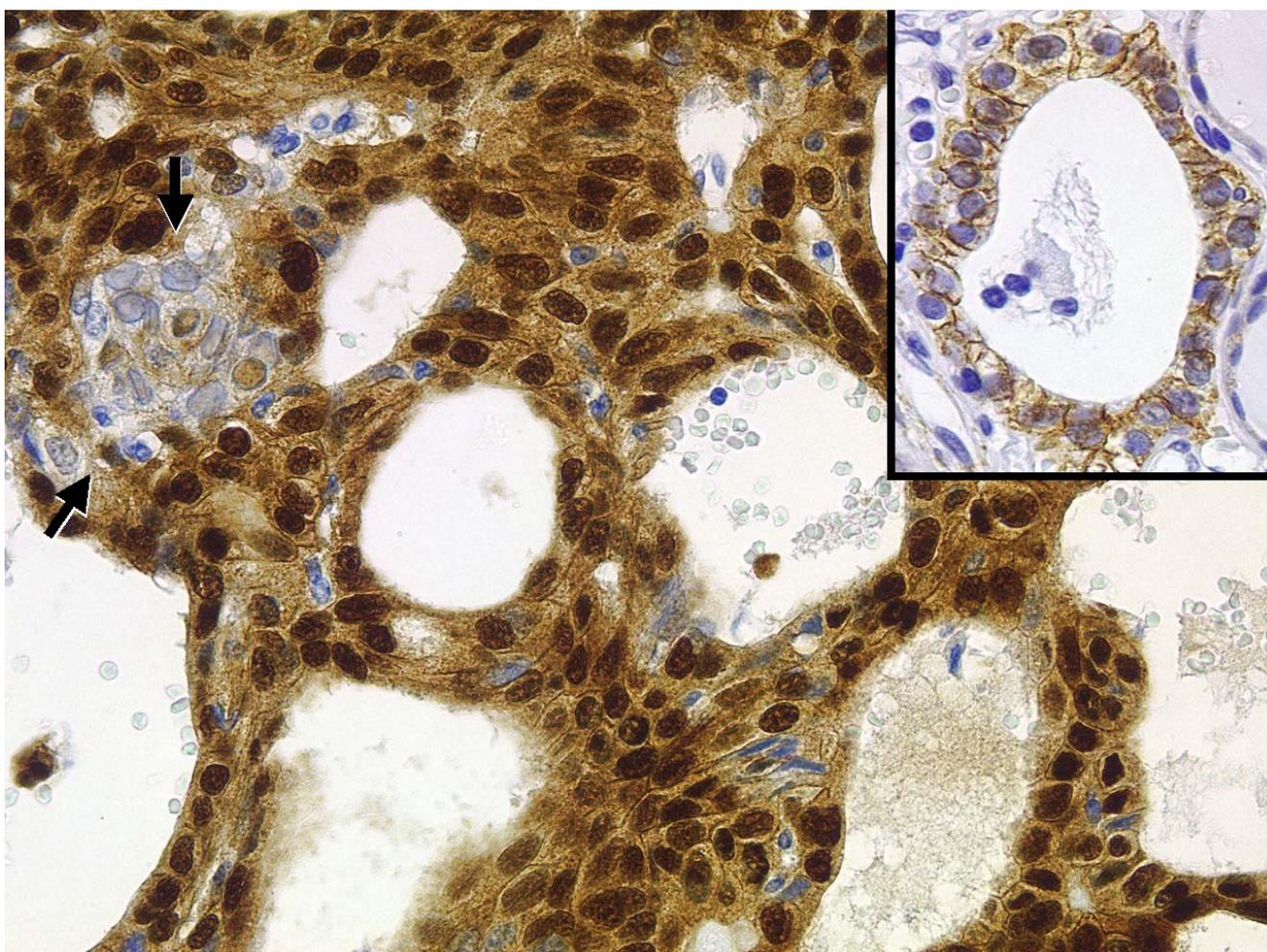


Figure 2. Immunohistochemical stain for β -catenin in a case of the CMV of PTC. Strong cytoplasmic and nuclear expression of β -catenin appears in tumor cells. Less intense positivity is seen in one morule (*arrows*). Normal follicular cells (*inset*) in the same case show a normal pattern of reactivity for β -catenin with no nuclear staining (x400).

FAP is caused by germline mutation of the adenomatous polyposis coli (*APC*) gene, a tumor suppressor gene mapped to 5q21 (11). *APC* forms a complex with glycogen synthase kinase-3 β (GSK-3 β), β -catenin, and Axin, and is involved in the WNT transduction signalling pathway, sequestering β -catenin and targeting it for degradation (12, 13) (Figure 3). The binding of β -catenin by

APC requires phosphorylation of β -catenin by GSK-3 β on specific serine and threonine residues, and aminoacids adjacent to them, all of which are encoded by exon 3 of the β -catenin gene (*CTNNB1*). GSK-3 β binds to and phosphorylates several proteins in this pathway and is instrumental to the down-regulation of β -catenin. Unphosphorylated β -catenin accumulates in the cytoplasm, and translocates to the nucleus. In the cell nucleus, β -catenin forms a complex with the T-cell factor/lymphoid enhancer factor family of transcriptional activators, that results in the activation of development-related genes.

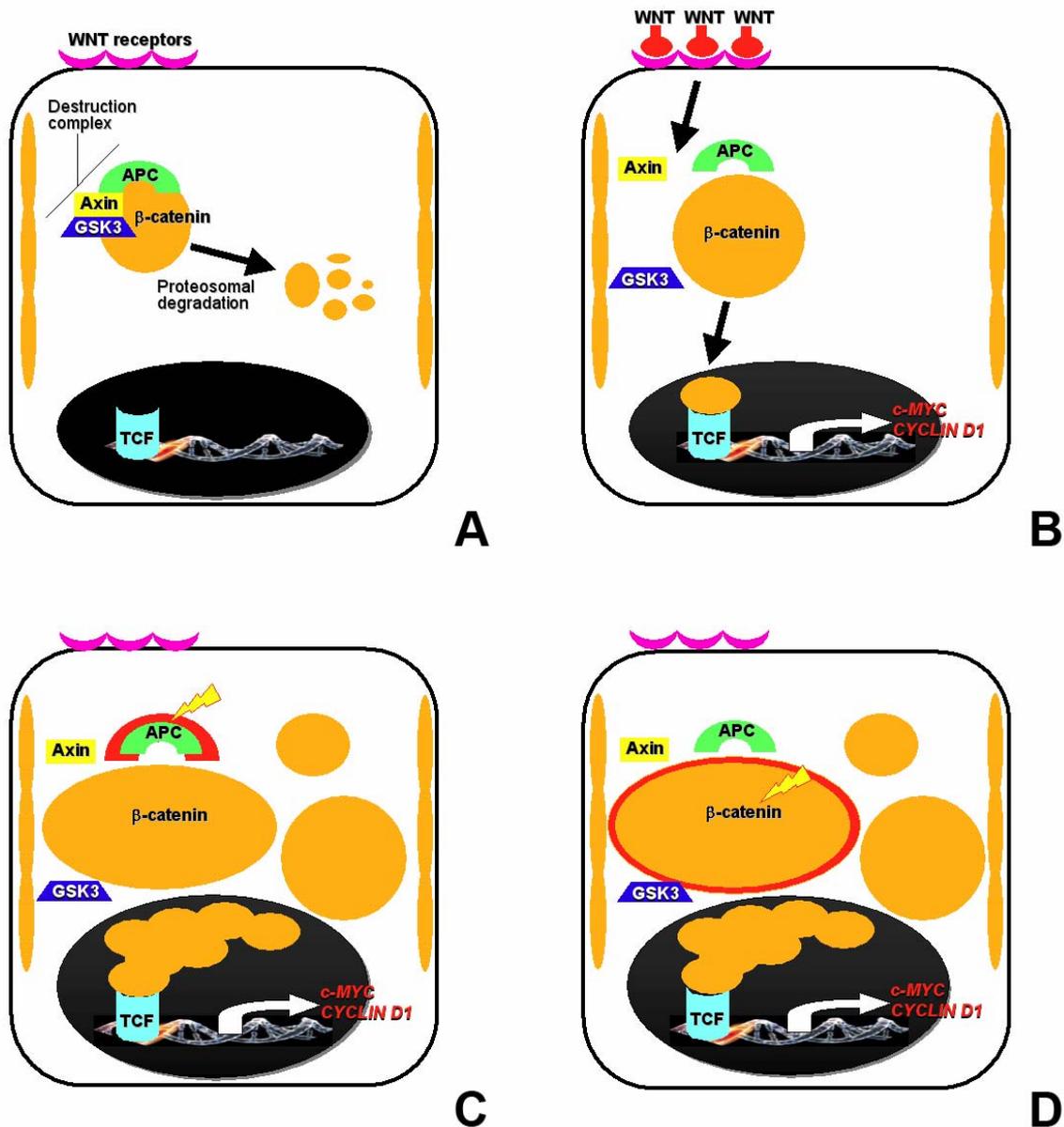


Figure 3. Schematic representation of WNT pathway in normal cells (A and B) as well as in tumor cells of the CMV of PTC with *APC* gene mutations (C) or *CTNNB1* gene mutations (D).

Mutations in the *APC* gene characteristically lead to a truncated APC protein that lacks the capacity to degrade β -catenin. Alternatively, mutations in exon 3 of the *CTNNB1* gene, which prevent phosphorylation of serine and threonine residues, result in activation of this WNT pathway because of the increased cytoplasmic β -catenin. The peculiar morphological features of the CMV of PTC are associated with the permanent activation of the WNT pathway, and consequently, with the aberrant nuclear and cytoplasmic immunoexpression of β -catenin (7, 9, 10, 15) (Figure 2). It has been shown that germline mutation of the *APC* gene, somatic *APC* gene mutation, and/or somatic mutation of the β -catenin gene (*CTNNB1*) lead to this permanent activation of the WNT pathway, explaining the uniform morphology of the CMV of PTC (7). In fact, the aberrant nuclear translocation of β -catenin as well as the presence of morular structures are characteristic features of a peculiar group of tumors of various organs, all of them sharing alterations in the APC/ β -catenin pathway (15, 16).

Regarding the genotype-phenotype correlations in patients with FAP and CMV of PTC, it has been reported that more than 85% of germline mutations of the *APC* gene were in exon 15 in the same genomic area associated with congenital hypertrophy of the retinal pigmented epithelium (CHRPE) (codons 463 to 1387) (17); interestingly, more than 90% of these germline mutations were outside the mutation cluster region (MCR) (codons 1286 to 1513), currently considered the hot spot mutation area. The majority of these mutations occurred before codon 1220 and outside the MCR (17-24). The mutation at codon 1061 has been found to be a hot spot for both thyroid carcinoma and hepatoblastoma (17). The difference in the incidence of germline mutations before and after codon 1220 between PTC and non PTC FAP patients was significant ($P < 0.05$) for both patients and kindreds ($P = 0.005$ and $P = 0.049$, respectively); so that, in patients with PTC, restriction of the mutational analysis of the *APC* gene to the MCR will detect germline and/or somatic mutations in <20% cases (6, 17). Cetta et al (17) recommended intensive screening for thyroid nodules after the age of 15 years if a single patient or entire kindred have ocular patches (CHRPE) and/or mutations in the 5'-portion of exon 15. The mean size of the thyroid tumors in patients with FAP was 1.3 cm (0.9 cm-3.5 cm), and the majority (75%) were multifocal and bilateral (6). In patients with germline *APC* mutation and multiple thyroid tumors, each thyroid tumor in the same patient usually showed a

somatic *APC* mutation that was different from the germline *APC* mutation as well as from the other somatic *APC* mutations (18, 23). Due to these different somatic mutations, this multicentricity of thyroid carcinoma formation is analogous to the multiplicity of colorectal tumor formation (23). A possible pathogenetic role of the common p.Thr1493Thr variant of the *APC* gene in CMV of PTC has recently been reported (7, 24).

For some cases of the sporadic form of CMV of PTC with somatic *APC* mutation, a dominant negative effect of the *APC*¹³⁰⁹ mutation has been proposed as the second hit (Knudson two-hits hypothesis) to explain the development of the tumor (8). Mutations in exon 3 of the *CTNNB1* gene with aberrant nuclear expression of β -catenin have also been reported by Xu et al (9) in sporadic cases of the CMV of PTC.

In addition to genetic alterations in the WNT pathway, *RET/PTC-1* and *RET-PTC-3* rearrangements have been found in this variant, supporting the concept that this tumor may be considered a subtype of PTC (7, 20, 25). No *BRAF* mutations have been found to date (7, 10, 26).

The CMV of PTC generally involves encapsulated or locally advanced tumors without distant spread, and usual treatment consists of total/near total thyroidectomy with or without radioiodine therapy (27). Although this tumor type is generally associated with a good prognosis, 6 out of 126 reported cases (5%) died of the neoplasia (7). More recently, an especially aggressive (poorly differentiated) case of familial CMV of PTC, showing neuroendocrine differentiation has been reported (7).

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Resistance to Thyroid Hormone (RTH) in the Absence of Abnormal Thyroid Hormone Receptor (TR) (nonTR-RTH)

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ABSTRACT

Resistance to thyroid hormone (RTH) is an inherited syndrome of reduced end-organ responsiveness to thyroid hormone (TH). It is characterized by elevated TH levels and nonsuppressed serum TSH in the presence of a goiter. As the term implies, subjects with RTH have impaired responsiveness to TH manifested to variable degrees in different tissues. TH action is mediated by the TH receptors (TR) β and α . The etiology of RTH is usually due to a mutation in the *TR β* gene. The mutant TR β proteins have impaired TH binding and/or cause impaired activation of TH-responsive genes. However, 15% of subjects with a clinically identical RTH phenotype have no demonstrable mutations in the *TR β* gene or in *TR α* gene, when examined. These subjects are classified as nonTR-RTH. The lack of *TR* gene mutation has been confirmed by sequencing both cDNA and gDNA and, in 4 families, *TR β* mutations have additionally been excluded by linkage analysis. We have identified 39 affected individuals belonging to 29 kindreds with nonTR-RTH. This relatively large number of individuals has allowed us to appreciate subtle differences in the demographics of nonTR-RTH compared to RTH with *TR β* mutations, including a female preponderance in the former (2.5:1). However, the key component to the phenotypes, namely TH and TSH levels, do not differ from RTH caused by *TR β* gene mutations. Despite the discovery of nonTR-RTH 15 years ago, the molecular basis for this condition has remained elusive.

Key-words: nonTR-RTH; thyroid hormone receptor β ; thyroid hormone receptor α ; goiter; TSH

TH Action and Reduced Sensitivity to the Hormone

TH action requires more than 30 different cofactors which involve several distinct processes. The first step in TH action is for the hormone to enter the cell. This is achieved through active cell membrane transport. T_4 and T_3 transport is mediated by an active transport process through a family of TH transporters, including the monocarboxylate transporter 8 (MCT-8) (1). In the cell T_4 is either activated by 5' deiodination to form T_3 or inactivated by 5-deiodination to form reverse T_3 . One mode of TH action is through rapid, non-genomic pathways, which are exerted at the level of the plasma membrane and cytoplasm (2). However, the principal, best-studied and characterized effect requires the translocation of the hormone into the nucleus where it interacts with TRs to activate or repress transcription of specific target genes. These genes contain nucleotide sequences at or near their promoter regions (TH response elements or TREs) recognized by TRs for binding. In the absence of TH, TRs homodimerize and associate with nuclear corepressors. These complexes have silencing effect on genes positively regulated by TH. T_3 binding to TRs produces conformational changes, which trigger a chain of processes, including release of the corepressor, often heterodimerization of TR with the retinoid X receptor (RXR) and recruitment of coactivators and a large number of other proteins. In positively controlled genes by TH, this results in making the DNA more accessible for transcription (3). If any of the above molecules (transporters, TH activating enzymes, repressors, activators, etc.) were dysfunctional, a form of reduced TH sensitivity could ensue some sharing the phenotype of RTH. However, since some of the accessory molecules serve in more than one pathway, the phenotype resulting from a defect cannot be predicted.

Clinical Features of RTH and Course of the Disease

The cardinal features of RTH are: 1) elevated serum levels of free T_4 and often free T_3 ; 2) normal or slightly increased serum thyrotropin (TSH); and 3) absence of typical symptoms and metabolic consequences of TH excess (4, 5).

The precise incidence of RTH is not known as it is usually not detected by routine neonatal screening for hypothyroidism, using blood spot TSH determination. A limited screen for high T_4 values found a prevalence of 1:40,000 live births (6).

Characteristic of the RTH syndrome is the paucity of specific clinical manifestations. When present, they are variable from one patient to another (4, 7) Presenting symptoms and signs are goiter, hyperactive behavior, learning disabilities, developmental delay and sinus tachycardia. The finding of elevated serum TH levels in association with nonsuppressed TSH usually leads to suspect the diagnosis.

The majority of subjects maintain a normal metabolic state at the expense of high TH levels. This compensation for the hyposensitivity to TH is variable not only among individuals but also in different tissues. As a consequence, clinical and laboratory evidence of TH deficiency and excess often coexist. For example, delayed growth and bone maturation and learning disabilities, suggestive

of hypothyroidism, can be present along with hyperactivity and tachycardia, compatible with thyrotoxicosis. Common clinical features are given in Table 1. They occur with similar frequency in subject with *TRβ* gene mutations or without. Frank symptoms of hypothyroidism are more common in individuals who have received treatment to normalize their circulating TH levels.

Table 1. Clinical Features: Frequency of Symptoms and Signs

FINDINGS		TR-RTH FREQUENCY*	NonTR-RTH FREQUENCY**
Thyroid gland	Goiter	66-95	83
Heart	Tachycardia	33-75	58
Nervous system	Emotional disturbances	60	50
	Hyperkinetic behavior	33-68	60
	Attention deficit hyperactivity disorder	40-60	75
	Learning disability	30	50
	Mental retardation (IQ <70)	4-16	***
	Hearing loss (sensorineural)	10-22	5
Growth and development	Short stature (<5%)	18-25	N/A
	Delayed bone age >2 SD	29-47	N/A
	Low body mass index (in children)	33	N/A
Recurrent ear and throat infections		55	12
Autoimmune thyroid disease		23	14

IQ = intellectual quotient

*Data derived from (4,7,8)

N/A insufficient data available

**NonTR-RTH associated with: hypertension (2); obesity (4); atrial fibrillation (2); pectus excavatum (2)

***IQ testing is only available on 7 subjects, of these 4 had IQ <70

Goiter is by far the most common finding, reported in 66-95% of cases. Enlargement is usually diffuse. Sinus tachycardia is also very common, which, together with goiter, often lead to the erroneous diagnosis of autoimmune thyrotoxicosis.

About one-half of subjects with RTH have some degree of learning disability with or without attention deficit hyperactivity disorder (4). One-quarter have intellectual quotients (IQ) less than 85 but frank mental retardation (IQ <60) was found only in 3% of cases. Deaf-mutism and color blindness occurred in all three affected members of a single family with *TRβ* gene deletion (8).

The course of the disease is as variable as its presentation. Most subjects have normal growth and development, and lead a normal life at the expense of high TH levels and a small goiter. Others present variable degrees of mental and growth retardation. Symptoms of hyperactivity tend to improve with age. Goiter usually recurs after surgery. As a consequence, some subjects have been submitted to several thyroidectomies or treatments with radioiodide (4).

RTH and *TRβ* Gene Mutations

In the majority of cases, RTH is caused by mutations in the *TRβ* gene, located on chromosome 3. Mutations are found in the carboxyl terminus covering the ligand-binding domain and adjacent hinge domain of the *TRβ* protein (9-11). They are contained within three clusters rich in CG "hot spots", separated by areas devoid of mutations (cold regions). The latter are located between codons 282 and 310, and with the exception of 383, codons 353 and 429. No mutation has been reported upstream of codon 234. As cold regions are not devoid of "hot spots", the lack of mutations reflects the observation that mutations in the second cold region does not impair TR function and, therefore, is not expected to produce a phenotype (5)

TRβ gene defects have been identified in 473 families comprising more than 150 distinct mutations. The authors have found mutations in 148 families and a partial listing is available from <http://www.receptors.org/cgi-bin/nrmd/nrmd.py>. Though mostly missense, nucleotide deletion and insertions producing frameshifts have created nonsense proteins with two additional aminoacids or produced truncated receptors. In only one family complete *TRβ* gene deletion resulted in recessively inherited RTH. The mutant *TRβ* molecules have either reduced affinity for T₃ (9, 10) or impaired interaction with one of the cofactors involved in the mediation of thyroid hormone action (10, 12-14). As TR mutants are still able to bind to TREs on DNA and dimerize with normal TRs or the RXR partner, they interfere with the function of the normal TRs, explaining the dominant mode of inheritance. Therefore, it is not surprising that in the single family reported with a deletion of all coding sequences of the *TRβ* gene, only homozygotes manifest the phenotype of RTH (8).

No mutations in the *TRα* gene have been identified so far in humans. Based on observations in transgenic mice a putative *TRα* gene mutation should not cause typical thyroid function tests as seen in RTH.

nonTR-RTH: Definition and Demographics

In 1996, we reported a family in which RTH manifested in the absence of *TRβ* gene mutation and a *TRβ* gene transcripts of normal size and abundance (15). In addition abnormalities of TRβ were excluded in this family because of absence of phenotype cosegregation with the *TRβ* allele. Nevertheless, fibroblasts were resistant to the *in-vitro* effect of TH. Recombinant wild-type (WT) TRβ interacted aberrantly with nuclear extracts of fibroblasts from affected individuals of the family but not from normal individuals or subjects with complete *TRβ* gene deletion and Far Western analysis revealed an additional 84 kD band. More families with nonTR-RTH were subsequently reported (16-19).

We evaluated 39 affected subjects with nonTR-RTH and 139 unaffected first degree relatives from 29 different families. Comparison of the thyroid function test results of the 39 affected by nonTR-RTH with the corresponding 473 subjects with *TRβ* gene mutations showed no differences (Table 2). While RTH caused by *TRβ* gene mutations has equal gender incidence, nonTR-RTH is more common in females (2.5:1). The possibility of an autoimmune component was excluded by the absence of higher frequency of thyroid autoantibodies. NonTR-RTH occurred mostly sporadically with only 6 families having more than one affected subject. Recessive inheritance and mosaicism need to be considered and when possible excluded.

Laboratory Diagnosis of nonTR-RTH

The laboratory diagnosis of nonTR-RTH is similar to that previously published for RTH. No single test is conclusive and diagnosis of RTH must rest on a combination of test and observations: 1) the absence of an elevated serum concentration of the alpha pituitary glycoprotein subunit; 2) stimulation of TSH following the administration of TSH-releasing hormone (TRH); 3) absence of elevated serum sex hormone-binding globulin concentration (SHBG), reflecting a euthyroid state; and 4) ability to suppress serum TSH with supraphysiological doses of L-T₃.

The measurement of responses to the administration of incremental doses of TH is the best mean to assess the presence and magnitude of the hormonal resistance and obtain a clinical diagnosis of RTH. The rationale for the use of L-T₃ rather than L-T₄ is its direct effect on tissues, independent of variations in T₄ metabolism. The rapid onset of L-T₃ action reduces the period of hormone administration and the shorter half-life of this hormone decreases the duration of symptoms that may arise in hormonally responsive subjects. It involves the administration of three incremental doses of L-T₃, each for the duration of 3 days. Amounts range from just below to 3-times above replacement. Hospitalization for 11 days is required for the detailed study, which includes measurement of sleeping pulse, basal metabolic rate (BMR) and calorie balance for which food intake is controlled and urinary nitrogen excretion is measured (4). A TRH test is performed at baseline and at the time of the administration of the last L-T₃ dose of each increment. Blood samples drawn over

the period of 180 min are used to measure the TSH and prolactin responses as well as the nadir and peak of serum T_3 achieved with each incremental dose. Measurements of TG and T_4 assess the magnitude of thyroid gland suppression, while those of serum cholesterol, creatine kinase, ferritin, SHBG and osteocalcin (OC), the responses of peripheral tissues to the hormone. Whereas these tests can confirm or exclude RTH, they are unable to distinguish TR β -RTH from nonTR-RTH (Figure 1).

Differential Diagnosis of nonTR-RTH

The combination of non-suppressed (normal or slightly elevated) serum TSH with increased concentrations of T_4 , T_3 or both, is characteristic of the three syndromes of reduced sensitivity to TH. However, the most difficult differential diagnosis to make is between RTH due to *TR β* gene mutations and nonTR-RTH as appreciated from the overlapping phenotype and clinical characteristics. Gene sequencing of both cDNA and gDNA and ideally linkage data (when family size permits) can be very helpful to distinguish the two. In addition genetic analysis using several tissue as source of DNA can identify subject with mosaicism due to de-novo mutation.

1. MCT8 Mutation (Transport Defect)

Although the clinical presentation of TH cell transporter defects involving other cell-membrane transporters than MCT8, is unknown, the latter always presents in males accompanied by psychomotor abnormalities, including truncal hypotonia, limb spasticity, poor head control, dyskinetic movements and absent or garbled speech. However, presence of the characteristic thyroid test abnormalities is mandatory. Typical serum test abnormalities are high T_3 , low reverse T_3 and often slightly reduced T_4 concentrations.

The lowish serum T_4 concentration and psychomotor abnormalities should enable the physician to distinguish MCT8 from RTH (20). Sequencing of the *MCT8* gene in subjects with similar psychomotor manifestations but no characteristic thyroid test changes have yielded negative results (21).

2. SECISBP-2 Mutation (T_4 to T_3 Conversion Defect)

Elevated serum T_4 can be observed in subjects with defects in the conversion of T_4 to T_3 . Patients with defects in 5' deiodination are unable to generate sufficient amount of T_3 resulting in pituitary stimulation of TSH and increase in serum T_4 concentration. To date the only gene mutation

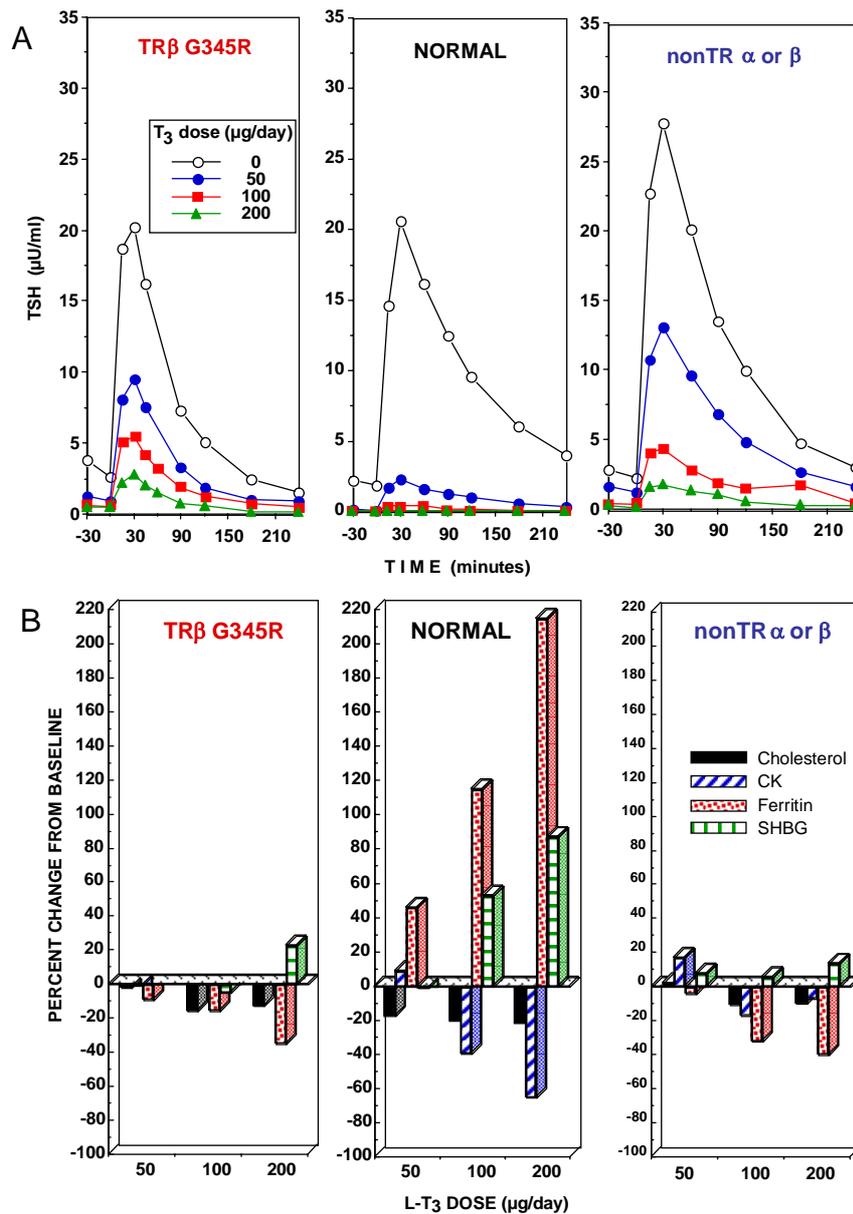


Figure 1. A. Thyrotroph responses to TRH stimulation at baseline and after the administration of graded doses of L-T₃. The hormone was given in three incremental doses, each for 3 days. Results are shown for patients with RTH in the presence (left) or absence (right) of a TRβ gene mutation, together with the unaffected mother of the patient with nonTR-RTH (center). B. Responses of peripheral tissues to the administration of L-T₃ in the presence or absence of mutations in the TRβ gene. Note the stimulation of ferritin and sex hormone binding globulin (SHBG) and the suppression of cholesterol and creatine kinase (CK) in the normal subject. Responses in affected subjects, with or without a TRβ gene mutation, were blunted or paradoxical. [Modified from www.thyroidmanager.org, chapter 16c].

found to result in a iodothyronine deiodinase defect is selenocysteine incorporation sequence-binding protein 2 (SECISBP-2). The defect causes a selective, though generalized reduction in the synthesis of selenoproteins. These subjects are easily distinguished from RTH subjects due to the low T₃ (22). Growth retardation in childhood and azoospermia in adulthood are common.

3. Binding Defects (TBG Excess; FDH)

RTH is characterized by elevation of usually both free T₄ and T₃ levels with non suppressed TSH. Subjects with familial dysalbuminemic hyperthyroxinemia caused by albumin gene mutations, or thyroxine binding globulin (TBG) excess present with elevated total T₄ and T₃, but the free hormone concentrations, when measured by equilibrium dialysis or ultrafiltration, are normal.

4. Mosaicism

Any subject expressing the RTH phenotype in whom no mutation can be demonstrated in a particular cell lineage may have mosaicism. If peripheral blood leukocytes (the most common source of DNA) are not found to harbor a *TRβ* gene mutation, DNA from skin fibroblasts, buccal epithelial cells, sperm (all easily accessible) or other available tissues should be analyzed (23). Such a patient was initially believed to have nonTR-RTH. In the list of subjects with nonTR-RTH (Table 2), the number of tissues examined are listed.

5. TSH Secreting Pituitary Tumor

Patients with TSH secreting tumors display thyroid function tests similar to those of subjects with nonTR-RTH and also have no detectable *TRβ* gene mutations. Pituitary microadenoma may be too small to be detected by imaging. More often a positive MRI may be associated with RTH. The finding of elevated serum α-subunit to TSH ratios and failure to respond to TSH releasing hormone (TRH) are useful tests to distinguish TSH secreting pituitary tumors from RTH, irrespective of the presence or absence of *TRβ* gene mutation. Furthermore, the presence of more than one family member with the same phenotype makes a TSH pituitary tumor unlikely. Rarely, somatic *TRβ* gene mutations can produce TSH secreting adenomas (24).

Treatment of nonTR-RTH

As treatment of RTH is not dictated by the presence and nature of the *TRβ* gene mutation, the therapeutic approach in nonTR-RTH is not different, being aimed at alleviating symptoms when present. Stigmata of TH deficiency are treated with L-T₄ and symptoms of TH excess are treated with β adrenergic blockers. It is important not to treat asymptomatic, fully compensated, individuals with the sole purpose of correcting the laboratory test abnormalities. Prior ablative treatment, resulting from misdiagnosis, requires the judicious administration of TH, often in supraphysiological doses.

Table 2. Genetic studies thyroid function tests and demographics of families with nonTR-RTH

Family ID	#Affected	#Normal*	Linkage to TR β	FT ₄ ** %ULN	FT ₃ ** %ULN	TSH** mU/L	n. of tissues	Ethnic origin	Country
Mm***	3	10	Excl	259	218	17.4	2	Norwegian/Irish/German	USA
Mal	3	21	Excl	189	222	2.1	2	Irish/Scottish/German	USA
Muna***	5	10	not Excl	131	98	5.9	1	Turkish	Turkey
Mpa**	2	2	nonInf	103	109	7.2	1	European	USA
Mlv**	2	3		147	92	4.2	2	Austrian/German (non Jewish)	Israel
Magc	2	7	nonInf	137	119	3.5	3	Turkish	Turkey
Mbz	1	2		114	124	5.5	1	Turkish	Turkey
Msn	1	9	Excl	193	183	3.0	1	German/Duch/Amerindian	USA
Mch	1	8	Excl	174	142	1.9	2	South Chinese (Han)	Hong Kong
Mgd***	1	3		170	84	15.3	1	Dutch/French/German	USA
Mli	1	2		154	137	3.3	1	European	USA
Mk	1	10		199	169	2.1	2	Ashkenazic/Russian/German	USA
Mry	1	0		142	117	3.1	1	European	USA
Msh	1	7		170	192	7.0	3	European	USA
Mby	1	4		190-	121	1.8	2	Irish	Ireland
Maf	1	3		211	144	1.0	1	Hispanic	USA
Mcap	1	1		177	209	4.1	1	European	USA
Mdig	1	2		124	121	1.9	1	Syrian/Irish/Scottish/Polish	USA
Mmg	1	3		180	172	4.7	1	European	USA
Mpe	1	2		117	139	7.6	2	Italian	USA
Mve***	1	6		142	70	143	1	Chilean (Hispanic)	Italy
Meg	1	6		145	156	5.1	2	European	USA
Mwm	1	5		135	110	6.1	1	African American	USA
Mdeb	1	4		253	185	2.6	1	French	France
Mkam	1	0		144	101	9.4	1	Asian Indian	USA
Mdor	1	0		143	112	1.8	1	Unknown	USA
Msz	1	5		174	154	5.2	1	Polish	USA
Mno	1	3		147	132	2.2	1	European	USA
Mer	1	2		154	114	4.3	2	Turkish	Turkey
Total	39	139		176±58	153±64	4.4±4.0			
RTH with TRβ mutations	473	803		189±43	188±45	3.6±3.5			

ULN = Upper Limit of Normal; † 1 = circulating white blood cells (WBC); 2 = WBC and fibroblast; 3 = an additional tissue.

* Including relatives by marriage.

** For more than one affected family member, average value is given.

*** Patients on antithyroid drugs or after thyroidectomy are not included in the calculation of the overall mean.

Excl = excluded by linkage analysis; **nonInf** = non informative.

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The Central Regulation of Food Intake and Energy Expenditure by Thyroid Hormones

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ABSTRACT

For more than a century the essential role of thyroid hormones in the regulation of energy homeostasis has been recognised. In the past, these effects were attributed to thyroid hormone action in the periphery, however in more recent years the important effects of thyroid hormones in the central nervous system (CNS) have also been described, and there is now a substantial body of evidence from genetic and molecular biology studies delineating the CNS pathways through which thyroid hormones influence food intake and energy expenditure. Nutritional signals and neurotransmitters conveying information about the metabolic milieu converge on the hypothalamus effecting changes in neuronal activity culminating in alterations in food intake and metabolic rate. These effects are mediated in part by changes in thyrotropin releasing hormone expression, local CNS thyroid hormone levels, the hypothalamo-pituitary-thyroid axis and the sympathetic nervous system. The characterisation of these pathways will not only improve our understanding of thyroid physiology, but may also translate into targeted pharmaceutical treatments for obesity the archetypal outcome of an imbalance between food intake and energy expenditure. Given the increasing worldwide prevalence of obesity, this would clearly bring significant public health benefits.

Key-words: Thyroid; thyrotropin releasing hormone; TRH; energy; food intake; hypothalamus; paraventricular nucleus; arcuate nucleus; leptin; deiodinase

Introduction

It has long been recognised that thyroid hormone plays a major role in the control of metabolic rate and food intake. This is clearly demonstrated in pathological thyroid states. Hypothyroidism results in reduced basal energy expenditure (1), cold intolerance (2) and weight gain (3) whilst increased energy expenditure, and hyperphagia are characteristic of hyperthyroidism (4). The majority of hyperthyroid subjects lose weight, although, and despite the catabolic state induced by thyrotoxicosis, 5 to 10% of these patients have a sufficiently increased appetite to gain weight (5). This suggests that thyroid hormones may directly stimulate food intake.

Historically these thyroid hormone mediated effects on food intake and energy expenditure have been attributed to the actions of the hormone in the periphery. However, in more recent years, the important role of thyroid hormones and thyrotropin releasing hormone (TRH) within the central metabolic pathways of the hypothalamus has been recognised. This review examines the role of TRH, local CNS thyroid hormone action and the hypothalamo-pituitary-thyroid (HPT) axis in the regulation of energy homeostasis and their interplay with other hormonal, nutritional and neuropeptide mediators of food intake and energy expenditure.

Thyrotropin Releasing Hormone (TRH)

Thyrotropin Releasing Hormone (TRH) is a modified three amino acid peptide hormone (pyro-glutamate-histidine-proline-NH₂) (6, 7, 8, 9). Mature TRH is generated by the post-translational cleavage of pre-TRH which contains five TRH progenitor sequences. Each of these sequences is flanked by basic amino acids which act as cleavage sites for prohormone convertases 1 and 2 (10) and carboxypeptidase E (11). The importance of this post-translational modification is emphasised in mice with defective production of these enzymes which results in an obese phenotype with increased levels of the pro-hormone (11, 12).

TRH was first isolated from extracts of ventral hypothalamic tissue (7). High levels of pre-pro-TRH mRNA were subsequently identified in the hypothalamic paraventricular nucleus (PVN) (13) and neurons of the parvocellular division of the nucleus have been shown to both synthesise and secrete the mature tripeptide (14, 15).

TRH gene expression is regulated by thyroid hormones

The TRH neurons of the PVN are subject to transcriptional regulation by circulating thyroid hormones. The PVN is enclosed by the blood-brain barrier (BBB) and therefore in order for the two circulating forms of thyroid hormone tri-iodothyronine (T3) and thyroxine (T4) to gain access to the PVN these hormones are actively transported into the CNS by two thyroid hormone specific transporters, organic anion transporting polypeptide 1C1 (OATP1C1) and monocarboxylate transporter 8 (MCT8). OATP1C1 has a six fold higher affinity for T4 ($K_m = 0.18\mu\text{M}$) than for T3 (16). Its expression is increased in hypothyroidism and reduced in hyperthyroidism (16). The second thyroid hormone specific transporter, MCT8, has a higher affinity for T3 than for T4 (17).

Previously evidence indicated that OATP1C1 was expressed in the capillary and choroid plexus (16) whilst MCT8 was predominantly associated with target neurons (18) thus suggesting a role for OATP1C1 in the transport of thyroid hormones across the BBB and MCT8 in thyroid hormone neuronal uptake. Recently however the expression of both transporters has been demonstrated in the cerebral microvasculature of rodents and the choroid plexus epithelial cells of both humans and rodents with MCT8 localised to the apical and OATP1C1 to the baso-lateral membrane (19, 20).

Missense mutations or deletions of the MCT8 gene have been identified in humans with Allan-Herndon-Dudley syndrome (AHDS) a form of X-linked psychomotor retardation associated with normal to high circulating TSH and very high serum T3 levels (21). Interestingly, MCT8 null mice have similar abnormalities of the HPT axis but are neurologically normal (22). Recently it has emerged that there may be inter-species

differences in the tissue distribution of these transporters particularly with respect to their expression within the cerebral microvessels. Whilst MCT8 and OATP1C1 are both highly expressed in the mouse and rat CNS microvasculature MCT8 alone is found in human cerebral microvessels (20). Therefore it may be that the human CNS is heavily dependent upon MCT8 for thyroid hormone uptake whilst this may not be the case in mice because of functional redundancy or compensation by OATP1C1. This could explain the phenotypic difference between patients with AHDS and MCT8 knockout mice.

Following transport into the CNS, T₄ and T₃ following its conversion to T₃, regulate TRH expression (14, 23). TRH gene expression is negatively regulated by T₃ such that the transcription of TRH is suppressed by high levels of circulating thyroid hormone and increased when levels fall (14, 23). This negative feedback is mediated by the binding of T₃ to the beta2 isoform of the thyroid hormone receptor (TR β 2) which then binds to a negative thyroid hormone response element (TRE) in the promoter region of the TRH gene (24).

The importance of TRH in the regulation of the HPT axis is underscored by the phenotype of mice lacking TRH, TR β or both. Whilst TR β knockout mice have raised circulating TSH, T₄ and T₃, the double knockouts show a reduction in thyroid hormones and despite being hypothyroid fail to mount a rise in TSH, a phenotype which can be rescued by TRH administration (25).

The local regulation of T₃ and T₄ levels is orchestrated by tissue specific deiodinase enzymes

Two forms of thyroid hormone, T₄ and T₃ are released by the thyroid gland but it is the inactive pro-hormone T₄ which is the major secretory product. T₄ has low activity at thyroid hormone receptors (TRs) and must be converted to T₃ in order to exert its biological activity. The activation and the inactivation of thyroid hormones is catalysed by the deiodinases, a group of three thioredoxin fold enzymes which have a conserved selenocysteine containing

active site. It is the selective removal of iodine from T4 and its derivatives by the deiodinases that activates or inactivates these hormones.

Deiodinase 1 (D1) is predominantly expressed in liver and kidney cells with a preference for reverse T3 (rT3) and sulfated iodothyronines as substrates, however the physiological reason for this is not known (26). D1 can act as an outer ring deiodinase converting T4 to T3 or rT3 to T2. D1 can also deiodinate the inner ring of T4 thus converting it to rT3 (26). Although the physiological role of D1 is uncertain its renal and hepatic activity is thought to contribute to the circulating T3 level.

Deiodinase 2 (D2) has a high affinity for T4, with a K_m of 1nM for T4, which is three orders of magnitude higher than the affinity of D1 for T4 (27, 28). The majority of intra-cellular T3 is generated by the D2 catalysed outer ring deiodination of T4 to T3 (29). The expression of the deiodinase 2 gene (*Dio2*) and the activity of D2 increases markedly in hypothyroidism (30) and falls in response to T4 administration (29) thus protecting the tissue from the adverse effects of thyroid deficiency or excess (31). D2 is highly expressed in the CNS and in the periphery where it is found in brown adipose tissue, the anterior pituitary gland, skeletal muscle, cardiac myocytes (32) and the thyroid gland (33, 34).

In the anterior pituitary gland and the hypothalamus D2 plays a well defined role in the feedback regulation of the HPT axis. *Dio2* knockout mice have elevated serum levels of both T4 and TSH and in these animals TSH may be suppressed by the administration of T3 but not T4 demonstrating central T4 resistance (35). This suggests that the activity of pituitary D2 is critical in the regulation of TSH secretion. D2 is also essential in the thyroid hormone mediated feedback regulation of TRH production. D2 is not expressed in hypophysiotropic neurons (36) but is highly expressed in tanycytes (37), the specialised endothelial cells which line the third ventricle, which have been shown in mice and rats to express both MCT8 and OATP1C1 (20). Within tanycytes D2 converts circulating T4 to T3 which can then access the TRH neurons of the PVN. In mice the neuronal uptake of thyroid hormones is

thought to be mediated by MCT8 (18). This mechanism ensures that under basal conditions TRH production is regulated by the peripheral T4 concentration the importance of which is underscored by the phenotype of *Dio2* knockout mice described (35).

Deiodinase 3 (D3) catalyses the conversion of T4 to rT3 and T3 to 3',3'-diiodothyronine (T2) by inner ring deiodination. Both rT3 and T2 lack activity at TRs and thus D3 activity results in the local termination of T3 action. D3 has been identified within the adult CNS (38) where a small amount of D3 mRNA is detectable within the hypothalamus of euthyroid rats. Its expression within the CNS is regulated by the prevailing thyroid hormone level, being increased in hyperthyroidism (39) and reduced when local levels of thyroid hormones fall. The expression of D3 in other adult tissues is low although its expression may be induced in the liver and skeletal muscle of critically ill patients (40) and this activity could contribute to the pattern of low circulating T3 and high rT3 levels seen in the euthyroid sick syndrome.

It is therefore the relative expression and activity of D2 and D3 which governs the temporal and spatial level of intracellular T3 and this is important during both developmental and adult life.

D3 is highly expressed in the placenta where its activity regulates the transplacental passage of T4 and T3 from mother to fetus (41) thus protecting the developing fetus from maternal thyroid hormone levels which may be inappropriate for gestational age.

D3 has also been demonstrated to play a key role in retinal development during *Xenopus laevis* morphogenesis. In this species D3 is expressed in the dorsal ciliary marginal zone an area of the retina which contains cells that proliferate under thyroid hormone stimulation (42). This specific temporo-spatial activity of D3 results in the characteristic asymmetric growth of the frog retina.

The interplay of D2 and D3 expression in governing the tissue specific availability of thyroid hormones is also critical during cochlea development. In the mouse there is an increase in

local D2 expression in the cochlea in early postnatal life leading to an almost 100 fold increase in local T3 relative to the serum concentration (43). Despite normal circulating levels of T3, *Dio2* knockout mice have impaired hearing due to the retarded or abnormal development of the cochlear inner sulcus, sensory epithelium and tectorial membrane, a phenotype that can be rescued by the administration of high dose T3 to the nursing dam during the appropriate postnatal time period (44). Prior to this postnatal expression of D2, the cochlea is protected from exposure to T3 by the expression and activity of D3 (45). Cochlea development thus provides an elegant example of how the interplay between the expression and activity of the deiodinases allows an individual subpopulation of cells within a tissue to govern local exposure to thyroid hormones irrespective of the amount of the hormone present in the circulation.

The paraventricular nucleus (PVN) and arcuate nucleus (ARC) are key hypothalamic nuclei in the control of food intake and energy expenditure

The hypothalamus is a region of the brain that is important in the regulation of food intake and energy expenditure. The arcuate nucleus (ARC) of the medio-basal hypothalamus is located near the median eminence where the BBB is incomplete thus rendering it susceptible to the effects of circulating factors which signal nutritional status and the metabolic milieu (46) whilst the PVN is thought to be a second order nucleus.

Leptin is a protein synthesised and secreted by adipocytes signalling information about energy stores and nutritional status (47, 48). Serum leptin levels are normally proportional to fat mass (49) but after a short period of fasting circulating leptin levels fall markedly, a reduction that is out of proportion with the loss of adipose tissue (50). The peripheral or central administration of leptin results in a reduction in food intake, a fall in body weight and an increase in energy expenditure (48, 51, 52). Conversely, leptin deficient *ob/ob* mice which can be used as a model of perceived starvation, are hyperphagic, obese, and hypothermic with reduced energy expenditure (47).

When rats and humans are food deprived there is a rapid reduction in circulating T4 and T3. This in part may be explained by the down regulation of hepatic and thyroid gland Deiodinase 1 gene (*Dio1*) expression (53) and D1 activity (54) during fasting. However, part of this effect is due to a reduction in circulating leptin which results in a rapid down regulation of the HPT axis in a pattern consistent with central hypothyroidism (55, 56) with a reduction in pro-TRH mRNA in the PVN (Figure 1).

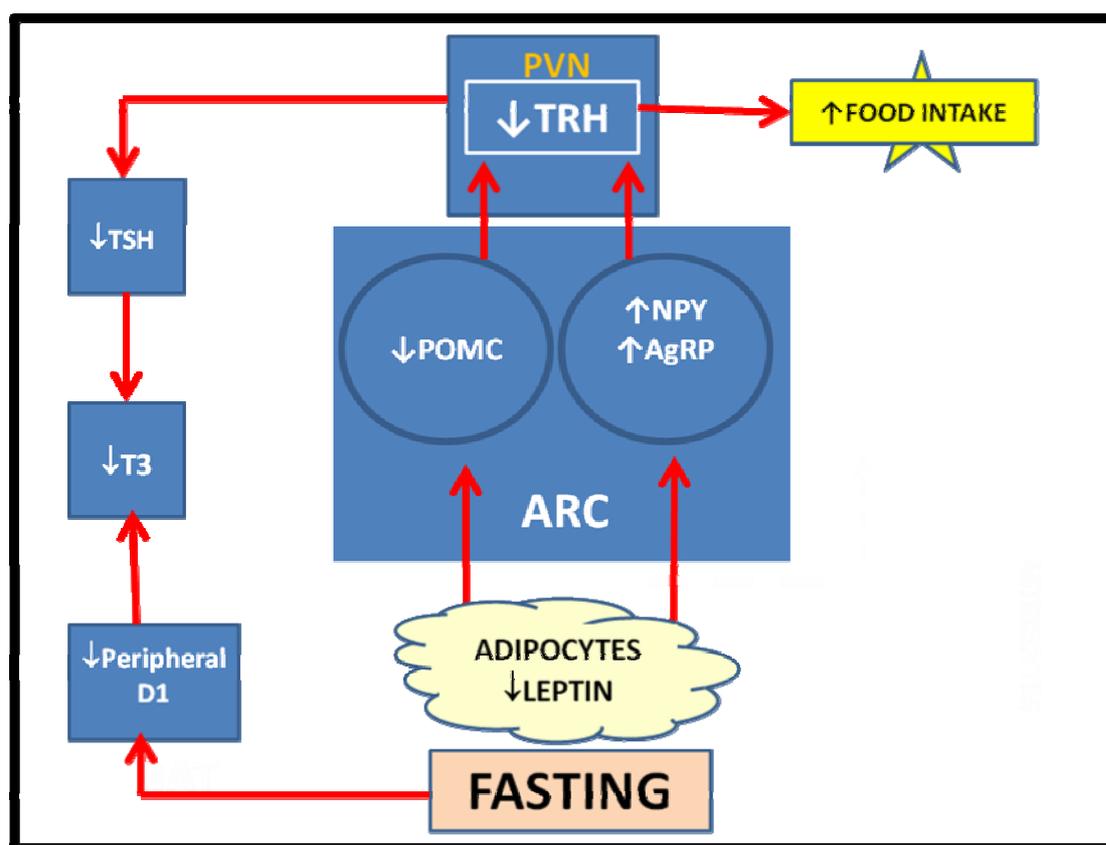


Figure 1 The response of the hypothalamo-pituitary thyroid axis to fasting

During fasting there is a fall in the plasma leptin, a peptide hormone produced by adipocytes, which conveys information about energy stores and nutritional status. Leptin acts on two neuronal populations of the arcuate nucleus (ARC) of the hypothalamus. One subpopulation expresses the anorectic peptide alpha-melanocyte-stimulating hormone (α -MSH) a product of the Pro-opiomelanocortin (POMC) gene which is down regulated in the face of low circulating leptin. The other leptin responsive neurons of the ARC express the orexigenic neuropeptide Y (NPY) and agouti-related protein (AgRP) which are up regulated in response to low leptin levels. These changes in ARC neuropeptide expression and release exert an effect on the thyrotropin releasing hormone (TRH) neurons of the paraventricular nucleus (PVN) of the hypothalamus. A fall in α -MSH and a rise in NPY and AgRP signalling reduces the expression of TRH. This fall in TRH leads to the systemic down regulation of the hypothalamo-pituitary-thyroid axis with a resultant reduction in the synthesis and release of thyroid

stimulating hormone (TSH) a reduction in peripheral deiodinase 1 (D1) expression and a fall in plasma tri-iodothyronine (T3). TRH may also exert a direct anorectic effect, thus the fall in its expression may lead to an increase in food intake.

This central down regulation of the HPT axis in response to food deprivation is due to both the indirect action of leptin on TRH neurons via effects on the ARC and the direct effect of leptin on the PVN.

These starvation induced changes of the HPT axis can be reversed by the administration of leptin (57, 58). Similarly, humans and mice with mutations of the leptin receptor or leptin itself have central hypothyroidism (59, 60), which is ameliorated in the latter case by leptin administration (61).

Leptin exerts direct effects on the PVN leading to up regulation of TRH gene expression

A subset of TRH neurons in the PVN express the leptin receptor (ObR) (62) and the administration of leptin *in vitro* to rat hypothalamic explants containing TRH neurons transfected with a reporter gene has provided a line of evidence for the direct stimulation of TRH neurons by leptin resulting in the increased synthesis and release of TRH (62).

Binding of leptin to the ObR leads to the activation of Janus tyrosine kinase and the subsequent phosphorylation and activation of signal transducers and activators of transcription (STAT) proteins (63, 64). Phosphorylated STAT proteins dimerise and translocate to the nucleus where they activate gene transcription (65). The peripheral administration of leptin leads to a rapid increase in STAT phosphorylation in a subset of TRH neurons of the PVN (66) and STAT3 binds to a STAT-binding site on the TRH promoter (67) which leads to the up regulation of TRH gene expression (68).

The actions of leptin on the HPT axis are mediated in part by the melanocortin system

The ARC contains two key leptin responsive neuronal populations which regulate food intake and energy expenditure. One group of neurons co-express the orexigenic peptides neuropeptide Y (NPY) and agouti-related protein (AgRP) (69). The other neuronal sub-population co-expresses the anorectic peptides alpha-melanocyte-stimulating hormone (α -MSH) derived from pro-opiomelanocortin (POMC) and cocaine-and amphetamine-related transcript (CART) (70).

The expression of the POMC gene is up regulated by leptin and reduced during food deprivation (71) whilst the mRNA expression of AgRP is regulated in a fashion that is inversely proportional to POMC mRNA in response to fasting and the administration of leptin (72, 73). Physiologically, α -MSH mediates a basal tonic inhibition of food intake by agonism at the melanocortin 4 receptor (MC4R). Conversely, AgRP which is an antagonist at the MC4R, stimulates food intake (74, 75).

The POMC and AgRP neurons of the ARC synapse directly onto the TRH neurons of the PVN (76, 77, 78) and both neuropeptides act at the MC4R which is expressed on TRH neurons (79).

The administration of AgRP into the PVN of rats reduces plasma TSH and T4 whilst the intracerebroventricular (ICV) administration of α -MSH increases plasma TSH in fasted animals and prevents the starvation induced fall in TRH expression (78, 80). These downstream effects of α -MSH are mediated by a cAMP-response element binding protein which can bind to the TRH promoter (79). The application of α -MSH to hypothalamic explants increases TRH release and this effect is opposed by coadministration of AgRP (80).

The ARC orexigenic neuropeptide NPY down regulates the HPT axis

NPY is a potent orexigenic neuropeptide (81). Large numbers of leptin receptor expressing NPY neurons are present in the ARC (82) and the administration of leptin leads to the

inhibition of NPY synthesis and release (83). Mice deficient in both leptin and NPY are less hyperphagic and have increased energy expenditure leading to a less obese phenotype compared to mice deficient in leptin alone (84). This confirms NPY's position as downstream of leptin in the central regulation of food intake and energy expenditure. The NPY deficient mouse with intact leptin does not have abnormalities of the HPT axis (85), however nor does this model show a phenotypic change in food intake or body weight (86) which highlights the redundancy within central appetite circuits.

ARC NPY neurons project directly to the PVN (87) where NPY fibres densely co-localise with TRH cell bodies (76, 88). When NPY is injected directly into the PVN of satiated rats the peptide markedly increases food intake in a dose-dependent manner (89).

NPY mediates its effects on food intake through the G_i protein-coupled Y1 and Y5 receptors (90, 91). The Y1 receptor is expressed by TRH neurons of the PVN (92) and the Y5 receptor is also highly expressed within the PVN (93). The ICV administration of NPY as well as highly selective Y1 and Y5 agonists leads to a reduction in plasma T3 and T4, an inappropriately low TSH and a fall in proTRH mRNA in the PVN (94, 95). This NPY mediated down regulation of the HPT axis may partly explain the fall in energy expenditure that occurs following NPY administration (96).

Taken altogether these findings show that leptin acts both on ARC orexigenic and anorectic neurons which project to the PVN and directly at the PVN leading to changes in TRH gene expression. This allows the HPT axis at the level of TRH to be influenced by nutritional status.

Thyroid hormones stimulate food intake

It has long been recognised that T3 stimulates food intake. In humans one of the characteristics of thyrotoxicosis is hyperphagia (97) and in rodents, the peripheral and central administration of T3 increases food intake (98, 99).

The peripheral administration of T3 leads to an increase in hypothalamic NPY mRNA and the ICV administration of an NPY Y1 receptor antagonist has been shown to blunt T3 induced hyperphagia, (99). In addition, T3 administration leads to a reduction in hypothalamic POMC and CART mRNA (99). However, changes in hypothalamic neuropeptide expression in response to the peripheral administration of T3 has not been reported by all investigators (98) which may reflect differences in the administered dose of T3.

The direct injection of T3 into the ventromedial nucleus (VMN) of rats but not the ARC leads to an increase in food intake (98), however the mechanism responsible for this observation is not clear and it is not known whether this hyperphagic effect of T3 is mediated within VMN neurons or through their post-synaptic connections in another hypothalamic or brain region.

Since the ARC is well established as a key hypothalamic nucleus in the regulation of food intake (100) an indirect effect on the ARC via the VMN is a possible mechanism by which intra-VMN T3 leads to an increase in food intake. Excitatory inputs from the VMN to POMC neurons have been described (101) which sits well with the classical lesioning experiments of the 1940s implicating the VMN as a 'satiety centre' (102). The possibility of excitatory output from the VMN is further supported by the large amount of the excitatory neurotransmitter glutamate identified in the VMN (103) as evidenced by the presence of vesicular glutamate transport proteins (VGLUTS) which are considered a specific marker of neurons which use glutamate as a neurotransmitter (104). It is therefore possible that T3 may inhibit glutamate synthesis and or release thus increasing food intake by interrupting the excitatory input from the VMN to the POMC neurons of the ARC. Interestingly, in the anterior pituitary gland, the VGLUT-2 isoform mRNA has been shown to be increased in hypothyroidism (105) however the converse has not been shown for hyperthyroidism. Further supporting evidence for this putative indirect inhibition of the POMC neurons comes

from studies of changes in ARC neuropeptide mRNA following peripheral T3 administration which leads to a marked reduction in hypothalamic POMC expression (99).

If T3 is exerting a direct orexigenic action within the VMN, one putative peptide which may be important in this effect is brain-derived neurotrophic factor (BDNF) which is highly expressed in the nucleus (106). Chronic ICV administration of BDNF into the brain of adult rats significantly reduces food intake (107) and food deprivation reduces BDNF mRNA by 60% in the VMN (108). BDNF acts via tyrosine kinase receptor (TrkB) and mice with a reduction in brain trkB expression develop hyperphagia and obesity (108). *In vitro*, T3 has been shown to reduce BDNF gene expression when applied to hypothalamic explants (109). The exact physiological role of BDNF however remains uncertain and of note the Cre-LoxP mediated BDNF excision within the leptin receptor expressing SF-1 neurons of the VMN does not produce an obese phenotype (110).

Local changes in T3 levels mediated by D2 and D3 influence food intake and energy expenditure

Within the CNS high D2 activity is found in the ARC and the median eminence (111) where it is expressed within astrocytes and tanycytes. The processes of the D2 containing tanycytes are in direct apposition to the NPY/AgRP neurons of the ARC (112) which also express the inner mitochondrial membrane uncoupling protein 2 (UCP2) (112). Compared with uncoupling protein 1 which plays a well characterised role in non-shivering thermogenesis (see below) UCP2 generates less marked changes in mitochondrial inner membrane permeability and its function is not dependent upon sympathetic activation (113).

During fasting there is a marked increase in hypothalamic D2 expression and activity (98). This not reversed by the systemic administration of T4 (114) but appears to be regulated by the fall in plasma leptin and rise in plasma glucocorticoids during food deprivation (115). Leptin receptors are not expressed on tanycytes but are expressed on astrocytes in the ARC (116) which are immunoreactive for STAT3. This fasting stimulated rise in ARC D2 activity

results in the tissue specific increase in T3 which in turn leads to increased UCP2 activation and mitochondrial proliferation within NPY/AgRP neurons (112). This latter effect may be critical for the ability of these orexigenic neurons to maintain an increased firing rate during fasting.

In *Dio2* null mice fasting fails to elevate NPY expression (112) which further emphasises the significance of intra-ARC T3 production in the response to food deprivation.

This central increase in T3 also results in the suppression of TRH expression in the PVN (117) which can be prevented by infusion of the D2 inhibitor iopanoic acid (114). The reduction in TRH in response to fasting may also be important as TRH itself has been shown to have a direct anorectic effect when administered by injection into the medial hypothalamus (118).

Evidence from studies on hibernating mammals suggests that in addition to D2, D3 may also play a role in the regulation of feeding. These animals show marked changes in food intake and energy expenditure depending on photoperiod and during short days which occur in the winter months enter a catabolic phase with a reduction in food intake and body weight and a fall in core body temperature (119). It is during these short photoperiod days that the expression and activity of hypothalamic D3 increases leading to a reduction in locally available T3 with a concomitant fall in food intake and body weight which is reversible by the implantation of a T3 pellet into the dorsomedial hypothalamus (120).

Therefore within the hypothalamus the expression and activity of D2 and D3 depends upon the prevailing homeostatic circumstances, resulting in tissue specific changes in hypothalamic T3 availability which in turn plays an important role in the regulation of food intake and energy expenditure (Figure 2).

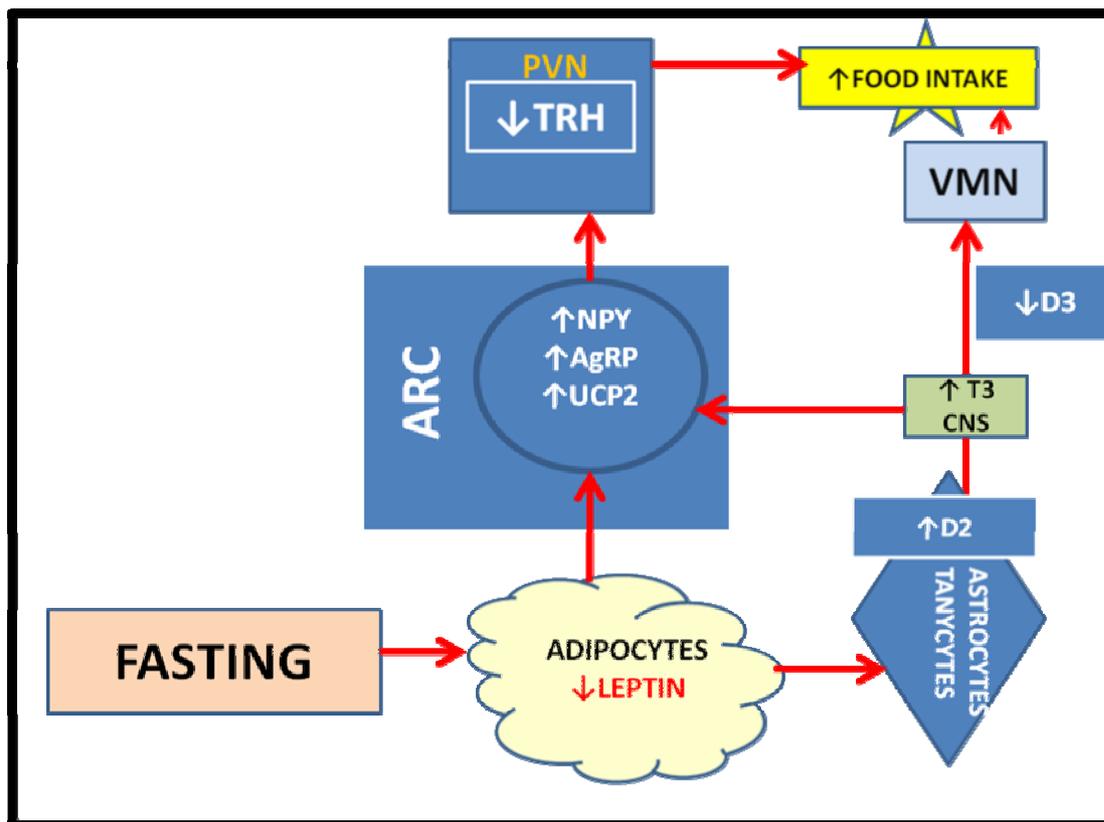


Figure 2 The response of deiodinase 2 (D2), deiodinase 3 (D3) and central nervous system (CNS) tri-iodothyronine (T3) to fasting

During fasting the fall in plasma leptin increases the activity of deiodinase 2 (D2) in the tanocytes of the third ventricle and the astrocytes of the mediobasal hypothalamus resulting in a local increase in tri-iodothyronine (T3) within the central nervous system (CNS). This results in an increase in the firing rate of the orexigenic NPY/AgRP neurons of the arcuate nucleus (ARC) in response to fasting through uncoupling protein 2 (UCP2) activation and mitochondrial proliferation within the neurons. In the paraventricular nucleus (PVN) NPY stimulation leads to down regulation of TRH expression and stimulates food intake. The increase in CNS T3 also exerts an orexigenic effect within the hypothalamic ventromedial nucleus (VMN) an effect which is augmented by a concurrent fall in hypothalamic deiodinase 3 (D3) activity.

The sympathetic nervous system (SNS) and the HPT axis act synergistically to promote thermogenesis in brown adipose tissue (BAT)

For more than a century the critical influence of thyroid hormones on basal metabolic rate (BMR) has been recognised (121). Obligatory thermogenesis is the heat produced as a consequence of BMR whilst adaptive thermogenesis describes the heat generated in

response to food intake or a fall in ambient temperature. These key intracellular processes consume a large amount of ATP and are therefore major determinants of an organism's energy expenditure. The relationship between adaptive thermogenesis and the HPT axis is clearly demonstrated by the prevention of the rapid rise in metabolic rate in cold exposed thyroidectomised rats (122).

The HPT axis acts in close synergism with the sympathetic nervous system (SNS), the mediator of the 'fight or flight' response which can be activated by a fall in ambient temperature or excess food intake (123). TRH neurons of the PVN are innervated by catecholaminergic neurons arising from the brainstem (124, 125) and respond to the released norepinephrine (NE) by the increased synthesis and release of TRH. This norepinephrinergic effect is mediated via the direct binding of catecholamines to adrenoceptors present on TRH neurons of the PVN through activation of the cAMP response element binding protein pathway (126). Centrally administered β -adrenoceptor antagonists block cold induced pro-TRH synthesis whilst α -adrenoceptor antagonists prevent cold-induced TRH release (126). This up regulation of the HPT axis in response to cold exposure or overfeeding results in an increase in circulating T4 which acts within brown adipose tissue (BAT), a key site of adaptive thermogenesis. Previously, it had been thought that BAT was confined to small mammals and human neonates. Recent work using fluorodeoxyglucose positron emission tomography (FDG PET) scanning has identified a significant amount of BAT in adult humans (127, 128) although further work is needed to define its distribution and physiological significance. BAT is a mitochondria rich tissue which receives dense innervation from the SNS (129). When BAT is stimulated by NE the tissue responds with the increased expression and activation of the inner mitochondrial membrane protein uncoupling protein 1 (UCP1). UCP1 activity results in the dissipation of the proton gradient across the inner mitochondrial membrane leading to heat rather than ATP production, an effect that is abolished in animals treated with propranolol (130) (Figure 3).

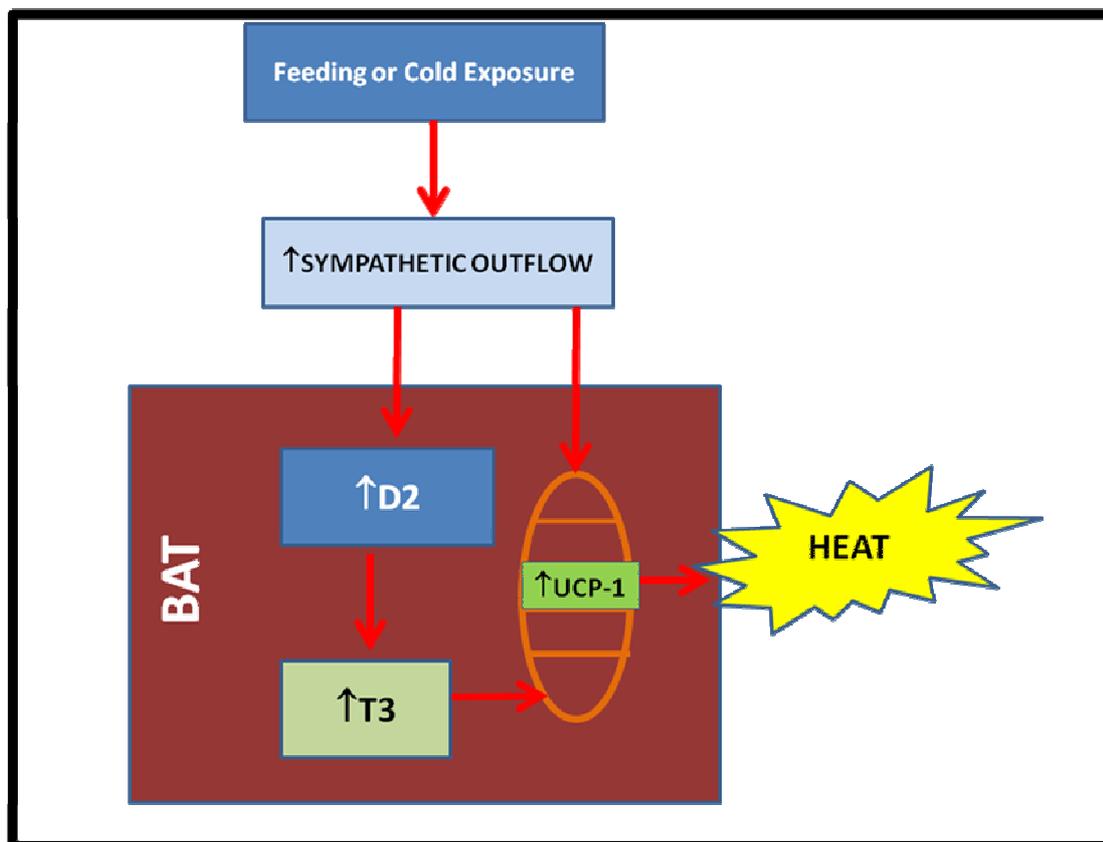


Figure 3 The interplay of tri-iodothyronine (T3), deiodinase 2 (D2) and the sympathetic nervous system in thermogenesis

Feeding or cold exposure leads to an increase in the sympathetic outflow from the paraventricular nucleus with a consequential increase in the sympathetic stimulation of brown adipose tissue (BAT). This leads to an increase in the activity of the enzyme deiodinase 2 (D2) within BAT which is required for the conversion of the pro-hormone thyroxine to the active hormone tri-iodothyronine (T3). A rise in intra-BAT T3 and an increase in the sympathetic stimulation of the tissue results in the increased expression and activity of mitochondrial uncoupling protein 1 (UCP1) which functions to dissipate the proton gradient across the inner mitochondrial membrane leading to heat rather than ATP production.

This sympathetic stimulation also leads to the activation of D2 within BAT and an increase in tissue T3 (131, 132). The importance of this D2 effect is underscored by the phenotype of the *Dio2* knockout mouse which shows impaired thermogenesis and can only respond to a cold stimulus by shivering (133). The T3 generated by D2 activation increases the

expression of UCP1 which has a TRE in its 5'flanking region (134). It is this synergism between the SNS and HPT axis which augments the metabolic response to a fall in ambient temperature or excess food intake.

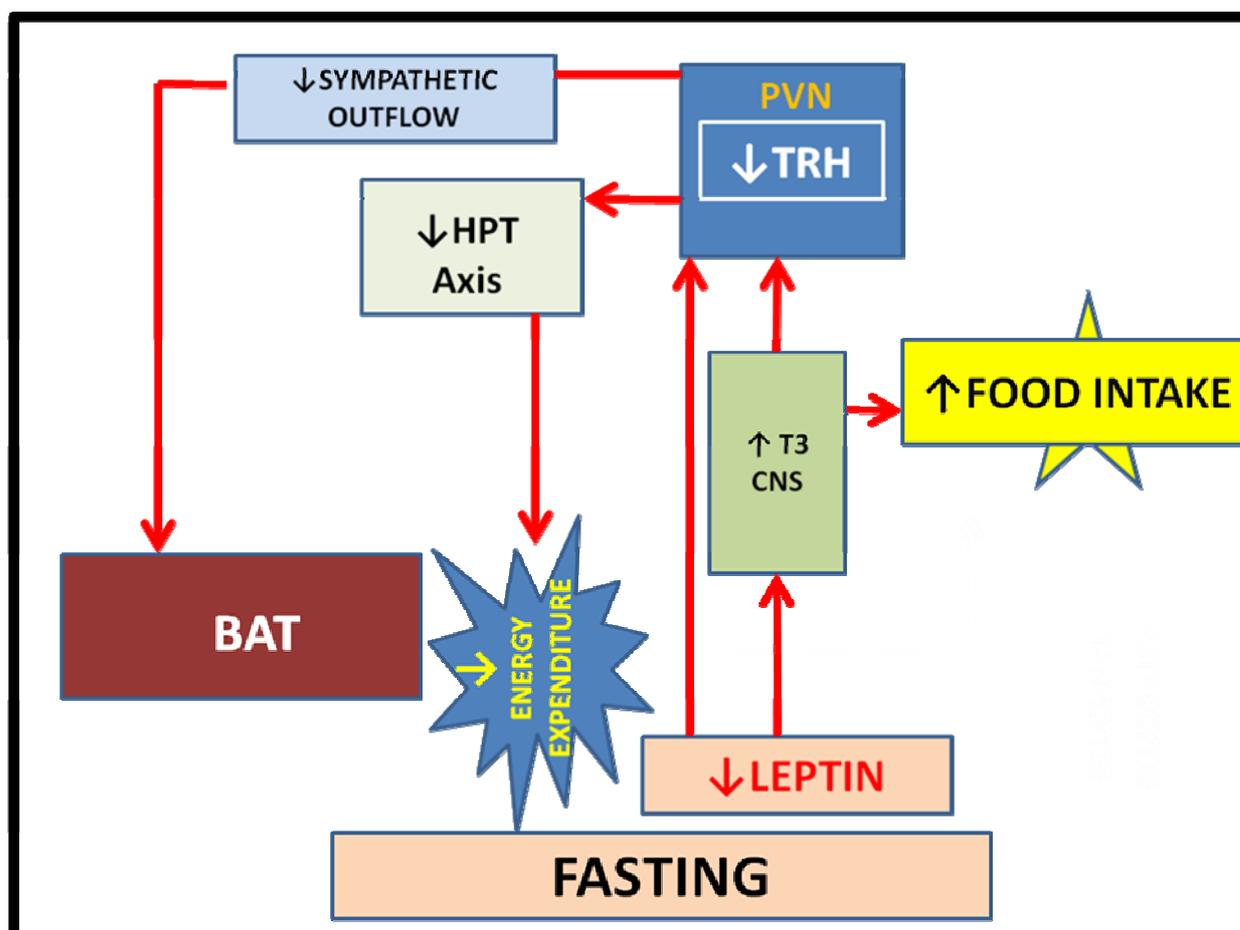


Figure 4 The regulation of food intake and energy expenditure by thyroid hormones

During fasting there is a fall in plasma leptin which results in a reduction in systemic T4 and T3 leading to a fall in energy expenditure but a local increase in T3 production in the CNS. Within the hypothalamus this latter corollary exerts a potent orexigenic effect. Tri-iodothyronine (T3); Central nervous system (CNS); Thyrotropin stimulating hormone (TRH); Paraventricular nucleus (PVN); Hypothalamo-pituitary-thyroid (HPT) axis; Brown adipose tissue (BAT).

Concluding Remarks

The interplay between nutritional status, the HPT axis and energy expenditure is tightly regulated facilitating the conservation of energy during food deprivation (Figure 4). In the

past two decades molecular biology and genetic studies have proffered mechanisms for *in vivo* and clinical observations related to the HPT axis, energy expenditure and food intake. The amassed evidence suggests that when food intake falls these complex neuronal pathways effect a reduction in systemic T4 and T3 leading to a fall in energy expenditure but a local increase in T3 production in the CNS. Within the hypothalamus this latter corollary exerts a potent orexigenic effect which may be mediated within the VMN. Conversely when nutrition is replete the HPT axis is up regulated. As our understanding of the regulation of food intake and energy expenditure by thyroid hormones develops, future work may lead to the translation of these physiological findings into targeted pharmaceutical treatments for the management of obesity the archetypal outcome of an imbalance between food intake and energy expenditure.

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Abbreviations

3',3'-diiodothyronine (T2)

Agouti-related protein (AgRP)

Allan-Herndon-Dudley syndrome (AHDS)

Alpha-melanocyte-stimulating hormone (α -MSH)

Anterior pituitary gland (PIT)

Basal metabolic rate (BMR)

Blood-brain barrier (BBB)

Brain-derived neurotrophic factor (BDNF)

Brown adipose tissue (BAT)

Central nervous system (CNS)

Cocaine- and amphetamine-related transcript (CART)

Deiodinase 1 (D1)

Deiodinase 1 gene (*Dio1*)

Deiodinase 2 (D2)

Deiodinase 2 gene (*Dio2*)

Deiodinase 3 (D3)

Deiodinase 3 gene (*Dio3*)

Fluorodeoxyglucose positron emission tomography (FDG PET)

Hypothalamo-pituitary-thyroid (HPT)

Intracerebroventricular (ICV)

Leptin receptor (ObR)

Melanocortin 4 receptor (MC4R)

Michaelis-Menten constant (K_m)

Monocarboxylate transporter 8 (MCT8)

Neuropeptide Y (NPY)

Norepinephrine (NE)

Organic anion transporting polypeptide 1c1 (OATP1C1)

Paraventricular nucleus (PVN)

Pro-opiomelanocortin (POMC)
Reverse T3 (rT3)
Signal transducers and activators of transcription (STAT)
Sympathetic nervous system (SNS)
Thyroid gland (THYROID)
Thyroid hormone receptor (TR)
Thyroid hormone receptor β (TR β)
Thyroid hormone response element (TRE)
Thyroid stimulating hormone (TSH)
Thyrotropin Releasing Hormone (TRH)
Thyroxine (T4)
Tri-iodothyronine (T3)
Uncoupling protein 1 (UCP1)
Uncoupling protein 2 (UCP2)
Ventromedial nucleus (VMN)

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Genetic Markers for Thyroid Cancer in the Context of Current and Future Therapeutic Applications

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ABSTRACT

There is increasing insight into the molecular changes that contribute to thyroid cancer development and progression. Concurrent to the recent advances in knowledge of the cellular processes central to thyroid carcinogenesis, there has also been a dramatic increase in clinical trials available for thyroid cancer patients. The majority of these promising agents in phase I & II trials belong to the class known as tyrosine kinase inhibitors (TKIs). These small molecule kinase inhibitors have multiple receptor and nonreceptor targets. Despite the addition of TKIs, there is no cure for thyroid cancer. Continued research is necessary to address clinical questions such as how to distinguish benign from malignant thyroid lesions on biopsy, predict which tumors will have a more aggressive outcome, and how to individualize treatment for maximal therapeutic response. This review will highlight research findings that have influenced the rationale behind using TKIs and other novel therapies and discuss how elucidating the mechanisms contributing to thyroid cancer may translate to future diagnostic and treatment strategies.

Introduction

Differentiated thyroid cancer, which arises from the epithelial follicular cells, is the most common endocrine malignancy and the subtypes papillary and follicular thyroid cancer make up the majority of cases (1). As no non-invasive test can yet accurately predict a benign from a malignant nodule, the majority of non-functioning thyroid nodules will undergo fine needle aspiration biopsy. Papillary thyroid cancer can be readily diagnosed on FNA; however follicular thyroid cancer can only be diagnosed by examination of the surgical specimen for the presence of capsular or vascular invasion, or extra-thyroidal extension (2). Thyroid cancer is one of the few cancers with an increasing incidence, with papillary thyroid cancer (PTC) representing the majority of cases; however this increase may be attributed to improved detection methods (3).

Most thyroid cancer patients have an excellent outcome and standard treatment usually consists of total thyroidectomy, often with lymph node resection, thyroid hormone suppressive therapy, and in more advanced staged disease, radioactive iodine (I-131) for either remnant ablation or therapeutic treatment (1, 4). These standard therapies are dependent on the tumor exhibiting a differentiated phenotype similar to normal thyrocytes consisting of responsiveness to the growth factor TSH via the presence of the TSH receptor and expression of the sodium-iodide symporter (5, 6). Surveillance for these patients typically consist of a combination of anatomical imaging such as neck ultrasound, radiiodine whole body scans, and serum measurement of the thyroid-specific protein thyroglobulin with anti-thyroglobulin antibody levels (2).

Thyroid cancer patients with recurrent or metastatic disease can have mortality rates approaching 50% (4, 7). Dedifferentiation of thyroid cancer may consist of loss of expression of the TSH receptor, NIS, and loss of thyroglobulin production. In the process of a tumor losing NIS expression, the clinician loses the ability to use radioiodine for monitoring and treatment. However this subset of tumors frequently become visible with 18F-fluoro-deoxyglucose positron emission tomography scans (FDG-PET) (8). Clinically, these FDG-PET positive, non-iodine avid tumors have limited treatment options which may include observation, additional surgery, external beam radiation, conventional chemotherapy like the US FDA-approved agent doxorubicin, and clinical trials. The recent introduction of targeted therapeutic agents that have multiple targets, including the receptor tyrosine kinases, nonreceptor tyrosine kinases, and serine-threonine kinases, has shown much promise in trials for thyroid cancer patients with advanced disease (9). However, tyrosine kinase inhibitors (TKIs) which were tested as a single agent in thyroid cancer trials, have only shown at best, a partial response in these patients (10-12).

One goal of molecular medicine is to be able to profile each patient's tumor in order to determine which treatments will achieve the maximal response with minimal side-effects. There have been

significant conceptual and technical advances in elucidating areas of tumor biology such as: epigenetic, genetic, and miRNA regulation, but a unifying theory for thyroid carcinogenesis is lacking. This review aims to provide a framework to understand the rationale of how selected research developments may segregate tumors into different therapeutic regimens.

Chromosomal Rearrangements

One of the earliest genetic changes identified in papillary thyroid cancer was chromosomal alterations involving the proto-oncogene RET (rearranged during transfection) (13). Chromosomal translocations of the 3' end of RET gene, located on chromosome 10q11.2, with various 5' partner genes lead to ligand-independent activation of this tyrosine kinase receptor (14, 15). Constitutive RET activity, mediated in part through autophosphorylation of tyrosine residue Y1062, results in increased signaling through the Ras/Raf/MAPK cascade and to activation of other pathways like PI3K-Akt (See Figure 1) (15). The partner genes are designated as "PTC" and RET/PTC1 and RET/PTC3 variants represent the majority of these rearrangements (14).

Clinically, RET/PTC variants are often found in radiation-associated PTC. The increase in pediatric thyroid cancers following the Chernobyl nuclear plant explosion in 1986 resulted in two groups of thyroid cancer. The first was an early appearing and aggressive solid variant of PTC which contained the RET/PTC3 rearrangement while a later onset of PTC, with a more classical phenotype and clinical course, in the Chernobyl survivors contained RET/PTC1 (16). Both transgenic mouse models and *in vitro* cellular work have shown these fusion proteins as capable of initiating PTC (16-18). RET/PTC's presence in some microscopic PTC also suggests its importance in the early steps of thyroid cancer development with different mechanisms of tumor development compared to thyroid tumors without RET/PTC rearrangements (19, 20). RET/PTC rearrangements are also less commonly found in undifferentiated thyroid cancers, suggesting that these tumors may be managed with conventional treatment (21). The utility of RET/PTC identification from FNA of thyroid nodules for diagnosis of PTC is beginning to be used (22). There are multiple drugs in trial with activity against RET, most notably ZD6474 (Vandetanib) and Bay 43-9006 (Sorafenib), but these agents also target other tyrosine kinase receptors (Figure 1) (9). As shown in Table 1, Sorafenib has now been shown in two phase II clinical trials to have achieved a partial response rate of 15 and 23% in patients (10, 23). Due to Vandetanib's ability to block RET signaling activity, it is primarily being used for medullary thyroid cancer patients; however there is an ongoing phase II trial including DTC patients (24).

In general, clinical trials for thyroid cancer patients are primarily limited due to the smaller number of patients with advanced disease compared to other malignancies. The majority of these targeted agents are in phase I or phase II trials and no randomized controlled trial has been

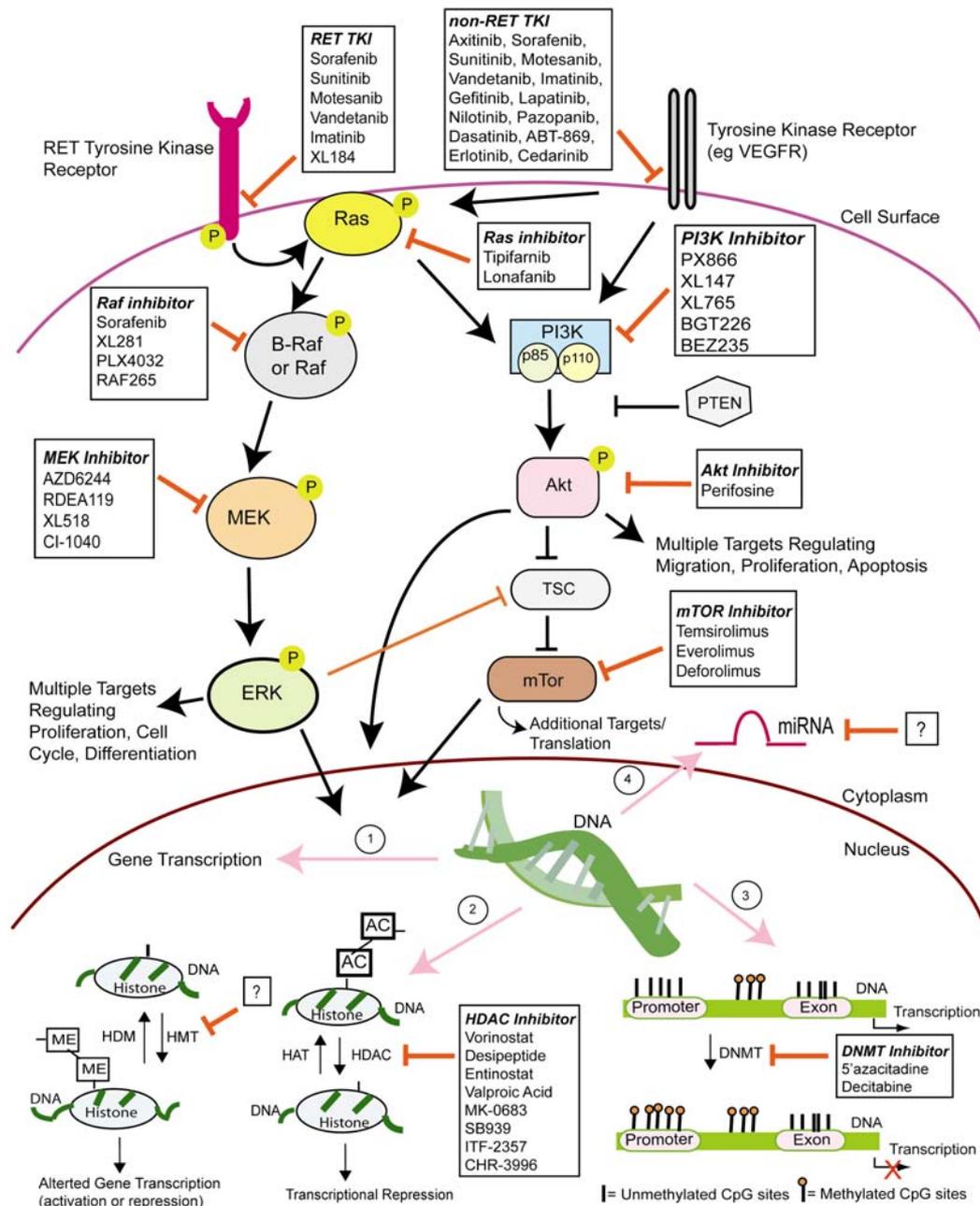


Figure 1: Molecular Targets for Thyroid Cancer

Schematic of two important pathways for thyroid cancer: Ras/Raf/MAPK and the PI3K-Akt pathway. Extracellular signals activate tyrosine kinase receptors leading to activation of the G-protein Ras. Ras can activate Raf leading to phosphorylation (P) of the mitogen activated protein kinase (MAPK) kinase (MEK) and activation of MAPKs such as ERK. PI3K-Akt indirectly activates mTOR (mammalian target of rapamycin) through inhibition of the tuberous sclerosis complex proteins (TSC). Each box lists agents currently in clinical trials that may target the receptor or downstream effectors. Note that not all agents have been tested for thyroid cancer. Pathway 1: Overactivation of either pathway results in altered gene expression which may facilitate tumor growth, survival, and/or metastatic potential. Pathway 2: Newer targets that may also be important in thyroid carcinogenesis include epigenetic regulation of genes through histone methylation (ME) regulated by histone demethyltransferases (HDM) and histone methyltransferases (HMT). Histone acetylation (AC) is regulated by histone acetyltransferases (HAT) and histone deacetyltransferases (HDAC). Pathway 3: Methylation of the promoter region by DNA methyltransferases (DNMT) leading to gene silencing is of genes is also a current target for thyroid cancer as it may cause redifferentiation of the tumor. Pathway 4: The role of microRNAs (miRNA) in translational control and tumorigenesis is becoming better delineated but no agents are in clinical trials. Adapted from a figure by Ma and Adeji (81).

completed to-date. Table 1 lists several additional targeted therapies that have activity against tyrosine kinase receptors and/or protein kinases (24-29). An alternative therapy to TKI, siRNA to RET/PTC mRNA may offer greater specificity and is currently under investigation (30). Therapeutic siRNA/shRNA for tumors may allow more direct targeting (i.e. inoculation directly into a lung metastasis versus systemic delivery). Understanding how RET/PTC tumors differ from non-RET/PTC PTCs would also lead to more targeted therapy for both groups.

For follicular thyroid cancers (FTC), the Pax8/PPAR γ rearrangement initially brought great excitement in finding a molecular correlate specific for FTC or the follicular variant of PTC (31). However these rearrangements have now also been found in benign follicular adenomas (21). The concept of downregulation of PPAR γ signaling pathways as important for FTC has been supported through mouse models (32), although a transgenic mouse with the Pax8/PPAR γ rearrangement has yet to be reported. Other fusion proteins with PPAR γ have been described with similar properties as the Pax8/PPAR γ rearrangement which leads additional evidence of the importance of PPAR γ . Further research needs to be done to clarify the role of these fusion proteins in FTC.

B-Raf^{V600E} and the MAPK Pathway

The evolutionarily conserved mitogen-activated protein kinase (MAPK) signaling pathway allows a cell to respond to external stimuli such as hormones and growth factors that interact with various receptors, including tyrosine kinase receptors like RET and G-protein coupled receptors like the TSH receptor (Figure 1). Extracellular signals such as TSH cause these cell surface receptors to activate the GTP-binding protein Ras which leads to Raf recruitment and activation. The serine-threonine protein kinase Raf then activates the MAPK kinases (MEK1/2) which ultimately lead to phosphorylation and activation of members of the MAPK family, such as: ERK1/2, p38, JNK, or ERK5 (Figure 1) (33). Dysregulation of this pathway is a common finding in many cancers. There are three known mammalian isoforms of Raf: A, B, and C-Raf (34). B-Raf is the most potent in activating MAPK and the activating point mutation T1799A results in a valine to glutamate substitution at position 600 (B-Raf^{V600E}); this mutation is found in many cancers, particularly in melanoma and is second only to Ras mutations in prevalence (35). B-Raf^{V600E} is also frequently found in PTC, particularly the aggressive tall-cell variant (36, 37). The B-Raf^{V600E} mutation is sufficient to induce PTC *in vivo* when expressed only in mouse thyrocytes (38). Recent work in understanding the genotype-phenotype correlations of B-Raf^{V600E} thyroid cancers suggest that B-Raf^{V600E} tumors are almost exclusively PTC, do not co-exist with RET/PTC mutations, accompanied by lymph node metastasis, recurrence, and may have a predisposition to become dedifferentiated as seen by loss of responsiveness to TSH and inability to take up iodine (36, 39-41).

B-Raf^{V600E} therefore serves as a good but negative prognostic marker for PTC. Research into using B-Raf^{V600E} to help with diagnosis is underway, but there are no clinical guidelines into whether mutation status, separate from pathologic staging, should modify follow-up in terms of more frequent imaging, use of I-131 for smaller tumors, or entrance into clinical trials. For patients with advanced PTC that is positive for the B-Raf^{V600E} mutation, many of the targeted therapies inhibit B-Raf^{V600E}, but are non-selective. More recently, two specific B-Raf^{V600E} inhibitors have been reported: PLX4720 which has been tested *in vitro* in melanoma and PLX4032 which has been evaluated in PTC cell lines with encouraging results and is currently in a Phase I trial for solid tumors (Figure 1) (35, 42, 43). Through complementary research utilizing mouse models, human cell lines and specimens, and clinical trials, new therapies may be designed to target B-Raf^{V600E}.

PI3K-Akt-mTOR Pathway

Follicular thyroid cancer remains a diagnostic dilemma given the inability to accurately determine by FNA whether a nodule is a benign follicular adenoma or FTC. In addition, a single mutation, such as B-Raf^{V600E} has not been identified for FTC. However the importance of the PI3K-Akt pathway in thyroid cancer, particularly FTC, has become more evident in recent years. The clinical observation of patients with Cowden syndrome, a genetic condition with a higher risk of development of thyroid nodules and FTC suggested a role of the PI3K-Akt pathway in FTC (44). Cowden syndrome is due to inactivating mutations of the tumor suppressor and phosphatase, PTEN which modulates PI3K-Akt signaling (44). Similar to the MAPK signaling pathway, PI3K is also a downstream effector of receptor tyrosine kinases. To transmit extracellular signals, the heterodimer protein PI3K, which is composed of a catalytic p110 subunit and regulatory subunit, typically p85, phosphorylates the phosphoinositide PIP2 to PIP3. PIP3 then recruits the kinase Akt to the plasma membrane where it undergoes phosphorylation at two sites: Thr³⁰⁸ and Ser⁴⁷³, via PDK1 and mTORC2, respectively (45, 46). One of PTEN's mechanisms of tumor suppression is dephosphorylating PIP3, thereby reducing its levels, to inhibit Akt activation (47).

There are three Akt isoforms: Akt1-3 with unique and redundant roles and all isoforms have been overexpressed in thyroid cancer (48). Akt has many targets, including the tumor suppressor TSC2; inactivation of TSC2 by phosphorylation leads to activation of the serine-threonine kinase mTOR and protein translation (Figure 1) (49). Along with decreased PTEN activity through either an inherited or sporadic LOH within tumors, other mechanisms that result in increased Akt activity such as activating mutations in receptor tyrosine kinases, activating or amplifying mutations of the p110 catalytic subunit have also been seen in thyroid tumors (50).

Examination of human thyroid cancer cell lines and tumor specimens documents that Akt overactivation is common in thyroid cancer (51-53). The TRβ^{PV/PV} mouse model which harbors a

mutant thyroid hormone receptor beta and spontaneously develops FTC with lung metastases also has significant Akt activation in the tumors (52). In tailoring therapies focusing on PI3K-Akt, use of a selective PI3K inhibitor reduced mortality in TR β ^{PV/PV} mice (54). While single-agent therapy with a PI3K inhibitor are in clinical trials for other solid tumors (55), these agents may not be completely effective for thyroid cancer given the probable activation of other upstream pathways of PI3K as discussed below. Dual PI3K-Akt and mTOR inhibitors may lead to improved efficacy. Future investigations into the unique of overlapping roles of the PI3K-Akt cascade with other oncogenic signaling pathways will help guide which are the most critical targets in FTC.

One aspect of clinical follow-up for thyroid cancer patients which is not well understood by basic research is the finding that many dedifferentiated thyroid tumors that lose iodine avidity become positive on PET scan (8). PET-positivity is considered a negative prognostic factor for thyroid cancer patients, but it is unclear as to the mechanism of this phenomena. PET scans are frequently used with other cancers and the increased glucose uptake in tumors is believed to be due to a cancer's favoring of glycolysis versus oxidative phosphorylation to generate its energy source, ATP (56, 57). Both Ras and Akt are known to upregulate genes that favor glycolysis and future research may be able to help determine which oncogenes are activated in a tumor partly based on PET uptake. Trials are now including PET response of tumors (ie decreased uptake) as a biological marker for tumor response to a drug (Table 1).

Covergence of Ras, MAPK, and the PI3K-Akt-mTOR Pathways

With the knowledge that Ras activating mutations are found in both FTC, PTC, and dedifferentiated thyroid cancers, it is important to understand how the proto-oncogene Ras, which can activate both Raf and PI3K, contributes to thyroid cancer (58, 59). There are three Ras family members: H-Ras, K-Ras, and N-Ras (60, 61). N-Ras and H-Ras mutations may be more predominant in thyroid cancers while K-Ras point mutations account for the majority of Ras mutations in non-small cell lung cancer (60, 61). One study found that targeting K-Ras to mouse thyrocytes resulted in only a single incidence of thyroid cancer following TSH stimulation (62). However a more recent study showed that mice lacking PTEN in their thyrocytes when crossed with a mouse strain carrying an activated K-Ras mutation (Kras^{G12D}) developed aggressive FTC (63). In contrast, mice with only a single mutation (Kras^{G12D} or PTEN null) did not develop FTC. These double mutant mice also developed probable toxic nodules given the suppressed TSH compared to the other strains. Interestingly neither the single mutant K-Ras activation nor PTEN lacking mice show significant ERK phosphorylation as would be expected. Rather only the double mutant mice showed ERK phosphorylation (63). The additive effects of K-Ras and PI3K in a mouse model of thyroid cancer suggests that aggressive tumors may also have overactivation of several signaling pathways.

Table 1: Protein kinase inhibitors in clinical trials for differentiated thyroid cancer

Drug	Targets	Clinical Trial	Results (RECIST Criteria)	Notes	Ref.
Sorafenib (Bay 43-9006)	BRAF, B-Raf ^{V600E} , VEGFR2,3; RET, PDGFRβ; FLT-3, c-KIT, FGFR-1	Gupta-Abramson et al., 2008 phase II, open-label 400 mg BID, some with prior use of chemotherapy > 3wks since last dose. 30 patients with metastatic disease (18 papillary, 9 follicular/Hurthle, 1 medullary, 2 poorly differentiated or anaplastic) which was no longer responsive to I-131. 93% patients had uptake on FDG-PET scans.	PR: 7/30 (23%) Stable disease: 16/30 (53%)	Of the 7 patients with PR: 4 had PTC, 3 FTC/Hurthle. Serum thyroglobulin for 17/19 patients decreased (mean 70%). 1 patient died from liver failure.	10, 24
		Kloos et al., 2009 phase II, open-label, 2-arm study 400 mg BID. Arm A: 19 patients with metastatic PTC, chemotherapy naïve. All patients in Arm A that had FDG-PET scans had uptake. Arm B: 22 patients with metastatic PTC, some patients received chemotherapy in the past, 11 follicular/Hurthle, 4 anaplastic.	Patients with PTC: PR: 6/41 (15%) Stable disease: 23/41 (56%)	17/22 of Arm A patients had BRAF mutation. No PRs in non-PTC patients. Serum thyroglobulin decreased for 12/19 patients in Arm A. Biopsies before and after Sorafenib showed decrease in MAPK signaling, VEGF expression, and VEGFR phosphorylation in 4/10 samples.	23
Axitinib (AG-013736)	VEGFR1-3; PDGFRβ; c-KIT	Cohen et al., 2008 phase II, open-label to goal dose 10 mg BID, no prior antiangiogenesis treatment. 60 patients (30 PTC; 15 FTC/Hurthle; 11 medullary, 2 anaplastic, 1 insular, neuroendocrine)	PR: 18/60 (30%) Stable disease: 23/60 (38%)	All subtypes had a patient with PR; Majority of patients had a decrease in thyroglobulin. A decrease in serum VEGFR2 and 3 was noted.	11
Motesanib (AMG 706)	VEGFR1-3; RET; PDGFRβ; c-KIT	Sherman et al., 2008 phase II, open-label of 125 mg qDay. 93 patients (67 PTC; 32 FTC/Hurthle; 4 insular/poorly differentiated); 10 with BRAF mutation.	PR: 3/93 (14%) Stable disease: 33/93 (35%)	22% patients required an increase in their levothyroxine dose.	12
Sunitinib (SU11248)	VEGFR1-3; RET, PDGFR-α,β; FLT-3; c-KIT; CSF1R	Cohen et al., 2008 phase II, open-label of 50 mg qDay for 4 weeks then 2 weeks off, 43 patients (37 differentiated thyroid cancer, 6 medullary).	PR (2 cycles): 13% Stable disease: 68%		26
		Ravaud et al., 2008 phase II, open-label of 50 mg qDay for 4 weeks then 2 weeks off. At 3 months, 17 patients (8 papillary, 4 medullary, 1 anaplastic, 4 miscellaneous).	Of all patients evaluated (15): PR:1/17 Stable disease: 12/17		29
		Goulart et al., 2008 phase II, open-label of 37.5 mg qDay. 18 patients (15 differentiated thyroid cancer, 3 medullary), all with FDG-PET positive lesions. Repeat FDG-PET 7 days after treatment.	No clinical data yet. 7/16 patients had 20% decrease in FDG-PET uptake (44%). All had differentiated thyroid cancer.		27
Gefitinib (ZD1839)	EGF receptor	Pennell et al., 2008 phase II, open-label of 250 mg qDay. 27 patients (11 papillary, 7 follicular/Hurthle, 5 anaplastic, 4 medullary)	No PR in 25 patients evaluated, stable disease 48, 24, and 12% at 3, 6, 12 months evaluation	8 patients had decrease in tumor size but not meeting criteria for PR, 1 anaplastic had SD for 12 months.	28
Vandetanib (ZD6474)	VEGFR2, 3; RET; MET	In phase II trial for differentiated thyroid cancer			24
XL281	BRAF, B-Raf ^{V600E}	In phase I trial for solid tumors (including papillary thyroid cancer).			24

RECIST (Response evaluation criteria in solid tumors) (Reference #25):

Target lesion: >2 cm in largest diameter with conventional imaging or >1 cm with spiral CT, up to 5 lesions/organ maximum and 10 lesions in total

Complete response: Disappearance of all target lesions, confirmed at 4 wks

Partial response (PR): >30% reduction in the sum of the longest diameter of target lesions compared with reference, confirmed at 4 wks

Stable disease (SD): Neither PR nor PD criteria met

Progressive disease: At least 20% increase in the sum of the longest diameter of target lesions, reference.

Is the smallest sum of the longest diameter recorded since treatment started or appearance of new lesions

Future trials will likely focus on a combinatorial approach with targeted agents to have improved clinical response and circumvent the development of “resistance” to single agents.

Epigenetic Regulation in Thyroid Cancer

Recognizing that DNA is associated with histone proteins to form a condensed structure known as chromatin, research is now investigating how modifications in chromatin structure may contribute to carcinogenesis. Epigenetic modifications refer to heritable alterations of the DNA structure, histones, and/or in nucleosome remodeling, resulting in altered gene expression (64). Epigenetic changes have been described in thyroid cancer, most notably the altered DNA methylation patterns in the CpG islands of promoters of genes important in normal thyrocyte function such as the sodium-iodide symporter and the TSH receptor (Figure 1) (65-67). Increased promoter methylation by DNA methyltransferases (DNMT) leads to gene silencing and further dedifferentiation of the thyroid tumor. DNMT inhibitors such as 5'-aza-deoxycytidine are being evaluated as “re-differentiation” agents thereby allowing tumors to again become more responsive to conventional therapy such as radioactive iodine (Figure 1) (68). Identification of specific methylation patterns may also allow stratifying tumors that may no longer be responsive to thyroid hormone suppressive therapy and I-131.

Research on how posttranslational modification of histones may influence cancer has recently seen tremendous growth. The nucleosome, or basic structural unit of chromatin, consists of 147 bp of DNA wrapped around an octamer of four core histone proteins (H2A, H2B, H3, H4) (69). Histone modifications include methylation, acetylation, phosphorylation, and ubiquitination and may act in concert with DNA promoter methylation to modulate gene silencing (Figure 1) (70). Histone deacetylation typically leads to gene silencing while acetylation enhances gene expression with aberrant acetylation patterns being recognized in cancer (71). Utilization of histone deacetylase (HDAC) inhibitors to promote gene expression and differentiation of tumors has had most success in hematologic malignancies (71). Recent use of the HDAC inhibitor, Vorinostat, in a phase II trial in thyroid cancer patients showed no therapeutic benefit, but further research is needed in this use of such agents in thyroid cancer (72). Histone methylation at specific lysine or arginine residues is also critical in regulating gene expression, but agents targeting the histone methyltransferases are in the early stage of development and testing (Figure 1) (73). Epigenetic drug targets may play a more central role in cancer treatment in the future.

miRNAs and Thyroid Cancer

There has been increasing awareness of the importance of microRNAs (miRNAs) in tumorigenesis. MicroRNAs are short (20-25) nucleotides of non-coding RNA whose primary function is to repress translation of target mRNAs through binding to their 3' untranslated region.

Other classes of non-coding RNA include endogenous small interfering RNAs and the Piwi-interfering RNAs which are being investigated for their role in stem cell regulation (Figure 1) (74). Over 500 miRNAs have been discovered, but each miRNA may have more than 100 targets (75). In the context of cancer, miRNAs can either act as a tumor suppressor by repressing translation of an oncogene, or act as an oncogene by repressing translation of tumor suppressors. Several groups have published miRNA profiles comparing different subtypes of thyroid cancer with normal thyroid samples with some consistent findings clustering specific miRNA with specific mutations (eg RET/PTC) (76, 77). In particular, miRNA profiles could also be used for diagnostic purposes and risk stratification in thyroid cancer as they are in other malignancies. In 2005, Calin *et al.* proposed that a set of 13 miRNAs found in chronic lymphocytic leukemia cells could predict whether a patient would have disease progression or not; however there is still no consensus on the core group of siRNAs critical for this disease (78).

One exciting aspect of miRNAs is their potential therapeutic use in thyroid cancer. He *et al.* compared miRNA signatures in fresh and archived human papillary thyroid cancer tissue compared to normal thyroid tissue and found that a group of three miRNAs (miR-221, miR-222, and miR-146b) were specific for PTC (76). Weber *et al.* found that miR-197 and miR-346 were overexpressed in FTC compared to adenomas and that suppression of these miRNAs *in vitro* suppressed growth (79). In contrast, Visone *et al.* found a subset of miRNAs that were downregulated in thyroid cancers (80). Nikiforova *et al.* also found a similar overexpression of miRNAs in PTC compared to He *et al.*, but these were more restricted to PTC with BRAF mutations (77). This group also found that miR-187 was highly expressed in FTC and tumors with Ras and RET/PTC mutations and lacking BRAF mutations (77). Identification of signature miRNA profiles and their downstream targets in thyroid cancer may reveal novel therapeutic targets. With improved understanding of the miRNAs that promote carcinogenesis or repress oncogene expression, one can envision development of miRNA antagonists or miRNA mimics that may lead to a new class of therapeutic agents.

Conclusion

The growing interest in exploring the basic mechanisms of thyroid cancer development has led to a realization of the central role of the MAPK pathway in PTC and the PI3K-Akt pathway in FTC. There is also a clearer delineation of PTC aggressiveness through identification of the tumor's RET/PTC or B-Raf^{V600E} mutation status. Multiple new agents which selectively and non-selectively target predominantly receptor tyrosine kinase activity are in clinical trials and have shown promise. Improved understanding in the crosstalk among signaling pathways between different subtypes of differentiated thyroid cancer along with undifferentiated thyroid cancer will allow for further individualization of targeted therapy. Future therapies may also take advantage of our growing

knowledge of the contributions of epigenetic changes and miRNA to expand our treatment options. The age of truly targeted medical care in the terms of diagnosis, treatment, and follow-up for thyroid cancer patients is closer than ever.

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Concise Review**GENETIC ANTICIPATION AND TELOMERE-TELOMERASE COMPLEX DYSFUNCTION IN FAMILIAL NON MEDULLARY THYROID CANCER (FNMTC).**

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The clinical and genetic features of patients with familial non-medullary thyroid cancer (FNMTC) are heterogeneous and far from being defined. Familial predisposition in differentiated thyroid carcinoma is reported in 3-10% of the cases (1,2), in absence of recognized predisposing syndromes (Cowden syndrome, Werner syndrome, Carney complex, Familial adenomatous polyposis) and the risk of developing the same tumor in first-degree relatives of subjects with differentiated thyroid cancer (DTC) is significantly higher than in the general population (3,4). Until now, no specific genetic alterations have been demonstrated in the blood of FNMTC patients, apart from susceptibility loci found in a few pedigrees with FNMTC (5-7). Recent studies (8) reported that patients with FNMTC display the features of “*anticipation phenomenon*”, that is the tendency for children to develop clinical disease at an earlier age than the affected parents. In fact, after ruling out the bias of screening effect, patients in the second generation presented an earlier age at disease presentation at diagnosis and at disease onset compared to the first generation and their tumours were more frequently multifocal and bilateral, had higher rate of lymph node metastases at surgery and worse outcome compared to the first generation. The presence of genetic anticipation has been reported in several non-thyroidal diseases (Table1). In thyroid diseases the

occurrence of disease anticipation has been reported in a large multigenerational family with medullary thyroid cancer (9), but in this case the occurrence of anticipation is uncertain because it was biased by the introduction of genetic analysis as a screening procedure. Preliminary evidence of genetic anticipation has been also reported in familial cases of Graves' disease (10).

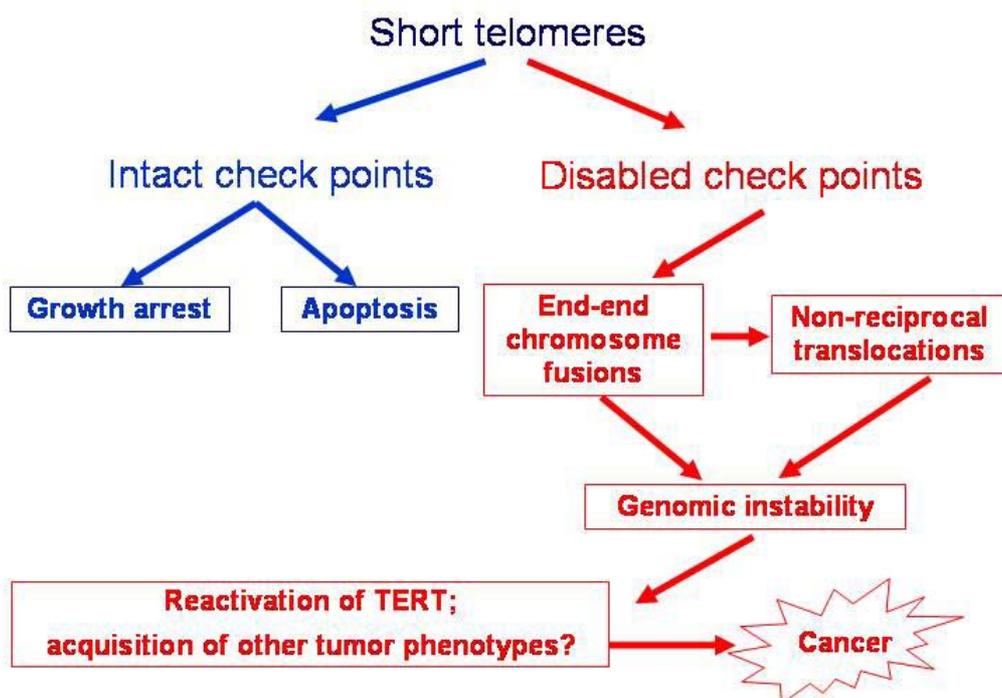
Table 1. Human diseases with demonstrated or suggested anticipation phenomenon.

Fragile X syndrome	Kronquist KE <i>et al.</i> Genet Med. 10:845-7, 2008
Myotonic dystrophy	Mahadevan M <i>et al.</i> Science 255:1253-5, 1992
Spinocerebellar ataxia	Moseley ML <i>et al.</i> Neurology 51:1666-71, 1998
Huntington disease	Ridley RM <i>et al.</i> J Med Genet 25:589-95, 1988
Familial leukemia	Horwitz M <i>et al.</i> Am J Hum Genet 59:990-8, 1996
Familial pancreatic cancer	McFaul CD. Gut 55:252-8, 2006
Bipolar disorders	McInnis MG <i>et al.</i> Am J Hum Genet 53:385-90, 1993
Diabetes type 2	Yaturu S <i>et al.</i> Med Sci Monit 11: 262-5, 2005
Graves disease	Brix TH <i>et al.</i> Thyroid 13:447-51, 2003
Crohn disease	Grandbastien B <i>et al.</i> Gut 42:170-4, 1998
Polycystic kidney disease	Peral B <i>et al.</i> Hum Mol Genet 5:539-42, 1996
Familial adenomatous polyposis	Presciuttini S <i>et al.</i> Ann Hum Genet 58:331-42, 1994
Familial medullary thyroid cancer	Fugazzola L <i>et al.</i> Clin Endocrinol 56: 53–63, 2002
Parkinson disease	Bonifati V <i>et al.</i> Can J Neurol Sci 22: 272-279, 1995
Dyscheratosis congenita	Vuillamy T <i>et al.</i> Nat Genet 36:447-449, 2004

Several molecular mechanism(s) possibly underlying genetic anticipation have been studied in familiar disorders, mainly benign, with particular attention to the telomere-telomerase complex. Telomeres are special structures consisting of a tandem repeats of the sequence TTAGGG at the ends of chromosomes that are maintained by telomerase, a specialized ribonucleoprotein complex that includes an RNA template (*TERC*) and a reverse transcriptase catalytic subunit (*TERT*). Telomerase expression is low or absent in most of human somatic tissues, while it is expressed in germ and stem cell compartments. Telomeric DNA is dynamic, being progressively lost with each cell division due to incomplete replication of the ends of linear DNA. When telomeres become critically short, the cells undergo senescence or apoptosis but if the integrity of checkpoints

mechanisms are altered genomic instability is triggered and leads to cycles of chromosome breakage and fusion, in a period called “crisis” that permit the acquisition of further genetic alterations. Although most cells die by apoptosis during the “crisis”, rare cells survive and maintain stable short telomere lengths through the reactivation of telomerase that facilitates cell immortalization (11). The strong association of telomerase re-activation with cancer provides evidence that this mechanism plays an important role in cancer development (Figure 1). RTL segregates in families (12,13) and a decrease in telomere length may play a role in age-related genetic instability (14). Interestingly, patients who have inherited or acquired genetic defects in telomere maintenance seem to have an increased risk of developing familial benign disease such as dyscheratosis congenital syndrome (15) and malignant diseases such as head, neck, lung, breast, and renal cancers (16).

Figure 1. In cells with intact signalling pathways, short telomeres trigger either senescence or apoptosis. In cells with disabled checkpoints, short dysfunctional telomeres trigger chromosome instability, perpetuated through recurrent breakage-fusion-bridge cycles. Few cells, through reactivation of a telomere maintenance mechanism, usually telomerase, stabilize telomere length and chromosome ends, resulting in cell immortalization. Upon additional somatic events, the latter may eventually acquire malignant phenotype (figure adapted from Londono-Vallejo. Biochimie 91:73-82, 2008).



In sporadic thyroid cancer telomerase re-activation is reported in nearly 50% of thyroid cancer tissues and in the past same authors proposed that the detection of telomerase activity may be helpful to distinguish between malignant and benign thyroid tumours (16-18), but the large majority of these studies were conducted on tumor tissues, while blood was not investigated. Recently, first evidence has been provided that patients with FNMTc have dysfunctional telomeres compared to patients with sporadic differentiated thyroid cancer, patients with benign thyroid diseases, healthy subjects and unaffected siblings of FNMTc patients (19). FNMTc patients had significantly shorter relative telomere length (RTL) in their peripheral blood cells, compared to sporadic cases and normal controls. hTERT gene was significantly amplified in FNMTc patients respect to control groups and it was significantly more represented in 2nd generation with respects to 1st generation. In addition, hTERT mRNA levels and the protein activity were significantly increased in FNMTc patients compared to control groups.

In conclusion, evidences have been provided for the presence in FNMTc of genetic anticipation at the clinical level and of short telomere lengths, hTERT gene amplification and increased telomerase expression in peripheral white blood cells at the molecular level. It is possible that patients born with short telomeres might reach earlier in life the threshold telomere length sufficient to trigger cancer development and this observation is consistent with the presence of genetic anticipation observed in FNMTc patients. If further confirmed in larger series, these results might propose measurement of RTL, as a marker of predisposition to FNMTc.

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CASE REPORT**COFFEE IMPAIRS INTESTINAL ABSORPTION OF LEVOTHYROXINE: REPORT OF ADDITIONAL CASES.**

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ABSTRACT

Several drugs inhibit the intestinal absorption of levothyroxine (L-T4) when taken simultaneously with the thyroid hormone or shortly later. Recently, in a study on 8 women, coffee has been reported to reduce the intestinal absorption of L-T4, so that L-T4 was added to the list of the medications whose intestinal absorption is decreased by coffee. We report another six adult patients, 5 women and 1 man, aged 38 to 62 years, who were observed during the last 18 months. All patients were referred because of failure of serum TSH to be normalized or suppressed by appropriate replacement (1.6-1.8 µg/Kg b.w.) or TSH-suppressive (2.0-2.2 µg/Kg b.w.) therapy with L-T4. In each of the six patients, serum TSH failed to be normalized or suppressed if L-T4 was swallowed simultaneously with coffee (or followed by coffee soon after). Correction of this habit, by drinking coffee 60 minutes after having swallowed L-T4 with water, resulted in normalization or suppression of serum TSH.

Key-words: Thyroxine; Thyrotropin; TSH; Coffee; Intestinal absorption.

Background.

L-T4 is widely used across the world; indeed, in 2007 it was one of the top 5 medications in the United States by units sold (1). In adults, the mean daily dose of L-T4 is 1.6 µg/kg body weight for replacement and 2.2 µg/kg b.w. for TSH suppression (2). As well known, L-T4 therapy is monitored based on serum TSH.

Recently, we have reported on the impaired intestinal absorption of L-T4 caused by drinking coffee within 10 minutes of swallowing the L-T4 tablet (3). These cases (8 women) were collected during a decade of clinical practise. Here we report six additional cases (5 women and 1 man), who were observed during the last 18 months, which suggests the degree of underestimation of the problem. None of the six patients was taking other known interferes of the intestinal absorption of L-T4.

Case reports.

Patient no. 1 had a 3-year history of multinodular goiter and had been treated with L-T4 at a daily dose of 132 µg (1.8 µg/Kg b.w.). However her TSH was never below 0.80 mU/L. Careful history taking disclosed that she used to ingest the L-T4 tablet with water, but followed by one full cup of coffee within the next 5-10 minutes. We suggested her to drink coffee at least 60 minutes after swallowing the L-T4 tablet. She returned to us six months later with a serum TSH of 0.20 mU/L, and after another six months with a serum TSH of 0.04 mU/L (Table 1).

Patient no. 2 had also been seen by other endocrinologists for multinodular goiter and was given L-T4 at a dose of 2.0-2.2 µg/Kg b.w./day. However, within the next 3 years serum TSH was never suppressed (1.1 to 1.7 mU/L). We found that she used to swallow the L-T4 tablet with a full tea-cup of barley coffee. She was instructed to postpone drinking barley coffee. Three months later, during which she took the L-T4 tablet with water 30 to 60 minutes prior to her barley coffee, serum TSH dropped to 0.62 mU/L: however, within the next 12 months, serum TSH dropped to from 0.19 to 0.02 mU/L, when the lag-time was prolonged to 60-90 minutes (Table 1).

Patient no. 3 had the nodular goiter variant of Hashimoto's thyroiditis, for which she underwent partial thyroidectomy 12 years prior to our observation. After thyroidectomy, she started taking L-T4 at a daily dose of 1.6 µg/Kg b.w., that over the years was increased up to 2.0 µg/Kg b.w., because serum TSH remained high. Even though serum TSH decreased (from 8.1 to 4.7 mU/L), it was still above normal. We found that she ingested L-T4 with one full cup of coffee. We instructed her to postpone drinking coffee by 60 minutes, and left her under the last regimen of L-T4 (1.8 µg/Kg b.w./day). Three and seven months later serum TSH was, respectively, 2.2 and 1.9 mU/L. She admitted, and her husband confirmed, that her compliance was partial (30 in lieu of 60 minutes) (Table 1).

Patient no. 4 was a nurse. She had undergone right lobectomy 16 years earlier and was put on a daily regimen of 1.7 µg/Kg b.w. of L-T4. In the last two years serum TSH had fluctuated between 0.94 and 2.0 mU/L. We found that two years prior to our observation, the patient bought an espresso machine, and most of the time she used to ingest the L-T4 tablet with one or two cups of espresso. We instructed her to postpone drinking espresso and, over the next six months, postponing espresso by 60 minutes, TSH fluctuated between 0.06 and 0.37 mU/L (Table 1).

Patient no. 5 had been seen by other endocrinologists for a nodular goiter and was given L-T4 at a daily dose of 1.6 µg/Kg b.w., but TSH was not suppressed (0.95 mU/L). We increased the dose to 2.1 µg/Kg b.w. and instructed her to postpone drinking coffee by 60 minutes. Over the next year, TSH decreased scantily (0.76 mU/L) when she persisted in her habit, but decreased substantially (0.43 mU/L) when she postponed drinking coffee by 60 minutes (Table 1).

Patient no. 6 was a man, who five years earlier was thyroidectomized for a multinodular goiter. After thyroidectomy, he started L-T4 treatment (2.1 µg/Kg b.w./day). The patient took the L-T4 tablet with water, but drunk a cup of coffee within the next 10 minutes. Serum TSH was 2.7 mU/L. We asked the patient to postpone the cup of coffee by one hour. Three months later, TSH dropped to 0.04 mU/L (Table 1).

Table 1. Serum TSH levels in 6 patients under L-T4 replacement or TSH-suppressive therapy based on the modality of swallowing the L-T4 tablet.

Case number	Age/Gender	Serum TSH (mU/L)*	
		L-T4 tablet with coffee or soon after coffee	L-T4 with water and drinking coffee postponed**
# 1	47/F	0.8 – 1.5	0.04 – 0.20
# 2	40/F	1.1 – 1.7	0.02 – 0.19
# 3	38/F	4.7 – 8.1	1.9 – 2.2
# 4	46/F	0.94 – 2.0	0.06 – 0.37
# 5	48/F	0.76 – 0.95	0.43
# 6	62/M	2.7	0.04

* Normal values of serum TSH (in mU/L) were, respectively, 0.4 – 4.0 (cases #1 and #6), 0.47 – 4.6 (case #2), 0.27 – 4.2 (cases #3 and #4) and 0.4 – 5.0 mU/L (case #5).

** Details on the lag-time (in minutes) are in the text.

Discussion.

In some circumstances, patients require greater daily doses of L-T4 to attain the desired fall in serum TSH concentrations. Interference can be related to a) patient characteristics, dietary habits

and compliance with the drug; b) the pharmaceutical characteristics of L-T4 and c) the interference of other medications (4). Several oral medications inhibit the intestinal absorption of L-T4 sodium when taken simultaneously with thyroid hormone. In most of these cases, the malabsorption of L-T4 is due to the binding of the hormone to medications, forming an insoluble or non-absorbable complex: these include cholestyramine (5), ferrous sulphate (6), aluminium hydroxide (7), calcium carbonate (8), sucralfate (9). Besides, L-T4 should be taken separately from food, since food adsorbs thyroid hormone, decreasing its availability for intestinal absorption (10-12): in particular, dietary fibres and herbal remedies reduce intestinal absorption of L-T4 (13,14).

Drugs are absorbed after oral administration as a consequence of interactions between the drug, its formulation and the gastrointestinal tract. Based on the *in vitro* studies described in ref. 3, at least one mechanism for the interference of coffee is physical sequestration of L-T4; however, we cannot exclude other possibilities. These possibilities should not include changes in gastric emptying, intestinal transit or pH, because these parameters have been reported to be unaffected by coffee (15,16). Coffee has been reported to impair the intestinal absorption of a number of medications and other substances (17-24) (Table 2), and very recently we added L-T4 to the list based on the said 8 cases observed and *in vitro* studies suggesting a sequestering effect (3).

Table 2. Impaired absorption of the most common drugs or substances caused by coffee*.

CLASS	DRUG/SUBSTANCE
Anticancer drugs	Camptothecin, Topotecan
Bisphosphonates	Alendronate, Risedronate, Ibandronate
Antipsychotics	<u>Butyrophenone</u> Haloperidol
	<u>Tricyclic antidepressants</u> Amitriptyline, Imipramine, Phenothiazines
	<u>Dibenzoxazepine</u> Loxapine
Others	Calcium, Iron , Zinc, L-thyroxine

- Based on references no. 3, 17-24.

Thus, regardless of type, coffee should act *in vivo* by sequestering L-T4 and reducing hormone availability for uptake by the intestinal epithelium (Fig. 1).

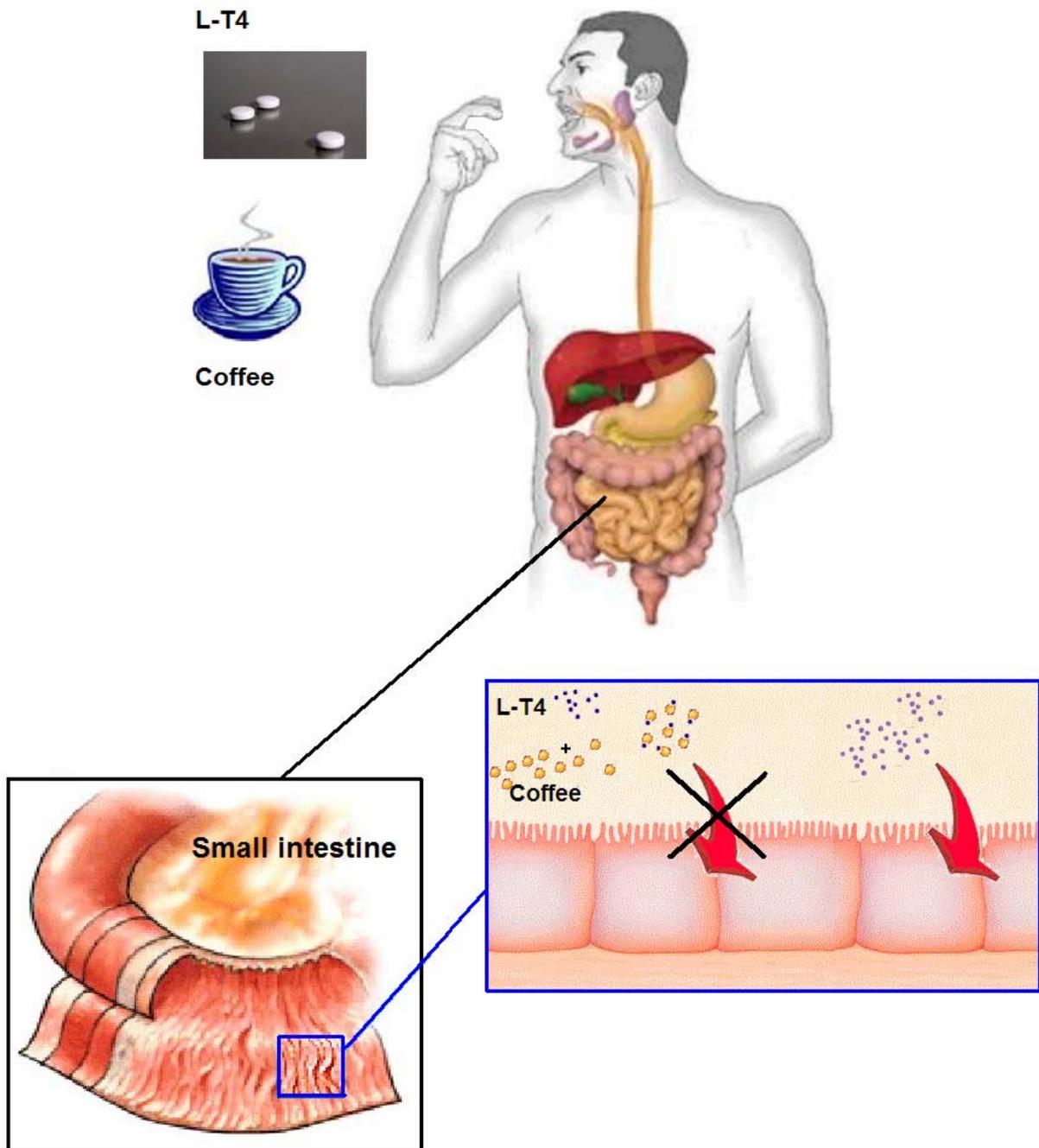


Fig. 1 Sequestering activity of coffee reduces intestinal absorption of L-T4.

Conclusion.

Here, we have reported that patients with impaired L-T4 absorption due to coffee are also men, and we have also reported the first patient in whom the interferer is barley coffee.

In conclusion, considering the wide consumption of coffee and the frequent prescription of L-T4 for either replacement or TSH-suppressive purposes, patients with inappropriately high or non-suppressed serum TSH should be questioned routinely about the possible impairment of L-T4 absorption caused by coffee drinking. Accordingly, patients should be instructed to take coffee at least one hour following the intake of L-T4 tablets. Furthermore, it would be desirable that the leaflet contained in pharmaceutical packaging be sufficiently informative about the reduced absorption of L-T4 caused by coffee.

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GENE EXPRESSION IN NORMAL AND TUMOR THYROID CELLS AND TISSUES

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The Authors declare no conflict of interest related to this work.

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ABSTRACT

Thyroid carcinoma is the most frequent endocrine malignancy. Since a few years, we have defined in molecular terms the pathways involved in the control of proliferation of normal thyroid cells and in the perversion of this process in thyroid tumors. We have used the microarray technology to characterize the fundamental biology of thyroid tumors, to better understand their physiopathology and to classify them according to their molecular phenotype. We have analyzed hyperfunctioning autonomous adenomas and papillary thyroid carcinomas and *in vitro* model systems: human thyroid cells in primary culture and human thyroid cancer cell lines. Prolonged *in vitro* stimulation of the cAMP or the MAPK pathway in normal thyrocytes in primary culture shows a convergence of gene expression with chronic results of mutagenic events in the corresponding *in vivo* tumors, respectively the autonomous adenoma and the papillary carcinoma. Analysis of gene expression in the primary cultured thyrocytes shows an induction of multiple specific negative feedback proteins for the cAMP and for the growth factor pathways. Some of these feedbacks do not operate in the corresponding tumors, suggesting that they constitute pathway-specific tumor suppressor genes. We also show that thyroid tumor cell lines, often used as models to investigate tumorigenesis, have evolved into a common, dedifferentiated phenotype. These results raise the very general

point that cancer cell lines represent the outcome of an adaptation and evolution *in vitro*, and therefore that their use as such as models for human tumors should be considered with caution.

Key-words: thyroid, microarray, adenoma, papillary, post-Chernobyl, primary culture, cell lines

Introduction

Tumors originating from thyroid follicular cells are the most frequent endocrine tumors and comprise a spectrum of well defined phenotypes with variable rates of growth, differentiation and biological aggressiveness (1-3). The major ones are the autonomously hyperfunctioning and cold follicular adenomas, both benign encapsulated tumors, and the malignant carcinomas. These can be further subdivided in follicular or papillary carcinomas, still partly differentiated, both of which may evolve in anaplastic carcinoma, totally dedifferentiated. Thyroid carcinogenesis is considered as a very interesting multi-step process where normal epithelial follicular cells evolve through a more and more dedifferentiated and aggressive phenotype. The coexistence of the different tumor types results from the perversion of different mitogenic cascades: constitutive activation of the TSH receptor/cAMP cascade causes hyperfunctional tumors (4), whereas oncogenic activation of growth factor pathways (RET/PTC rearrangements, B-Raf mutations) is associated with less differentiated thyroid papillary carcinomas (5,6). A causal effect of environment has been demonstrated: irradiation is the cause of “post-Chernobyl” thyroid papillary carcinomas, and iodine deficiency increases the relative proportion of follicular vs papillary carcinomas (7). While the differentiated papillary and follicular carcinomas have a relatively good prognosis and can be treated with I^{131} , the derived, very aggressive, anaplastic carcinomas are generally lethal within six months and do not respond to any therapy (chemotherapy, I^{131}) (8).

A major public health problem of thyroid tumors is the high frequency of tumors (nodules) discovered by ultrasound (up to 40 % of the population above the age of 50 years) of which only 5% are cancers (9). Even with modern technologies, such as fine needle aspirate histological analysis, 20 to 25% of these nodules appear suspect and many therefore are operated, three out of four unnecessarily. There is thus a need to develop fully reliable diagnostic tools to avoid many

unnecessary surgical interventions or dangerous delays, and reliable drugs to treat anaplastic carcinomas.

Since a few years, we have defined in molecular terms the pathways involved in the control of proliferation of normal thyroid cells and in the perversion of this process in thyroid tumors. In this review, we concentrate on the work of our group and do not attempt to review the whole literature on the subject.

Gene expression in normal thyroid cells

We have first studied normal dog thyroid cells by differential screening: a thyroid cDNA library was prepared from a methimazole and propylthiouracil treated dog and differentially screened with probes derived from control or stimulated thyroids. This allowed us to identify 3 new proteins: C5fw, a novel phosphoprotein, C3vs, and p76^{RBE} (rhopilin 2), a novel activated RhoB binding protein (10,11). The latter could play a key role between RhoB and potential downstream elements needed under stimulation of the thyrotropin/cAMP pathway in thyrocytes and responsible for intracellular motile phenomena such as the endocytosis involved in the thyroid secretory process (12). Rhophilin 2-deficient mice were generated and their thyroid structure and function analyzed. Their phenotype was normal, suggesting that rhophilin 2 does not play a unique role in thyroid physiology (13).

In a second approach, we used filter macroarrays to investigate genes induced by the TSH/cAMP dependent pathway in dog thyroid cells. The major and most interesting gene was ID3 transcription factor, identified as an early response protein, downregulated in papillary thyroid carcinomas, and thus a tumor marker for these carcinomas (14).

The cDNA-AFLP technique is a third approach which allowed us to identify differentially expressed transcripts in thyrotropin stimulated dog thyroid cells, among which 5 clones encoding known proteins: thrombospondine-1, TNFR1, RhoE, RalB, and annexin A2. These regulations provide

molecular counterparts of *in vivo* physiological effects of TSH: angiogenesis (decreased thrombospondin-1), decreased apoptosis (decreased TNFR1) and actin filament disruption, macropinocytosis and thyroid hormone secretion (decreased RhoE) (15).

Gene expression in thyroid tumors and their *in vitro* experimental models

New technologies to probe the global gene expression profiles of normal and cancer tissues, such as microarrays, have recently reached widespread use. Microarray analysis can be used to classify tumors according to their molecular signatures, and also to indicate the presence of previously unidentified molecular subtypes. It may also provide invaluable information on the underlying biology, disease progression, resistance to treatment, and may help identify novel potential drug targets or individualized therapeutic approaches. We have implemented the biochemical and bioinformatical tools for this methodology (16,17), in order to define gene expression profiles in different thyroid tumors and in their *in vitro* experimental models.

Hyperfunctioning autonomous adenomas

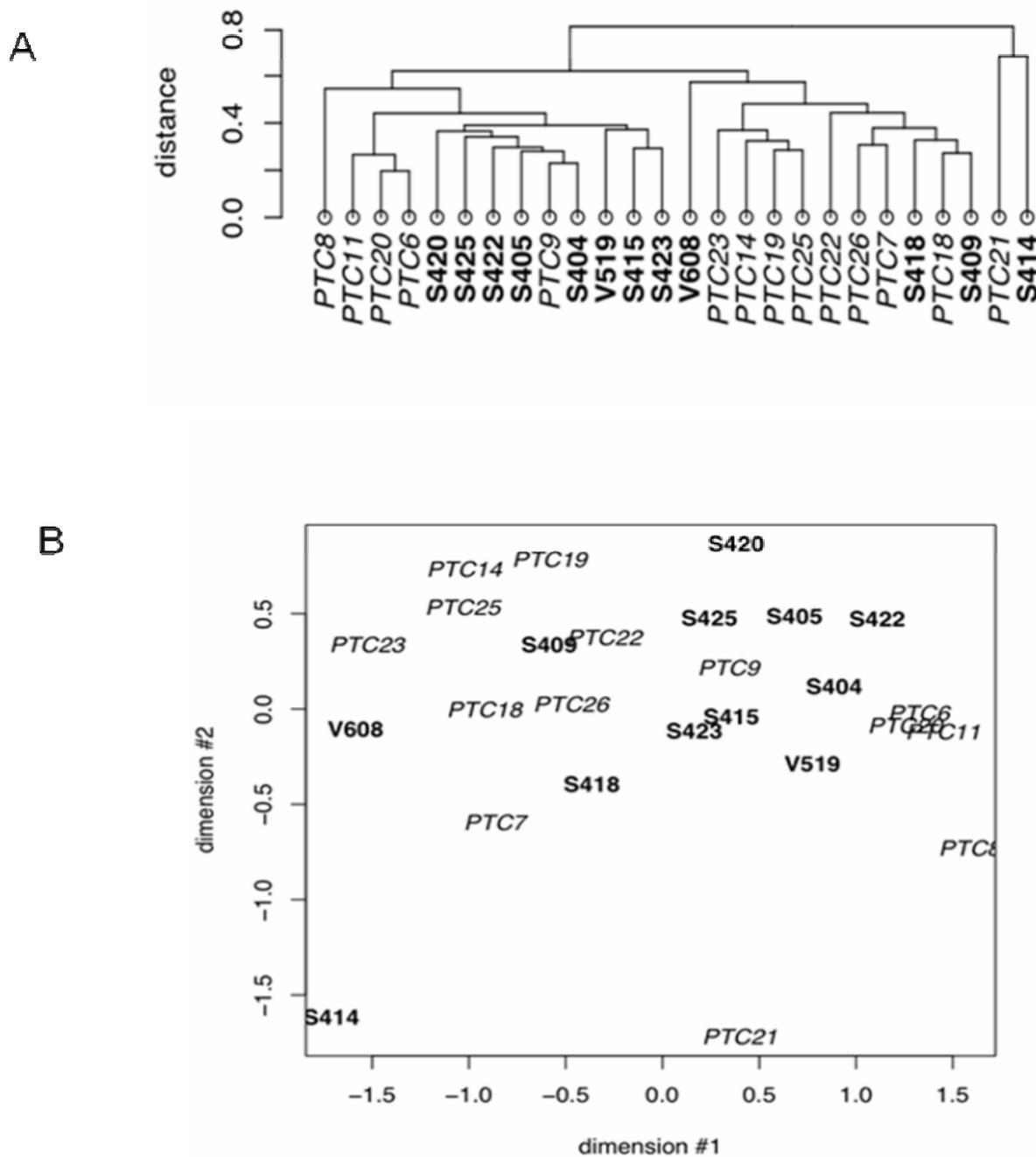
Autonomous thyroid adenomas are monoclonal encapsulated benign tumors that grow, metabolize iodide, and secrete thyroid hormones independently of the normal thyrotropin (TSH) control (18,19). They result from the constitutive activation of the TSH/cAMP-dependent mitogenic cascade, largely through mutations in the TSH receptor (50 to 80%) or in $G_{s\alpha}$ (8%) (20-24), and are thus a well-defined example of the results of long-term stimulation by the physiological TSH/cAMP-dependent pathway. We have defined gene expression profiles of these tumors, and our data show that several physiological and morphological characteristics of these adenomas can be explained by their transcriptional program. In particular, our results show 1) a change in the cell populations of the tumor with a marked decrease in lymphocytes and blood cells and an increase in endothelial cells. The latter increase would correspond to the establishment of a close relation between thyrocytes and endothelial cells and is related to increased N-cadherin expression; 2) an homogeneity of tumor samples, correlating with the clonality of these lesions and their common

physiopathological mechanism: the constitutive activation of the TSH/cAMP cascade; 3) a low proportion of regulated genes consistent with the concept of a minimal deviation tumor: the cells are intrinsically stimulated, but their functional behavior is normal ; 4) a higher expression of genes coding for specific functional proteins, consistent with the functional hyperactivity of the tumors; 5) an increase of phosphodiesterases gene expression which explains the almost normal cAMP levels measured in these tumors; 6) an overexpression of antiapoptotic genes and underexpression of proapoptotic genes compatible with their low apoptosis rate; 7) an overexpression of N-cadherin and downregulation of caveolins which casts doubt about the use of these expressions as markers for malignancy (25).

Papillary thyroid carcinomas

Thyroid cancers have been the main medical consequence of the Chernobyl accident (26). On the basis of their pathological features and of the fact that a large proportion of them demonstrate RET/PTC translocations, these cancers are considered as similar to classical sporadic papillary carcinomas. We analyzed gene expression in post-Chernobyl cancers, sporadic papillary carcinomas and autonomous adenomas and showed that, while hyperfunctioning autonomous adenomas and papillary carcinomas are easily distinguishable on the basis of their gene expression patterns, post-Chernobyl, radiation-induced, papillary carcinomas have the same molecular phenotype as sporadic papillary cancers (27). Increasing the number of samples and of genes analyzed by microarray confirmed that post-Chernobyl and sporadic PTC have similar overall expression profiles. They both represent the same disease (Figure 1). However, further studies have shown that subtle expression differences are exploitable to accurately classify these tumors according to their origin. Part of these expression differences includes genes involved in the differential response to H₂O₂ and radiation, and genes involved in homologous recombination. So, although sporadic and radio-induced PTCs represent the same disease, they are distinguishable with molecular signatures reflecting specific responses to γ -radiation and H₂O₂. These signatures could reflect the susceptibility profiles of the patients (28).

Figure 1: Global expression profiles of post-Chernobyl and sporadic papillary thyroid cancers. A) hierarchical clustering on the basis of all genes. B) Multidimensional scaling on the basis of all genes. Post-Chernobyl tumors are in bold, sporadic tumors in italics.



These data led us subsequently to correlate the molecular phenotype of PTC with their biological pathology. We combined our dataset with 2 other microarray studies (29,30), to produce a platform- and study-independent list of PTC-associated genes. Analysis of this list led to several conclusions: 1) there is a change in cell population with an increased expression of genes involved in the immune response, reflecting lymphocyte infiltration in the tumor compared to the normal

tissue; 2) the JNK pathway is activated by overexpression of its components; 3) the activation of ERK1/2 by genetic alterations is supplemented by activation of the EGF but not of the IGF signaling pathway; 4) there is a downregulation of immediate early genes, as in autonomous adenomas (25); 5) we observed an overexpression of many proteases and adhesion matrix proteins in accordance with the important remodelling in PTC, and suggested a probable role of S100 proteins and annexin A2 in this process; 6) numerous overexpressed genes (cadherins, claudins, connexins, integrin subunits, proteases) favor the hypothesis of a collective migration mode of tumor cells (31).

In vitro models of human thyroid tumors

Many differences in gene expression between normal and tumor tissues reflect differences in cell population rather than changes in the tumor cells themselves. The true differences between normal and tumor cells have been validated by their reproduction in primary cultures of human thyrocytes. These cultures contain only thyrocytes and therefore thyrocyte-specific gene expression can be studied without interference of other cell types. Thyrocytes in primary culture (32) are expected to be better models than immortalized cell lines that are already well on the way to transformation.

In a first study, we investigated the genes that are modulated by the cAMP signaling pathway in thyrocytes *in vitro* treated with their physiological stimulus TSH. These gene expressions were then compared with the chronically stimulated autonomous adenomas. Human primary cultures of thyrocytes were treated for different times with TSH to characterize modulations in gene expression using microarrays. This kinetic study showed a clear difference in expression, early (1.5 and 3 hr) and late (16 to 48 hr) after the onset of TSH stimulation. This suggests a progressive sequential process leading to a change of cell program. The gene expression profile of the long-term stimulated cultures resembled autonomous adenomas, but not papillary carcinomas (Figure 2A). The molecular phenotype of the adenomas thus confirms the role of long-term stimulation of the TSH-cAMP cascade in the pathology. TSH induced a striking upregulation of different negative feedback modulators of the cAMP cascade. The induction of these proteins

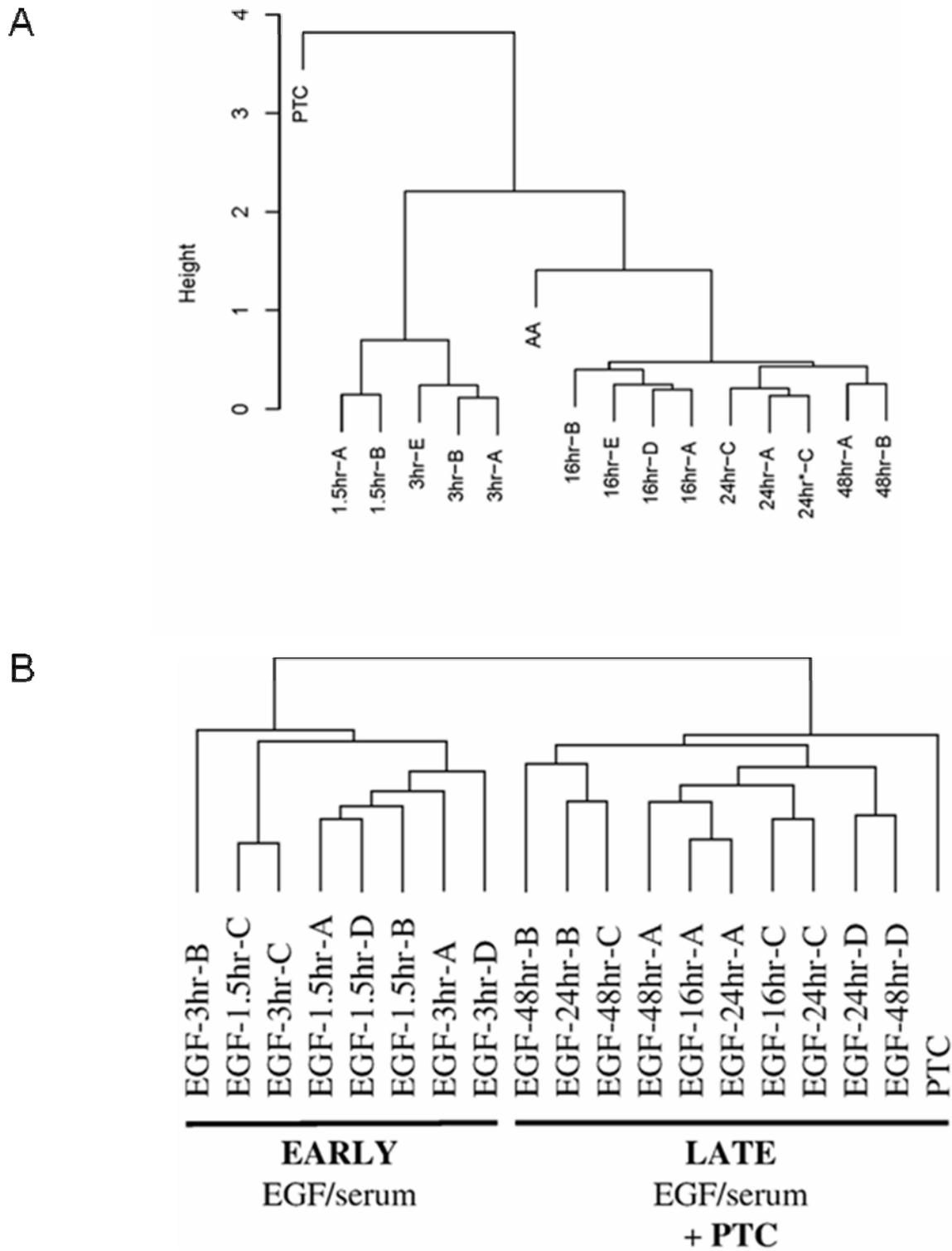
demonstrates a remarkable fail-safe control on the system: with these feedbacks, continuous growth stimulation has little chance to occur. Several of these inductions were downregulated or non-regulated in the autonomous adenoma, suggesting a loss of negative feedback control in the tumors. These results suggest that in tumorigenesis, activation of proliferation pathways may be complemented by suppression of multiple corresponding negative feedbacks, i.e. of specific tumor suppressors (33). Such inactivations may thus represent a general mechanism in cancer (34).

A similar study was conducted with human thyrocytes treated for different times with epidermal growth factor and serum (EGF/serum), which stimulate the MAPK cascade, constitutively activated in PTC (6). Gene expression profiles were obtained by microarrays and compared to the expression profiles of PTC. Similar to what was observed for TSH-treated thyrocytes, an evolution from short-term to long-term EGF/serum-treated cells was found, i.e. a program change showing a distinction between gene expression profiles of short-term and long-term EGF/serum-treated cells. The pattern of gene expression in long-term EGF/serum stimulated thyrocytes converged to the pattern of the *in vivo* PTC (Figure 2B). Overall gene expression profiles of EGF/serum-treated thyrocytes and PTC were distinct from TSH-treated cells and autonomous adenomas but showed an overlap in a number of immediate early genes, mostly transcription factors (35). There are thus commonalities between the initiation steps of even divergent programs.

Thus, prolonged, but not short term, *in vitro* stimulation of the cAMP or the MAPK pathway in normal thyrocytes in primary culture shows a convergence of gene expression with chronic results of mutagenic events in the corresponding *in vivo* tumors, respectively the autonomous adenoma and the papillary carcinoma. This shows the adequacy of long-term stimulations of the two pathways *in vitro* to mimic to some extent the effect of a chronic stimulation *in vivo*.

Human thyrocytes in primary cultures present a useful alternative to the two other *in vitro* models presently used: the human cancer cell lines which result from a long-term *in vitro* evolution from the initial tumor (see below), and transient or permanently oncogene expressing rat cell lines which present, in addition, the problem of species specificity (36).

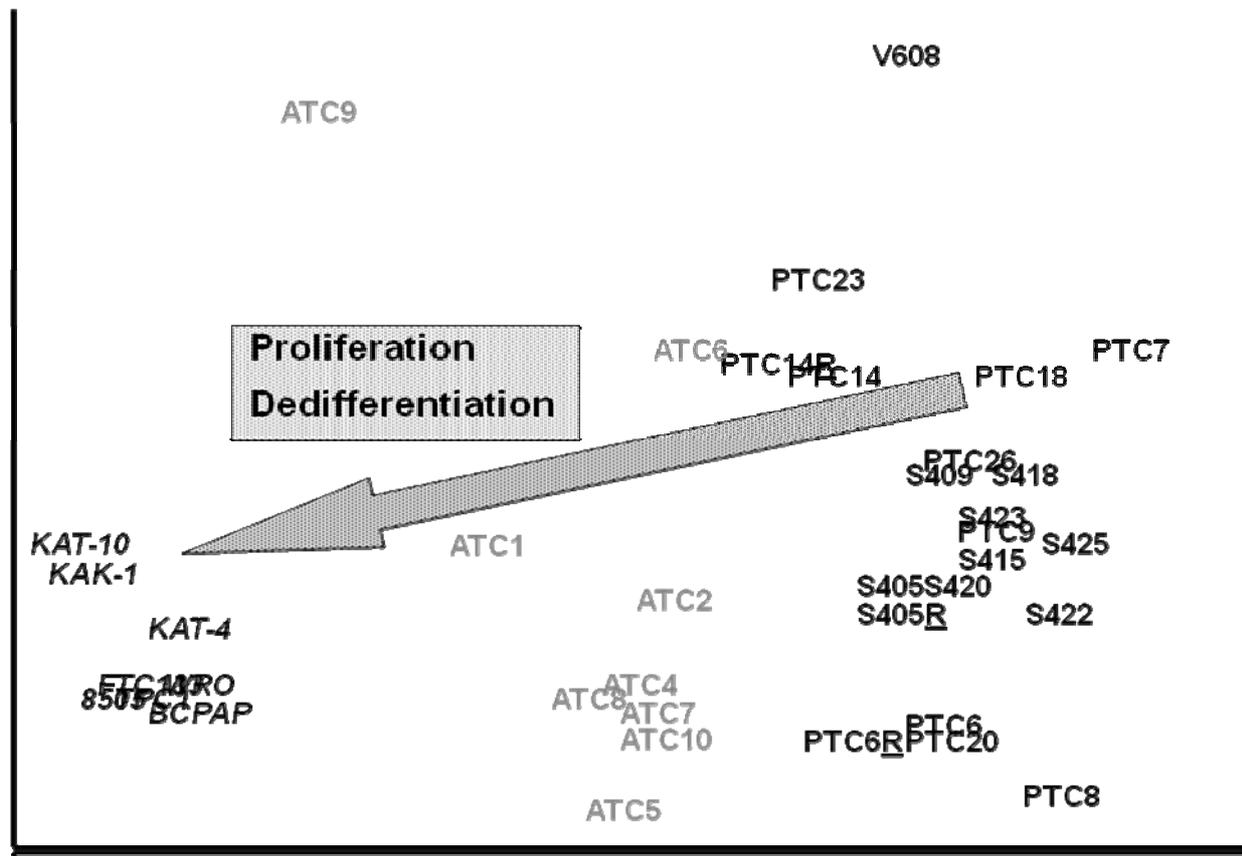
Figure 2: A) Hierarchical clustering of the microarray data from five independent human primary thyroid cultures, labeled A-E, treated with 0.3 mU/ml TSH for 1.5 (1.5hr), 3 (3hr), 16 (16hr), 24 (24hr) and 48 hours (48hr) or with 10^{-5} M forskolin for 24 hours (24hr*). In addition, the expression profiles of a pool of autonomous adenomas (AA) and of papillary tumors (PTC) are shown. Clustering was made based on considering only differentially expressed genes in the primary thyroid cell cultures selected by SAM (q -value < 0.05). B) Hierarchical clustering of the microarray data from four independent human primary thyroid cell cultures, labeled A–D, treated with 25 ng/ml EGF and 10% serum for 1.5, 3, 16, 24, and 48 h and of the microarray data from the averaged papillary thyroid carcinoma (PTC) data from 16 tumors.



Tumor-derived cell lines are extensively used in cancer research as models to elucidate mechanisms of tumorigenesis, and serve as tools for cancer treatment screenings, implying that numerous *in vivo* characteristics of the tumor are still represented *in vitro*. During the past years, a number of thyroid tumor cell lines from different pathologic origin have been developed. These commonly used cell lines were derived from one follicular adenoma (KAK-1), two FTCs (FTC-133 and WRO), three PTCs (B-CPAP, KAT-10, and TPC-1), and two ATCs (8505C and KAT-4). To investigate whether these thyroid tumor-derived cell lines are representative *in vitro* models, their characteristics were investigated using microarrays, expression of differentiation markers, and karyotyping (37). Gene expression profiling indicated that these cell lines, derived from differentiated and undifferentiated tumor types, significantly diverged from their original tumor they are derived from. They have evolved *in vitro* into similar phenotypes with gene expression profiles closest to *in vivo* undifferentiated tumors (Figure 3). Accordingly, the absence of expression of most thyrocyte-specific genes, including TSHR, NIS, TG, TPO, and ThOX2, their nonresponsiveness to thyrotropin, as well as their large number of chromosomal abnormalities, suggest that these cell lines have acquired characteristics of fully dedifferentiated cells. Their differentiation status was further explored by comparing their gene expression profiles with those of differentiated cells: human primary cultured thyrocytes treated with TSH, and autonomous adenomas (33). We found, as might be expected, that the genes that were upregulated in the differentiated thyrocytes and in the cell lines are more likely involved in proliferation, whereas the genes upregulated in the primary cultured thyrocytes but downregulated in the cell lines are more likely involved in differentiation.

Our results thus suggest that the cell lines have evolved into fully dedifferentiated cells. They represent the outcome of an adaptation and evolution *in vitro*, which questions the reliability of these cell lines as models for differentiated tumors. However, they may represent useful models for undifferentiated cancers, and by their comparison with differentiated cells, can help to define the genes involved in the differentiation/dedifferentiation process. The use of any cell line as a model for a cancer therefore requires prior careful and thorough validation for the investigated property. This work has been generalized to cancers and cancer cell lines of many other tissues (38).

Figure 3: Global gene expression multidimensional scaling of human thyroid tumor cell lines (in italics) and a panel of PTC (in black) and ATC (in grey). Cell lines and PTCs were hybridized on in-house–manufactured slides. Three PTC samples (noted R), already hybridized on homemade slides, and ATCs were hybridized on Affymetrix slides. Analysis was made based on all the genes in common between the homemade and the Affymetrix platforms. Comparison of the same PTC samples between the two platforms showed that their gene expression profiles were highly similar. Thus, gene expression profiles from all samples can be compared regardless of the platform. The arrow indicates increased proliferation and dedifferentiation.



Conclusion

Gene expression profiling is a powerful tool to analyze the complexity of cancer biology. It provides insights into the identification of molecular pathways that may be affected by tumorigenesis and helps to understand the physiopathology of the tumors, with potential therapeutic applications. It also allows to define signatures of the tumors and to identify new diagnostic markers. We have analyzed gene expression in human tumor tissues and in model systems, such as human thyroid cells in primary culture, and human thyroid cancer cell lines. By generating these data, we have contributed, together with other researchers in the field (39), towards a better understanding of thyroid cancer biology, although still very incomplete. The combination of this technology with other

high throughput techniques aimed to investigate, at RNA level, the miRome, or at DNA level, the methylome and the genome, will further allow to fully define an integrated molecular phenotype and genotype of thyroid tumors.

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CASE REPORT**Remission of Graves' Disease in a female patient with isolated methimazole-dependent febrile agranulocytosis.**

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ABSTRACT

I report the case of a patient affected by Graves' Disease and treated with an antithyroid drug such as methimazole. During the drug treatment she presented a methimazole dependent agranulocytosis and the drug was immediately withdrawn. Amazingly, thyroid hyperstimulation vanished during withdrawal of anti-thyroid therapy and the patient underwent a remission. The role that stress factors may exert on the autoimmune process directed against the thyroid gland is discussed.

Key-words: Graves' disease – thyroid – autoimmunity – methimazole - agranulocytosis

INTRODUCTION

Thyrotoxicosis is a common endocrine disorder. It affects mainly women and is approximately five times more common in females than in males. Usually the patients are treated with antithyroid drugs such as methimazole and carbimazole. A rare and potentially fatal complication of the therapy is the agranulocytosis, ranging from 0.3% to 0.6%. The stress factors seem to be important to trigger the onset of the disease. In literature no role is usually reported of the stress such as a

possible cause of hyperthyroidism recovery. We describe an unusual remission of Graves' Disease following the onset of a febrile agranulocytosis induced by methimazole.

CASE REPORT

In March 2008 we observed a 52 years old female patient with tachycardia and fine tremor. Elevated free triiodothyronine (FT3), free thyroxine (FT4) and TSH receptor antibodies (TRAbs) with suppressed thyrotropin (TSH) were observed. Anti-thyroglobulin and anti-thyreoperoxidase was slightly positive and no significant time dependent modifications were observed. Thyroid scintigraphy showed a diffuse ¹³¹I distribution with an increased uptake. No ophthalmopathy was present. The thyroid ecography showed a slight increase of volume, a hypoechoic gland with an increased vascularization at Doppler study. The patient started a methimazole (MMI) treatment with 20 mg/die, after progressively reduced to 15 and 10 mg.

Suddenly, in June, after two months of MMI treatment, the patient showed sore throat and fever (temperature, 39,5°C), the blood cell count indicated a marked reduction of white blood cells (WBC) and neutrophils (N), compatible with the diagnosis of an isolated agranulocytosis. The values of the other blood cells were normal. The MMI treatment was immediately withdrawn and the patient admitted to hospital. No hepatic and kidney failure was observed. Anti-nuclear antibodies were negative while anti-granulocyte antibodies was not evaluated. The fever and the sore throat solved after three days of antibiotic and antimycotic treatment and a progressive improvement of WBC and N was observed. No steroid treatment was started. After nine days the antibiotic treatment was withdrawn and the patient was discharged. We tested every ten days the FT3, FT4 and TSH in order to identify the hyperthyroidism relapse. Amazingly, the hormonal status and the TRAbs levels showed a progressive normalization without MMI treatment (Table 1). The patient did not attend the subsequent clinical controls (she lives in a rural area), but did not refer manifestations indicative of hyperthyroidism relapse in the following 3 months. In consideration of the impossibility to use antithyroid drugs, radiometabolic treatment was recommended in case of possible future relapse of hyperthyroidism.

DISCUSSION

Many papers discuss the importance of the stress to induce the onset or the worsening of the GD (1). On the contrary, in the case here reported, the stress dependent on a febrile agranulocytosis, might represent an unusual cause of GD remission.

Self-limited transient forms of GD are described after delivery, surgery of Cushing's disease, the withdrawal of antithyroid drugs or such as the spontaneous transient Graves' thyrotoxicosis (2). But the clinical characteristics could not assign this patient to one of these categories. Indeed, the long-lasting duration of MMI withdrawal guaranteed that the resolution of the hyperthyroidism was not dependent upon a drug effect. However, other factors, such as the TRAbs levels, the age and the goiter size, indicated that our patient was more prone to undergo a remission of hyperthyroidism, according to Vitti et al (3).

Table 1. Laboratory values detected at different times and laboratory normal ranges (n.r.). In bold the values at the moment of the hospital admission and MMI suspension. Not done (n.d.)

	01/03/08	21/03	25/05	12/06	26/06	15/07	26/08	n.r.
FT4	2.65	2.22	1.5	1.78	1.14	1.19	1.45	0.93-1.71 ng/dL
FT3	16.17	10.16	5.5	3.9	4.11	4.07	4.4	3.1-6.8 pmol/L
TSH	0.02	0.02	0.09	0.13	0.12	0.26	0.65	0.27-4.2 mu/L
TRAbs	20.8	n.d.	n.d.	n.d.	1.1	n.d.	1.0	<10 IU/L
WBC	n.d	5.0	4.2	1.5	5.6	4.8	5.0	4.0-11.0 10 ³ /mm ³
N	n.d	47.0	48.5	4.0	48.6	47.9	51	50-80 %

Usually, an immunosuppressive mechanism of the antithyroid drugs has been hypothesized to explain the remission in GD (4). Anyway, many evidences does not support this point of view, evidencing that the remission (secondary to TRAbs normalization) in GD is independent of dose, kind of antithyroid drug or surgical treatment (5). The restored euthyroidism, independently if secondary to the drug or surgical treatment, seems to be the main factor inducing the remission of GD, breaking the vicious circle existing between autoimmunity and hyperthyroidism (6).

It is well known that a significant stress, such as a sepsis, can induce a decrease in thyroid hormone bioavailability (7) and a severe immunosuppression (8). Conceptually, the decreased bioavailability, mainly, of FT3 and the immunosuppression are, theoretically, able to induce the normalization of TRAbs with consequent remission of the autoimmune process and hyperthyroidism in GD.

Moreover, in consideration that antithyroid drug-induced agranulocytosis and aplastic anemia are dependent on autoantibodies directed, respectively, against granulocyte and bone marrow (4), another possible hypothesis is that a similar autoimmune process could suppress the source of TRAbs responsible of the hyperthyroidism.

CONCLUSIONS

On the basis of the reported findings, it is tempting to speculate that the stress of the febrile agranulocytosis could have played an important role in disrupting the vicious circle between hyperthyroidism and autoimmunity in this patient. Then, should we consider that stress factors may potentially be able to induce opposite (negative or positive) effects on the autoimmune processes affecting the thyroid gland?

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Peripheral Regulation of Energy Metabolism by Thyroid Hormones

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ABSTRACT

Thyroid hormone is long known as an important regulator of metabolism. It exerts general effects such as increased cycling of metabolites and stimulation of ATP turnover in spite of reduced efficiency of oxidative phosphorylation, but also very specific effects in peripheral tissues. This article reviews the most relevant metabolic effects of thyroid hormone in peripheral tissues, including the specific contributions of the two different thyroid hormone receptor isoforms. Special focus is put on the thermogenic effects of thyroid hormone in muscle and brown adipose tissue as well as the exclusive role of the thyroid hormone receptor β in hepatic cholesterol metabolism.

Keywords: thermogenesis, brown adipose tissue, liver, glucose, metabolism, cholesterol

Introduction

It is known for over 100 years that thyroid hormone (TH) has a major impact on metabolism (1), and the metabolic rate has long been used as an indicator for the thyroid state. The connection between TH and metabolism becomes most evident in patients substituted with thyroxine (T4): after initiation of the treatment increased oxygen consumption is registered after 24 hours, reaching maximal levels after 4 days (2). Moreover, minimal dose changes, which do not move the serum free T4 out of the normal range, are readily detectable as changes in the metabolic rate (3). The higher energy expenditure caused by TH is usually accompanied by increased appetite; if food is restricted, weight loss occurs (4). This has led to the idea to use TH as diet pill, but the unwanted side effects such as tachycardia, muscle loss and osteoporosis outweigh the beneficial effects.

TH and its receptors

Two forms of TH are secreted from the thyroid gland: the prohormone T4 and the receptor-active form T3. Within the target tissue, T3 levels are fine-tuned by the activation or inactivation through deiodinating enzymes. While it has been assumed for long time that TH enters the cell due to its lipophilic properties, the view has changed with the recent discovery of specific TH transporters such as MCT8 or OATP14 (for review see (5)).

Within the cell, most effects of TH are mediated by nuclear TH receptors (TRs), which are encoded by the two distinct genes TR α and TR β . It is still a matter of controversy whether the TR α 1 and TR β isoforms can fully compensate for each other; however, the degree of redundancy seems to depend to a large extent on the level of TR isoform coexpression within a given celltype. The expression of TR α 1 is high in e.g. brown adipose tissue, skeletal muscle, brain and heart, while TR β is predominantly expressed in liver and kidney (for review see (6)).

A special feature of TRs is the ability to actively repress or activate target genes as aporeceptors in the absence of the ligand T3. This activity is the reason for the relatively mild phenotype of TR knockout mice (7-9) compared to mutants lacking TH or carrying mutations that impair TH binding to the TRs (10-14). The aporeceptor-function and the widespread partially overlapping expression

of the TRs make the interactions of TH with peripheral metabolism extremely complex including simultaneous actions at many different levels.

Metabolic Lessons from Animal Models

In most of the available animal models, targeting of the TR isoforms occurs in all tissues, thus the corresponding phenotypes are difficult to interpret. For instance, mice carrying a mutation in TR β which abolishes binding of T₃, or transgenic mice expressing a mutant human TR β both exhibit reduced body weight and size, but also high levels of TH due to the impaired negative feedback control of the pituitary (15, 16). Therefore, the metabolic effects are difficult to assign to a specific tissue and can be caused by either the high levels of TH acting on the intact TR α 1 or by the mutant TR β itself.

The situation is likewise complex for TR α 1. So far, four different mutations have been introduced into TR α 1, and the phenotypes range from metabolically lean (17), dwarfism with impaired adipogenesis (18, 19) or reduced fat content (20), to animals with visceral adiposity and increased fasting glucose and insulin (21, 22). As serum TH levels are surprisingly normal in these animals, the differences seem to be caused by the location of the mutation within the TR α 1 itself, which may affect interactions with cofactors or nuclear receptors such as PPARs (21).

However, hypothyroid mice as well as mice lacking all functional TRs show decreased metabolism (23). Furthermore, TR α 1^{-/-}-TR β ^{-/-} double mutants do no longer respond metabolically to TH. These observations indicate that the metabolic effects of TH are mediated largely by TR α 1 and TR β (23) and underline the importance of TH for the maintenance of normal metabolism.

Overall Effects of TH

TH generally enhances the turnover of lipids, carbohydrates and proteins, sometimes even simultaneously in reverse metabolic pathways (metabolic cycling). While fatty acids can be used up in this process, no protein is used for calorogenesis and the excretion of nitrogen as well as renal gluconeogenesis remain constant (2). Metabolic cycling, however, accounts for only 15% of

the increase in resting energy expenditure after TH administration (2), indicating that other processes such as ATP wasting and reduced efficiency of oxidative phosphorylation play a more important role in mediating the metabolic effects of the hormone.

The main energy waste is achieved by a combined increase of ATP consuming proteins, such as Na^+/K^+ - or Ca^{2+} -ATPases, and a stimulation of the ATP synthesizing machinery (3). TH enhances the mitochondrial oxidation capacity by e.g. increasing the amount of the adenine-nucleotide translocase 2 (ANT2), which transports ADP in and ATP out of the mitochondria (24), and the cytochromes c and c1 (25), which are part of the oxidizing machinery.

Simultaneously, the efficiency of the ATP synthesis itself is reduced; consequently, more fuel is needed for the same amount of biochemical work. In certain tissues such as the brown fat, this is achieved by a reduction of the coupling efficiency between mitochondrial proton gradient and ATP production through Uncoupling Protein 1 (UCP1). This protein generates artificial leaks in the mitochondrial membrane; however, UCP-independent mechanisms have also been reported (26). Moreover, at the cytosolic level, TH increases the expression of glycerol-3-phosphate dehydrogenase (24), which participates in one of the two shuttle systems that deliver NADH to the mitochondria. This shuttle system, which only yields 2 ATP molecules per NADH, is consequently preferred over the malate-aspartate shuttle, which generates 3 ATP per NADH (3). Thus, the efficiency of ATP production is additionally hampered.

Effects on the Adipose Tissue

In adipose tissue, TH stimulates lipolysis and lipogenesis simultaneously, which is in line with the concept of metabolite cycling. Lipolysis is enhanced by TH through a raised activity of hormone-sensitive lipase and an increased sensitivity of the adipose tissue to adrenergic stimulation, leading to higher levels of free-fatty acids in the serum. The expression of lipogenic enzymes such as malic enzyme, spot14 or fatty acid synthetase is also increased already after a few hours; the first de novo synthesis of fatty acids is detected about 10 hours later (2).

Interestingly, the $\text{TR}\beta$ -selective agonist GC1 does not induce fat loss to the same extent as T3, despite a similar increase in oxygen consumption (27). This demonstrates an important role for

TR β in the initial raise in energy expenditure, which differs from TR α 1-dependent adipose tissue activation. Indeed, the activity of the adipose tissue accounts for less than 5% of the increase in oxygen consumption after T3 administration, suggesting that the effects of TH on this tissue are a more long-term metabolic response. This correlates with the fact that the compensating food intake after TH treatment does not occur until 4 days later, most probably as a consequence of the reduced secretion of the satiety hormone leptin from the shrinking adipose tissue.

The effects of TH differ in the two types of adipose tissue. While the white adipose tissue is mainly a fat store and the induction of lipolysis generates fatty acids for the export, the brown adipose tissue (BAT) uses lipolysis, de novo synthesis and import of free fatty acids as fuel to maintain body temperature. TH does not induce thermogenesis by itself, but it is essential for the proper activation of this tissue (26). The activity of the BAT is mainly controlled by sympathetic signaling via the β 3-adrenergic receptor and completely dependent on UCP1; in its absence there is almost no sympathetic inducible thermogenesis (28). As there is good evidence that BAT also exists in humans (29), the thermogenic role of TH in mice and men will be elucidated in greater detail.

Thermogenic Effects of TH

TH stimulates obligatory thermogenesis (generated by basal metabolism) and is essential for facultative thermogenesis (generated by specialized mechanisms with the purpose to maintain body temperature) (3, 30). The latter is induced, if obligatory thermogenesis is not sufficient to maintain body temperature, and is divided into a fast response by e.g. muscle shivering and a slow but more long-lasting response through the BAT. The BAT response is based on heat production through uncoupling; mice lacking UCP1 loose more than 10°C body temperature when exposed to cold, whereas mice with an ablation of 60-70% of BAT are still cold resistant (31).

Interestingly, TR α 1^{-/-}-TR β ^{-/-} double knockout mice and hypothyroid mice such as the *hyt/hyt* mouse cannot survive cold at all (23, 32). While the BAT response is severely impaired in hypothyroid animals (33), UCP1 is inducible by adrenergic signaling in the TR α 1^{-/-}-TR β ^{-/-} double mutants (23). However, this is still not sufficient for survival in the cold; thus, proper TH signaling seems to be more important than BAT functionality alone.

Furthermore, TR aporeceptor activity plays an important role in this process: the T3 induced relief of apo-TR mediated repression seems required for sympathetic stimulation. While the UCP1 induction is restored by T3 and GC1 in hypothyroid mice, the sympathetic response is only rescued with T3. This indicates that TR β is involved in controlling UCP1 expression, while the adrenergic signaling is modulated mainly by TR α 1 signaling (33).

Vice versa, the adrenergic activation enhances TH signaling by inducing the T4 activating enzyme deiodinase type II (D2) (34), which in turn produces enough T3 to saturate all TRs in BAT (35). In the absence of D2, BAT adrenergic response is impaired. Consequently, D2 $^{-/-}$ mice develop hypothermia if exposed to cold, but still survive in contrast to hypothyroid animals, as the thermogenesis by shivering is not affected (36).

In summary, TRs exert well-defined tissue- and isoform specific roles in maintaining body temperature (Table 1). However, as mutations in TR α 1 also affect the sympathetic output from the brain (17), the central effects of TH on thermogenesis and metabolism might be underestimated to date. In addition, yet unknown TH effects on the vascular system might contribute to explain the reduced body temperature despite increased oxygen consumption in some animal models.

Animal Model	Body Temperature (relative to controls)	Comment
hyt/hyt	-2.5°C	severely hypothyroid, cold intolerant
TR β $^{-/-}$	normal	increased TH levels, not cold sensitive
TR α 1 $^{-/-}$	-0.5°C	not cold sensitive
TR α 0/0	-0.4°C	no facultative thermogenesis at room temperature, but higher O ₂ consumption
TR α 2 $^{-/-}$	+0.4°C	overexpress TR α 1
TR α 1R384C	-0.9°C	despite higher O ₂ consumption
TR α 1P398H	-0.5°C	obese
TR α 1L400R	normal	lean dwarfs
TR α 1 $^{-/-}$ -TR β $^{-/-}$	-0.4°C	increase metabolism and UCP1 upon cold, but still cold intolerant

Table 1: Body temperature of animal models with impairments in TH signaling (9, 17, 20, 22, 23, 32, 37-39)

Effects on the Muscle

The muscle is a versatile tissue regarding its use of metabolites. While the resting muscle consumes fatty acids, the active muscle requires large amounts of glucose. TH mainly affects muscle glucose metabolism; it increases glycolysis and almost doubles the amount of the insulin dependent glucose transporter GLUT4 on the cell surface (40). Consequently, the import, the flux through the Krebs-Cycle, and the oxygen consumption are all elevated; however, as the mitochondrial efficiency is also reduced by TH, the overall ATP production remains almost constant (41). Moreover, TH promotes further waste of energy by increasing the protein Ca^{2+} -ATPase, which transports Ca^{2+} from the cytosol into the sarcoplasmic reticulum (SR), and simultaneously raising the levels of the ryanodine receptor, which mediates the Ca^{2+} release from the SR back into the cytosol (42, 43). This mechanism alone accounts for an almost 2-fold increase in oxygen consumption between a hypo- and a hyperthyroid muscle (43), which raises the question for a physiological function as the muscle is dependent on an efficient ATP supply for proper function. However, as the muscle is also the first line of defence against hypothermia and TH actions in the muscle are absolutely required for survival in the cold, in the eye of TH the muscle is predominantly seen as a thermo- rather than a movement generator.

Effects on the Heart

It is well known that the consequences of thyrotoxicosis on the heart such as tachycardia closely resemble those of catecholamine excess; but surprisingly, catecholamine levels are normal if not lowered in hyperthyroidism. This suggests that TH increases cardiac responsiveness to catecholamines; however, not at the level of the adrenergic receptors (44).

The direct molecular effects of TH include an increase in HCN2 ion channels as well as an elevation of myosin heavy chain and SR- Ca^{2+} -ATPase 2, thus leading to an enhanced cardiac output and decreased the relaxation time (45). These effects are not observed with the $\text{TR}\beta$ selective compound GC1, underlining that $\text{TR}\alpha 1$ of major importance in the heart (27, 46). Correspondingly, mice lacking $\text{TR}\alpha 1$ have a decreased heart rate, while those overexpressing

TR α 1 exhibit an increased heart rate (9, 39). As for the BAT, any central effects of TH might additionally affect cardiac function e.g. via changes in the autonomic nervous system.

Effects on the Liver

As the liver acts at the crossroads of many metabolic pathways – the most important one being glucose homeostasis - it is not surprising that more than 5% of all genes expressed in the liver are regulated by TH (47, 48). These targets mediate general T3-effects such as increased oxygen consumption and ATP turnover (49), but also shift metabolic processes from glycogen synthesis to glycogenolysis and from glycolysis to gluconeogenesis, thus enhancing the endogenous hepatic glucose production (50). Moreover, T3 stimulates enzymes involved in lipogenesis such as malic enzyme, glucose-6-phosphate dehydrogenase and fatty acid synthetase. Although many of these target genes show redundant function for TR α 1 and TR β in the rodent liver (51), some such as spot14 (52) are predominantly regulated by TR β , the isoform accounting for 80% of hepatic T3 binding capacity (53).

One pathway exclusively regulated by TR β is cholesterol metabolism. Again, TH affects both ends: it induces the rate-limiting enzyme HMG-CoA reductase, thus stimulating de novo cholesterol synthesis, but also increases the expression of the LDL-receptor and CYP7A, the rate-limiting enzyme in bile-acid synthesis from cholesterol (54, 55). Together, this leads to a better clearance of serum LDL-cholesterol and an increased cholesterol breakdown.

Consequently, hypothyroidism is associated with hypercholesterolemia in men and mice due to a reduced clearance of serum LDL-cholesterol and a reduced bile-acid production by CYP7A (56). The TR β dependency of this process can be used to ameliorate this condition by the administration of the TR β selective compound GC1, which reduces serum cholesterol by 25% and leads to an increased faecal excretion of bile-acids (46, 57). Moreover, it reverses fatty livers and reduces the hepatic triglyceride content, indicating an important exclusive role of TR β also in fatty acid metabolism (58). Surprisingly, GC1 was found to be even more efficient than T3 in this context (46), which suggests opposite roles of TR α 1 and TR β in hepatic lipid metabolism. A similar reverse

effect of the TRs was observed in the regulation of PEPCK, the rate-limiting enzyme in gluconeogenesis (Vujovic, Vennström & Mittag, unpublished observations). These unique isoform-specific effects might be partially caused by the zoned hepatic expression of the TRs: TR β is limited to areas around the central vein, while TR α 1 is more widespread and the only periportal TR isoform (59-61). As a consequence, the isoforms cannot compensate for each other and might interact with different cellular cofactors in the different hepatic zones.

Despite the many well investigated direct effects of TH on hepatic gene expression, it should be kept in mind that TH also affects several hepatic metabolic pathways in an indirect manner, e.g. via the autonomic nervous system (62) or by enhancing the effects of other hormones such as insulin, glucagons or glucocorticoids (25).

Concluding Remarks

Besides the overall and long-known effects such as increase metabolic cycling and ATP turnover, many specific effects of TH in peripheral metabolism were identified over the last years, using e.g. novel TR β selective compounds and animal models with defective TH signaling (see Table 2).

Tissue	TR isoform	T3 Effect
Overall		1. metabolite cycling \uparrow 2. ATP use and production \uparrow 3. efficiency of ATP production \downarrow
White Fat	TR α 1+TR β	lipolysis, lipogenesis, export of FFA \uparrow
Brown Fat	TR α 1	adrenergic responsiveness, heat \uparrow
	TR β	uncoupling, lipolysis, heat \uparrow
Skeletal Muscle	TR α 1 \gg TR β	Ca ²⁺ cycling, glucose & ATP use \uparrow oxygen consumption, heat \uparrow
Heart	TR α 1 \gg TR β	tachycardia, cardiac output \uparrow
Liver	TR β (80%) ¹	lipogenesis (malic enzyme, spot14, FAS) \uparrow gluconeogenesis (PEPCK) \uparrow glycogenolysis \uparrow glycolysis (pyruvate kinase) \downarrow glycogensynthesis \downarrow cholesterol \downarrow
	TR α 1 ²	gluconeogenesis \downarrow ?
Other	TR α 1	required for proper development and function of the autonomic nervous system
	TR α 1, TR β	effects of glucocorticoids, insulin, glucagons, catecholamines \uparrow

Table 2: Role of TH and TRs in different tissues; ¹perivenously ²periportally; FAS = fatty acid synthetase; FFA = free fatty acids; PEPCK = phosphoenol pyruvate carboxykinase

This identification of TR isoform specific functions opened the possibility to target defined metabolic pathways. The TR β selective compounds KB2115 and GC1, for instance, were shown to be very efficient in reducing serum cholesterol in different animal models without the characteristic cardiac effects of T3 (63, 64). Unfortunately, the Holy Grail, a TH based diet pill, is still not achieved, as weight loss is usually accompanied by a compensatory increased food intake and weight regain on drug withdrawal. However, recent discoveries of novel TH dependent pathways, such as the actions of bile acids on TH-dependent activation of the BAT (65) or the hypothermic effects of the TH-derivate 3-iodothyronamine (66), impressively demonstrate the outstanding role of TH in the regulation of metabolism and reminds us, in spite of the many novel metabolic regulators, not to forget the “old” players.

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**MANAGEMENT OF GRAVES' ORBITOPATHY: THE CONSENSUS STATEMENT
FROM THE EUROPEAN GROUP ON GRAVES' ORBITOPATHY (EUGOGO)**

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Graves' orbitopathy (GO) is the main extrathyroidal expression of Graves' disease and represents a major clinical and therapeutic challenge (1,2). Although it is generally associated with hyperthyroidism, GO may develop also in patients with no present or previous history of hyperthyroidism, or even in hypothyroid Hashimoto's patients (3,4). The pathogenesis of GO still is incompletely understood, but the link between the thyroid and the orbit has important clinical and therapeutic implications (5, 6). GO is often mild and self-limiting, but may become sight-threatening in 3-5% of cases (3, 4). A complex interplay of endogenous and exogenous (correctable) factors concurs to the expression and severity of GO (7, 8). Suboptimal management of patients with GO seems to be widespread (2), while optimal management should be based on a coordinated approach to thyroid disease and orbital disease (9). This requires a strict collaboration and interaction between endocrinologists and ophthalmologists (9).

This report is based on our detailed manuscripts simultaneously published in the *European Journal of Endocrinology* (1) and *Thyroid* (2). It is a consensus statement agreed upon by the Members of the European Group on Graves' Orbitopathy (EUGOGO) (www.eugogo.org) after several meetings and review of the available literature. It should not be considered a guideline, because current evidence based on randomized-controlled clinical trials in the field of GO is too limited. The main conclusions reached by EUGOGO and related recommendations published in the two papers (1, 2) are summarized as follows:

1. Referral of patients with GO to specialist centres

All patients, with exception of the mildest cases, should preferably be referred to combined thyroid-eye clinics (or to physicians particularly expert in managing GO) for optimal diagnostic assessment and therapy (9). Referral is urgent if there are signs/symptoms suspicious for sight-

threatening dysthyroid optic neuropathy (DON) or corneal breakdown, such as unexplained deterioration in visual acuity, changes in intensity or quality of color vision, globe subluxation, corneal opacity, lagophthalmos with visible cornea, or disc swelling (1). All other cases with symptoms/signs related to less severe inflammation or corneal exposure (photophobia, lacrimation, grittiness, swollen eyelids, pain in or behind the eyes, lid retraction, lagophthalmos without visible cornea) or extraocular muscle dysfunction (diplopia or tilting of the head to avoid it) can be referred non-urgently (1).

2. Management issues that should be addressed by both non-specialists and specialists (Table 1)

Smoking: Patients should be informed that smoking bears a high risk for GO, because it contributes to its development and deterioration (7, 8, 10, 11), reduces the effectiveness of treatment (12) and enhances the likelihood of GO progression after radioiodine therapy for hyperthyroidism (13, 14). Patients should be urged to refrain from smoking, because this action may be associated with a better outcome of GO (15). If advice is not enough, referral to smoking cessation clinics or other smoking cessation strategies should actively be pursued.

Thyroid dysfunction: Euthyroidism should be restored promptly and maintained stably in all patients, because both hyper- (16) and hypothyroidism (17) are associated with more severe forms of GO. There is no evidence that antithyroid drugs and thyroidectomy influence the course of GO (18); radioiodine therapy can cause progression of eye disease in about 15 % of cases (13, 14), particularly in smokers (12). To prevent this untoward effect, patients given radioiodine therapy should have post-radioiodine hypothyroidism promptly corrected with L-thyroxine replacement (19) and should be given a short course of oral glucocorticoids (GC) (14), unless their GO is inactive (19) and other risk factors for GO progression (particularly smoking) are absent (20).

Simple measures that alleviate symptoms: Lubricants are useful for symptoms due to corneal exposure, particularly in patients with severe lid retraction; nocturnal ointments are of benefit in patients with incomplete eyelid closure (lagophthalmos); prisms may control intermittent or constant diplopia. Sleeping with head up may reduce eyelid swelling. Diuretics are rarely useful. Botulinum toxin injection may decrease upper lid retraction, but this procedure should be performed in specialized centres.

3. Management issues that should be addressed in specialist centres

Grading severity and activity of GO: Grading of both severity (1; see www.eugogo.org) and activity of GO, using the Clinical Activity Score (21), are crucial for therapeutic decision making. Patients with a CAS $\geq 3/7$ points are considered as having active GO. Regarding severity, patients have *sight-threatening* GO if DON and/or corneal breakdown are present; *moderate-to-severe* GO if eye manifestations have an impact on quality of life sufficient to justify the risks of

immunosuppression (active GO) or surgical intervention (inactive GO); and *mild* GO if the impact on daily life is limited. Patients with mild GO usually have one or more of the following features: lid retraction <2 mm, mild soft tissue changes, exophthalmos <3 mm above normal limit, transient or no diplopia, corneal exposure responsive to lubricants.

Management of sight-threatening GO: Intravenous (IV) GC and orbital decompression are the only treatments of proven efficacy for DON. High-dose IV GC is the preferred first-line treatment (22), but a poor response over 1-2 weeks mandates urgent surgical decompression (Table 2). Sight-threatening corneal breakdown should be treated as an emergency by topical lubricants, moisture chambers, tarsorrhaphy or other temporary measures; if these measures are ineffective, IV GC or orbital decompression should be considered.

Management of moderate-to-severe GO: IV GC are the first line treatment for moderate-to-severe and active GO (Table 2). Oral GC are less effective than IV GC (23, 24); subconjunctival or retrobulbar GC are less effective than oral GC. Acute liver damage has been reported in few cases in association with very high cumulative doses of GC (25). Accordingly, the cumulative dose of methylprednisolone in one course of therapy should not exceed 8 g (26). Patients to be treated with high-dose IV GC should first be screened for liver dysfunction, hypertension, diabetes, urine infections, and glaucoma, and then monitored for side effects. Although guidelines recommend bisphosphonates when long-term (>3 months) oral GC therapy (average daily dose >5 mg prednisone or equivalent) is used, antiresorptive agents should be considered also for patients treated with IV GC. Orbital radiotherapy should be considered in patients with active GO who have diplopia or restricted motility (27). Combination of oral GC and orbital radiotherapy is more effective than either treatment alone (3), but it is unclear whether IV GC combined with orbital radiotherapy are more effective than IV GC alone. Orbital radiotherapy should be avoided in patients with diabetic retinopathy and/or severe hypertension due to the risk of retinopathy (28, 29); it is also prudent to avoid it in patients younger than 35 years because of theoretical concerns about carcinogenesis. Other non-surgical treatments, such as somatostatin analogues, azathioprine, and IV immunoglobulins are of unproven value. Cyclosporine may be useful to reduce the dose of oral GC (30).

Rehabilitative surgery (orbital decompression, squint surgery, eyelid surgery) for moderate-to-severe GO is indicated when GO is inactive (31) (Table 2), but orbital decompression can be considered also in patients with active GO, who are intolerant of or non-responsive to IV GC. Surgical management should proceed in the following order: orbital decompression, squint surgery, eyelid surgery, since side effects of the preceding step can interfere with the step that follows.

4. Management of mild GO

GC and orbital radiotherapy may potentially be of benefit also for mild GO (32), but they are not recommended because the risks outweigh the benefits. Watchful waiting is appropriate for the

majority of patients, if quality of life, assessed by the EUGOGO questionnaire (www.eugogo.org) is satisfactory. If, however, the mild ocular involvement has a profoundly negative impact on psychosocial functioning and quality of life (33, 34), then treatment as for moderate-to-severe GO can be offered to these patients as well, after careful evaluation and discussion of risks and benefits (Table 2).

5. Special situations

Diabetes and hypertension: Diabetes and/or hypertension should not be considered contraindications to GC or surgical treatments, but close monitoring of glycemic control and blood pressure is essential. Thiazide or loop diuretics should be used cautiously during IV GC therapy because of the risk of hypokalaemia. Orbital radiotherapy may increase the risk of retinopathy in diabetic and hypertensive patients. Diabetic retinopathy and/or severe hypertension are absolute contraindications for orbital radiotherapy. Diabetes without retinopathy is a relative contraindication, but evidence is less clear (28, 29).

GO in childhood: GO in childhood is rare (because Graves' disease is less frequent than in adults) and usually milder than in adults (35). Euthyroidism should be restored promptly and stability maintained as in adults. Exposure to active (and, possibly, passive) smoking is as detrimental as in adults (36). Simple, local measures to address specific symptoms can be used as in adults. GC should be avoided as much as possible because of their negative impact on growth. Orbital radiotherapy is contraindicated in children. Orbital decompression may be required in cases of severe exophthalmos, but a conservative and expectant strategy is sufficient for most young patients.

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Table 1: General measures for Graves' orbitopathy (GO).

Referral	<ul style="list-style-type: none"> -Except for mildest cases, refer to combined thyroid-eye clinics or specialized centres -<u>Urgent referral</u>: Suspicion of sight-threatening manifestations (dysthyroid optic neuropathy, corneal breakdown) -<u>Non-urgent referral</u>: all other cases
Smoking	<ul style="list-style-type: none"> -Inform the patient on the multifaceted, negative influence of smoking on GO -Urge the patient to quit smoking -Refer to smoking cessation clinics or strategies if advice is not enough
Thyroid dysfunction	<ul style="list-style-type: none"> -Correct both hyper- and hypothyroidism promptly -Maintain euthyroidism stably -If radioiodine therapy is given, cover with steroid prophylaxis unless GO is inactive and other risk factor for its progression are absent

Table 2. Management of Graves’ orbitopathy (GO).

Severity of GO	First-line treatment
Sight-threatening	-Intravenous glucocorticoids (IV GC) -If response is absent/poor in 1-2 weeks: orbital decompression
Moderate-to-severe	- <u>Active</u> : IV GC (with or without orbital radiotherapy). Avoid using >8 g methylprednisolone cumulative dose per course - <u>Inactive</u> : Rehabilitative surgery. Sequence of needed surgical interventions: 1) orbital decompression; 2) squint surgery ; 3) eyelid surgery
Mild	-None, except for local measures (lubricants, ointments, dark lenses, prisms) -If quality of life is significantly impaired, manage as moderate-to-severe GO

HxT Thyroidology

RECOMBINANT HUMAN TSH (rhTSH) AUGMENTED RADIOIODINE TREATMENT OF BENIGN MULTINODULAR GOITRE

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Introduction

Despite iodization programmes, simple goitre - defined as a euthyroid goitre that is not associated with thyroid autoimmunity or malignancy – still constitutes a major diagnostic and therapeutic challenge. Such goitres can be diffuse, uni- or multinodular (1). Most goitres have a nodular structure, particularly in the elderly (1). According to current thinking, they are caused by an interaction between genetic susceptibility (2) and environmental triggers, iodine deficiency being the most important (3). Here we focus on therapeutic aspects of simple multinodular goitre (MNG), and highlight recent data on recombinant human TSH (rhTSH) augmented radioiodine (^{131}I)-therapy in MNG.

Clinical manifestations of nodular goitre are related to those of growth or functional autonomy. The annual growth rate is in the range of 0-20 % and the five year incidence of hyperthyroidism is approximately 10% (3). Symptoms are typically those of local pressure (dysphagia, globulus sensation, hoarseness, dyspnoea) or cosmetic complaints, and are difficult to evaluate objectively. It is mandatory to rule out malignancy, for which purpose fine-needle aspiration biopsy (FNAB) is the golden standard (4). There is no simple relationship between goitre size and symptoms (5), which often makes the decision whether to treat or not difficult. In asymptomatic subjects with relative small goitres observation is acceptable, although many have a growth potential. There is no ideal treatment, as reflected by the lack of consensus in questionnaire surveys (6-10). Surgery is recommended when facing a large goitre or when malignancy cannot be ruled out (3;4). At best levothyroxine (L-T4) suppressive therapy has a minor effect on the appearance of new nodules but does not shrink existing nodules (11), and is not recommended in recent guidelines and reviews

(3;4;12). In fact, our own recent study suggests that, using exclusion criteria from these guidelines, the vast majority of Danish MNG patients are ineligible for L-T4 therapy (13).

In MNG, conventional (non rhTSH stimulated) ^{131}I -therapy results in an approximately 40% goitre size reduction within one year (14). The effect may be attenuated by a high dietary iodine intake or by an inhomogeneous and often decreased radioiodine uptake (RAIU).

The recent advent of rhTSH has opened new avenues for ^{131}I -therapy of MNG since it can double the RAIU even using minute quantities (15). This potentially allows either increased goitre size reduction, when using standard ^{131}I doses (16), or ^{131}I dose reduction without compromising efficacy (17). In the following we summarise available data on rhTSH stimulated ^{131}I -therapy, and offer suggestions for future studies in order to optimize effect and limit potential side-effects in the treatment of MNG.

Radioiodine therapy with rhTSH pre-stimulation

Effect on thyroid radioiodine uptake

The success of radioiodine treatment in reducing the size of a MNG depends on many factors (18). Foremost a certain RAIU is necessary (above 20%) for ^{131}I therapy to be feasible, depending on goitre size and rules for maximum outpatient radioactivity. The thyroid RAIU is dependent on the general iodine load and the extent of nodular autonomy (3). As a consequence of the widespread iodine enrichment programs low thyroid RAIU is often encountered in MNG, thus high doses of radioactivity are needed to obtain acceptable goitre volume reductions.

Optimal rhTSH dose for enhancement of thyroid RAIU

The optimal dose of rhTSH for stimulation of thyroid RAIU has not yet been established. Defining a lower dose limit that increases RAIU sufficiently is paramount, since adverse effects, especially the induction of transient thyrotoxicosis, is dose dependent (19-21). The changes in thyroid RAIU following rhTSH doses between 0.01 and 0.9 mg have been investigated in both healthy individuals and patients with MNG. In our opinion studies conducted in healthy individuals should not readily be applied to MNG patients, since the morphological changes in MNG may indicate an

altered and/or delayed physiological response. Generally, studies are difficult to compare due to huge variations in iodine load, baseline RAIU and goitre size. The lack of a control group in many studies also complicates the interpretation. Despite these shortcomings, studies in MNG patients (17;19;22-26) and healthy individuals (20;21;27) document an approximately two-fold increase in RAIU, at large independent of rhTSH dose. There is a trend however, towards a positive correlation between the dose of rhTSH and the increase in RAIU, as documented by two studies in MNG patients. The first study reported a relative increase in mean RAIU of 76% when using 0.01 mg of rhTSH compared to 91% when using 0.03 mg (19). In agreement with this finding, another study found a relative increase in mean RAIU of 85% with 0.01 mg of rhTSH compared to 145% with 0.03 mg (17). Favouring the use of rhTSH is the concurrent finding, that the increase in RAIU is inversely correlated with the initial RAIU. Considering that an upper physiological barrier for thyroid RAIU probably exists, this is not surprising.

The importance of the iodine load was investigated by Lawrence et al. (27), who investigated the effect of 0.9 mg of rhTSH in healthy iodine loaded individuals. Although the thyroid RAIU doubled (from 3 to 6%), rhTSH far from normalized the RAIU.

Only one study has investigated changes in absorbed thyroid dose. Following 0.3 mg of rhTSH the retained thyroid ¹³¹I-dose was increased by 75% compared to placebo (28). With regard to minimization of adverse effects, the optimal rhTSH dose is probably 0.1 mg or perhaps even lower, since doses down to 0.01 mg have proven effective for enhancing RAIU(17;19;20). It is unclear whether large goitres demand higher doses of rhTSH for optimal increase in RAIU.

The effect of rhTSH on the inhomogeneous RAIU has been studied in a couple of trials (22;29). There is a trend toward a more homogenous distribution of ¹³¹I, judged by scintigraphy and perhaps even an altered regional uptake, evidenced by cold areas becoming warm, an vice versa (29). Whether this results in a more pronounced nodule reduction, instead of paranodular tissue destruction, is uncertain. The higher prevalence of hypothyroidism with the use of rhTSH suggests that this may not be the case(16).

Timing of rhTSH administration

Although sparsely investigated the optimal time interval between administration of rhTSH and ^{131}I -therapy seems to be 24 hours. However this may be heavily influenced by the characteristics of the study/treatment population. One trial, studying 15 MNG patients, documented that an interval of 24 h (relative mean increase in RAIU 76%) was more effective than 2 h (relative mean increase in RAIU 40%) (19). Considering that the sodium-iodide-symporter is stimulated by rhTSH with some time delay (30), this is of no surprise. So far intervals longer than 24 h have only been studied in healthy individuals, using 0.1 mg of rhTSH (20). This indicates that 24 hours is the optimal interval, since an interval of 24h resulted in a significantly higher mean increase in 24h RAIU from 25 to 47 % (relative increase 88%) compared to the 30 to 41% (relative increase 36 %) using a 48h interval. Interestingly, the 24 h RAIU was not significantly different from baseline when an interval of 72 h was used (20). Variance analysis revealed a borderline significant difference in the RAIU increase between the 24h and 48h interval ($p=0.057$). These findings need to be confirmed in MNG patients.

Repetitive injections of rhTSH have been investigated in two trials (22;31). In one trial 0.1 mg of rhTSH was administered 0 h and 24 h, respectively, before the ^{131}I -tracer, resulting in a 4-fold increase in 24 h RAIU (22). The second injection was probably too close to the tracer administration to significantly influence the RAIU. The pronounced increase in RAIU in this study (22) may very likely be due to a low baseline RAIU (12.3%), more than due to repetitive rhTSH administration. From the aforementioned, we conclude that a 24 hour interval between rhTSH injection and subsequent ^{131}I -therapy seems to be the optimal interval for obtaining an approximately doubling of RAIU in MNG patients, independent of the rhTSH dose.

Effect on goitre volume reduction

The potential benefits of rhTSH when combined with ^{131}I therapy are those of increased goitre volume reduction (GVR) or reduced ^{131}I -dose and extrathyroidal irradiation. Combined with ^{131}I therapy rhTSH increases the goitre volume reduction (GVR) in MNG patients by approximately 50%. This effect has been documented in three randomized controlled trials (RCT) depicted in

table 1 (16;32;33). In small to medium sized goitres (median goitre volume 51 ml, range 20-99 ml), we found that 0.3 mg of rhTSH improved the mean GVR by 33 % after one year (16). In another study using 0.3 mg of rhTSH, but in large goitres (median goitre volume 160 ml, range 99-440 ml), we demonstrated an even more pronounced effect. The average GVR was increased by 56.3% compared to standard ^{131}I therapy (32). In consistence with these two studies, Silva et al. (33), treating large goitres (median goitre volume 219 ml, range 82-728) with a fixed ^{131}I dose, demonstrated a 46% increase in mean GVR after pre-treatment with 0.45 mg rhTSH and a 12 month follow-up. The remaining non-controlled studies (Table 1) have demonstrated considerable GVR between 35% and 53 % (17;22-24;26;31) Although lacking a control group, some of the latter studies (22;26) were carried out in patients with relatively low baseline RAIU, thus demonstrating the feasibility of ^{131}I therapy in such patients when employing rhTSH. One study, in 18 MNG patients with a baseline RAIU of 12%, documented a mean GVR of 39% after 6 months, and 53% after 2 years (22;34). Attaining acceptable GVR in patients with a low baseline RAIU is promising, since a high proportion of MNG patients are expected to have low RAIU due to iodization programmes. Before eradicating simple goitre by such programmes, the treatment of patients with existing goitre is in fact impeded

Another approach is to reduce the ^{131}I dose with a factor corresponding to the increase in RAIU obtained by rhTSH stimulation. This strategy was investigated in 22 MNG patients, using 0.01 or 0.03 mg of rhTSH (17). Pre-treatment with rhTSH allowed a 50% reduction of ^{131}I dose, while still achieving a GVR of 40 %, after one-year. In addition a reduced absorbed radiation-dose was documented in extra thyroidal organs and tissues, especially bladder and stomach (35). Such a reduced ^{131}I dose is desirable both in terms of lowering the theoretical risk of late occurring extrathyroidal malignancy, and in terms of being able to treat patients on an out-patient basis. Minimizing in-patient treatment reduces the economic burden to society and the inconvenience for the patient.

Three studies have published follow-up results beyond one year (22;26;33). Two small observational studies (22;26) found insignificant GVR between one and two years of observation (34). In a randomized trial (33), further GVR, in both the rhTSH and the placebo group, was

reported, between one and four years of follow-up. The difference between the groups at one year was maintained after four-years, but was not more pronounced (36).

In our placebo-controlled study in patients with a very large goitre it was shown that rhTSH-augmented ^{131}I therapy, compared with ^{131}I therapy alone, resulted in a greater improvement of the inspiratory function due to a diminished tracheal compression (Bonnema, S.J., personal communication).

Table 1: Studies on the effect of rhTSH combined with ^{131}I therapy, in patients with benign multinodular goitre.

Author (year)	n	Design	rhTSH dose (mg) and time interval	Goitre size estimation	Iodine dose	Mean goitre volume reduction	Myxoedema prevalence at follow-up
Nieuwlaat <i>et al.</i> *(17) (2003)	12	Observational /	0.01	MR-scan	Adjusted	35% / 1 year	33%
	10	non-controlled	0.03		Equality study	41% / 1 year	40%
Duick <i>et al.</i> ** (24) (2003)	6	Observational /	0.3	Palpation	Fixed	30-40% / 7 months	50%
	10	non-controlled	0.9		(30 mCi)	30-40% / 7 months	60%
Silva <i>et al.</i> ** (33) (2003)	34	Randomized/ placebo-controlled	0.45 24 h	CT-scan	Fixed	rhTSH: 58% / 1 year placebo: 40% / 1 year P<0.05, between groups	rhTSH: 65% placebo: 21%
Albino <i>et al.</i> ** (22) (2005)	18	Observational / non-controlled	2 x 0.1 24/48 h	CT-scan	Fixed (30 mCi)	39% / 6 months 53% / 2 years	65% 72%
Cohen <i>et al.</i> ** (23) (2006)	17	Observational / non-controlled	0.03 24 h	CT-scan	Fixed (30 mCi)	34% / 6 months	18%
Giusti <i>et al.</i> ** (31) (2006)	12 8	Observational / with matched controls	2 x 0.2 0 24/48 h	CT-scan	Fixed (10- 15mCi)	rhTSH: 44% /20 months controls: 25% /22 months	rhTSH:17% controls:62%
Nielsen <i>et al.</i> ** (16) (2006)	57	Double-blinded/ randomized/ placebo-controlled	0.3 24 h	US-scan	100 Gy adjusted	rhTSH: 62% / 1 year placebo: 46% / 1 year P=0.002, between groups	rhTSH: 61% placebo: 11%
Bonnema <i>et al.</i> ** (32) (2007)	29	Double-blinded/ randomized/ placebo-controlled	0.3 24 h	MR-scan	100 Gy adjusted	rhTSH: 53% / 1 year placebo: 34% / 1 year P<0.001, between groups	rhTSH: 21% placebo: 7%
Paz-Filho <i>et al.</i> ** (26) (2007)	17	Observational / non-controlled	0.1 24 h	CT-scan	Fixed (30 mCi)	46% / 1 year 52% / 2 years	53% -

*Equality study: Reduced ^{131}I activity, aiming at the same absorbed dose as with conventional ^{131}I therapy.

** Superiority study: Aiming at increased thyroid irradiation.

Adverse effects of recombinant human TSH

RhTSH has been used in the treatment of differentiated thyroid cancer for more than a decade, and is generally well tolerated, even with repeated doses of 0.9 mg rhTSH. In large-scale clinical studies only a minority of patients had mild adverse reactions such as nausea and headache (37).

In combination with ^{131}I therapy for MNG, acute and long-term alterations in thyroid function and size constitute the main adverse effects in patients with the thyroid gland in situ.

Acute adverse-effects

The induction of transient hyperthyroidism is dose dependent. The effects of rhTSH on thyroid function, both in healthy individuals and in patients with MNG, have been studied by us and others (19-21;38;39). Although different doses of rhTSH (0.01, 0.03, 0.3 and 0.9 mg) were used, the same patterns in the various biochemical markers were observed. A clear dose-response exists, since a more pronounced response in serum levels of T4, T3 and Tg was observed when administering 0.3 mg rhTSH, compared to lower doses. A maximal stimulatory dose also seems to exist since 0.9 mg rhTSH did not stimulate thyroid function more than 0.3 mg, when administered to the same subjects (21). With rhTSH doses below 0.1 mg the response was blunted and most patients maintained thyroid hormone levels within the normal range (19). Thus, limiting rhTSH doses to 0.1 mg or less, the rise in thyroid hormones seems to be of little clinical relevance. Nonetheless caution is advised, especially when treating the elderly or patients with cardiovascular disease.

Swelling of the thyroid tissue within the first 48 hours has been documented in both healthy individuals and MNG patients. Thus 0.9 mg of rhTSH administered to nine healthy individuals resulted in a 35% increase in mean thyroid volume at 48 hours (38). One individual developed a very profound and tender thyroid enlargement, from 22 ml to 90 ml. Similarly, when 0.3 mg of rhTSH was administered to ten MNG patients, a mean volume increase of 24% was seen after 48 hours (39). Most likely this acute effect is caused by an exaggerated vascular response, possibly leading to an interstitial fluid accumulation. In susceptible individuals with a large obstructing goitre this may pose a serious threat in terms of respiratory problems. Until now only one study has evaluated whether rhTSH-augmented ^{131}I therapy results in a significant acute goitre swelling (32). In that trial, the goiter volume remained unchanged, on average, one week after ^{131}I therapy, but larger deviations from baseline were observed in patients pre-treated with rhTSH compared with placebo (32). The observations in this study are reassuring, but it should be kept in mind that the largest deviations in thyroid size are seen in the first 48 hours after rhTSH administration (38;39).

Although observed in healthy individuals, the acute swelling of the thyroid tissue is probably dose dependent since 0.1 mg of rhTSH resulted in a blunted (mean 10%) increase in thyroid volume, when administered to 25 healthy individuals (20). Other adverse reactions, like cervical pain or tenderness, typically occurring in the first three weeks after treatment, are more frequent with the use of rhTSH (16;32;33). Most likely these manifestations are a consequence of the increased thyroid irradiation.

Chronic adverse effects

More concerning than the acute and transient increase in thyroid hormones, is the up to five-fold increase in the rate of permanent hypothyroidism (16). In the three randomized controlled trials, permanent hypothyroidism was reported in 21% (32), 61% (16) and 65% (33) of rhTSH treated patients, compared to controls 7% (32), 11% (16) and 21 %, respectively (33). Life-long L-T4 therapy is needed in these individuals. In the recent years there has been increasing focus on the possibility that Levothyroxine substitution may result in reduced quality of life (40). Until this issue is clarified the higher prevalence of hypothyroidism should not withhold clinicians from using rhTSH-augmented ^{131}I -therapy. Whether the prevalence of hypothyroidism with rhTSH-augmented ^{131}I -therapy can be reduced without compromising efficacy (goitre size reduction) remains unclarified. It is unsettled whether the increased incidence of hypothyroidism is solely caused by the increased absorbed ^{131}I -dose, or if the rhTSH dose is an independent factor (28). As seen with conventional ^{131}I -therapy, the incidence of hypothyroidism is positively correlated to the GVR. Appearance of TSH receptor antibodies (TRAb) and/or anti-TPO antibodies has been reported in MNG patients following ^{131}I therapy (41). However, apparently the use of rhTSH does not increase this risk (42).

Unresolved issues and future perspectives

Based on the above we suggest that rhTSH may have a future role in ^{131}I treatment of MNG, especially in large goitres or in goitres with a low RAIU. A strong argument in favour of rhTSH is its potential to offer improved GVR or an equal effect with a reduced ^{131}I dose. The risk of subsequent

extrathyroidal malignancy is unknown. In theory, this risk is higher in euthyroid MNG patients than in hyperthyroid patients, since higher doses of radioactivity are employed. Although rhTSH amplifies the GVR, the failure to demonstrate an effect on patient satisfaction or quality of life (QoL) is problematic (16). This issue deserves further attention to clarify whether it is due to lack of sensitivity of the method for QoL determination. Acute swelling of the thyroid gland has been reported with rhTSH doses above 0.1 mg. This potentially serious side-effect is probably dose dependent, but further studies addressing this, and other side-effects, including the long-term effect of the increased hypothyroidism rate, deserves continued study. The optimal dose of rhTSH is most likely below 0.1 mg, but it should be noted that the serum concentration of rhTSH may have an effect beyond the increase in thyroid radiation caused by the increase in RAIU (16). The mechanism of such an effect, should it exist, could be an increased vulnerability of the rhTSH stimulated thyrocytes. This theory is supported by the finding that the negative correlation between initial goitre size and GVR (seen with conventional ^{131}I -therapy) is abolished by the use of rhTSH (22;32). The results of an ongoing phase 2 trial using low doses of rhTSH as well as studies using different applied ^{131}I doses to the thyroid will contribute to resolving these interesting issues.

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Key-words: Goitre, Thyroid nodule, Radioiodine therapy, recombinant thyrotropin

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CLINICAL UPDATE: GENOTYPE-PHENOTYPE CORRELATION OF RET MUTATIONS

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Concise Review invited by Dr. Dagmar Führer.

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Email: karin.frankraue@raue-endokrinologie.de**Abstract**

Multiple endocrine neoplasia type 2 (MEN2) is an autosomal dominant tumour syndrome caused by germline-activating mutations of the *RET* proto-oncogene. It is characterized clinically by the presence of medullary thyroid carcinoma (MTC), bilateral pheochromocytoma, and primary hyperparathyroidism (MEN2A) within a single patient or family. Three distinct clinical forms have been described: (i) classical MEN2A, (ii) MEN2B, an association of MTC, pheochromocytoma, and mucosal neuroma, and (iii) familial MTC (FMTC), which is associated with a very low incidence of other endocrinopathies. Each variant of MEN-2 results from a different *RET* gene mutation, with strong genotype–phenotype correlation. Genetic testing detects nearly 100% of mutation carriers and is considered the standard of care for all first-degree relatives of patients with newly diagnosed MTC. Recommendations on the timing of prophylactic thyroidectomy and extent of surgery are based on classification of RET mutations into three risk levels according to genotype-phenotype correlations. MEN-2 provides a unique model for early prevention and cure of cancer and for the stratified roles of mutation-based diagnosis of carriers.

Key-words: *RET mutation; Medullary Thyroid Carcinoma (MTC); Multiple Endocrine Neoplasia 2 (MEN2)*

Clinical syndromes of MEN-2

Multiple endocrine neoplasia type 2 (MEN2) (OMIM 171400) is an autosomal dominant tumour syndrome with an estimated prevalence of 2.5 per 100,000 in the general population.

MEN2 syndrome occurs in three clinically distinct varieties with medullary thyroid carcinoma (MTC) as a common manifestation. These three subtypes of MEN-2 differ with respect to incidence, genetics, age of onset, association with other diseases, aggressiveness of MTC, and prognosis (1).

MEN2A Syndrome

MEN-2A syndrome is characterized by MTC in combination with pheochromocytoma and/or multiple tumours of the parathyroid glands in a single patient, or the presence of two or more tumour types in multiple members of a single family. It is the most common form of all MEN2 syndromes, representing 55% of cases (2). The frequency of MTC is over 90% among patients with MEN2A, while the frequency of pheochromocytoma and multiple parathyroid gland

hyperplasia are 40-50% and 10-20%, respectively. MTC is generally the first manifestation of MEN2A and develops between the ages of 5 and 25 years. Rare variants of MEN2A exist, including MEN2A with cutaneous lichen amyloidosis and FMTC with Hirschsprung's disease.

MEN2B Syndrome

MEN2B syndrome is the most aggressive form of MEN2 and accounts for 5-10% of MEN2 cases. It consists of MTC, pheochromocytoma, an absence of hyperparathyroidism, visible physical stigmata such as raised bumps on the lips and tongue (due to cutaneous neuromas), ganglioneuromas, and a Marfanoid habitus with skeletal deformations and joint laxity. Patients with MEN2B typically have disease onset in the first year of life and have a more aggressive form of MTC with higher morbidity and mortality rates compared to MEN2A patients. Patients with MEN2B often do not have a family history of the disease, in which case the syndrome is due to a de novo mutation.

Familial MTC (FMTC) Syndrome

FMTC is the mildest variant of MEN2. It has been diagnosed more frequently in recent years and is reported to account for 35-40% of all MEN2 cases (3,4,5). With FMTC, there is a strong predisposition to develop MTC with a very low incidence of the other clinical manifestations associated with MEN2A. The diagnosis of FMTC can only be considered when 4 or more family members across a wide range of ages have isolated MTC. In general, the clinical course of MTC in FMTC is more benign than that seen in individuals with MEN2A and MEN2B, and FMTC typically has a late onset or no clinically manifest disease. FMTC carries a good prognosis; however, aggressive MTC tumours and even death due to MTC have been reported in cases harbouring codon 804 mutations. A family history is often inadequate in establishing the diagnosis of familial disease, and a more thorough evaluation by genetic and biochemical screening often reveals a family history of MTC in patients originally thought to have the sporadic form of the disease.

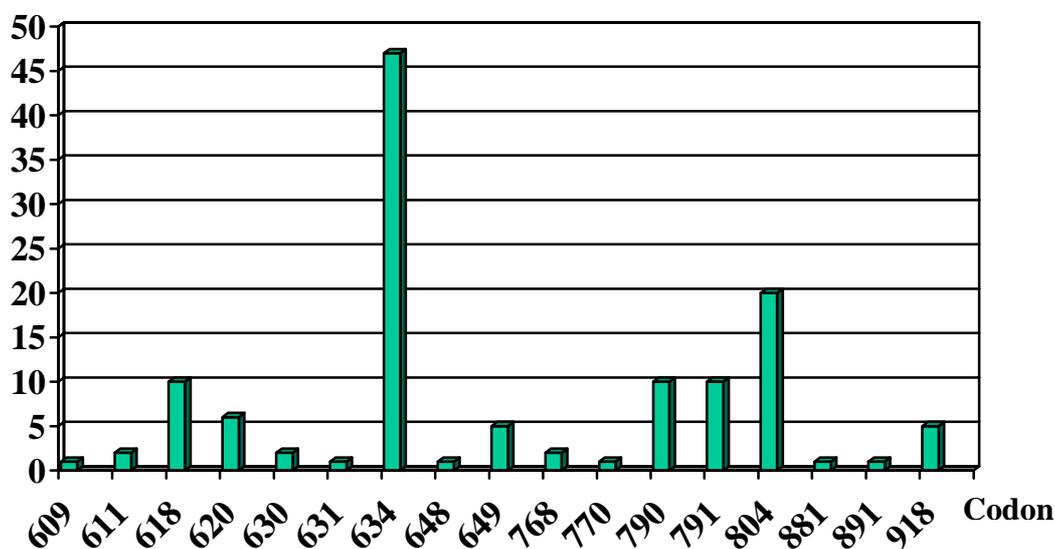
RET proto-oncogene: structure, function, and genetic abnormalities

The MEN2 gene was localized to centromeric chromosome 10 (10q11.2) by genetic linkage analysis in 1987. Subsequently, point mutations of the *RET* proto-oncogene were identified in MEN2A, MEN2B, and FMTC in 7 exons located near this region (exons 8, 10, 11, 13-16) (6,7). Analysis of *RET* in families with MEN2A and FMTC revealed that nearly 100% of these families have germline mutations, and that only those family members with the germline missense mutations have the disease. This discovery prompted major advances in our understanding of the molecular genetic basis of MTC and has significantly changed the clinical management of families with hereditary tumours. At present, mutation analysis has identified over 50 different missense mutations associated with the development of MEN2 (Fig.1) (5).

The *RET* gene has 21 exons and encodes a receptor tyrosine kinase that appears to transduce growth and differentiation signals in several developing tissues, including those derived from the neural crest. The protein consists of an extracellular segment with a ligand-binding domain, a cadherin (Ca²⁺-dependent cell adhesion)-like domain, and a cysteine-rich domain close to the cell membrane. It has a single transmembrane domain and an intracellular segment with two tyrosine kinase subdomains, TK1 and TK2. The RET protein is activated upon ligand-induced dimerization (8). RET is expressed in neuroendocrine cells including C-cells of the thyroid, the precursors of MTC, and in pheochromocytomas. Hereditary MTC is caused by autosomal dominant gain-of-function mutations in the *RET* proto-oncogene. Mutation of the extracellular cysteine at exon 11 codon 634 causes ligand-independent dimerization of receptor molecules, enhanced phosphorylation of intracellular substrates, and cell transformation. Mutation of the intracellular tyrosine kinase (codon 918) has no effect on receptor dimerization but causes constitutive activation of intracellular signalling pathways and also results in cellular transformation.

There is a significant age-related progression from C-cell hyperplasia (CCH) to MTC, which correlates with the transforming capacity of the respective *RET* mutations (9).

Fig. 1 Distribution of different RET mutations (130 families) diagnosed between 2000 and 2008 (Laboratory of molecular genetics, endocrine practice, Heidelberg)



MTC is generally the first neoplastic manifestation in patients with MEN2A because of its earlier age and higher rate of penetrance compared with pheochromocytoma or parathyroid hyperplasia. This indicates that C cells are more susceptible to oncogenic *RET* activation than adrenal medullary or parathyroid cells.

Genotype – phenotype correlation in MEN2

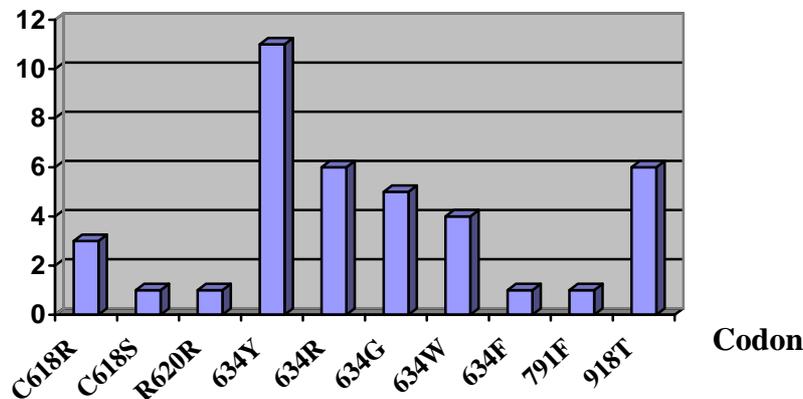
Clear associations are documented between specific *RET* mutations (genotype) and the age of onset and aggressiveness of MTC and the presence or absence of other endocrine neoplasm (phenotype), such as pheochromocytoma or hyperparathyroidism (10,11). Although some overlap exists between *RET* mutations and the resulting clinical subtype of MEN 2, 85 % of patients with MEN2A have a mutation of codon 634 (exon 11, cysteine –rich domain); mutations in codons 609, 611, 618, and 620 account for an additional 10% to 15% of cases. Hyperparathyroidism in MEN-2A is most commonly associated with codon 634 mutations, and in particular with the C634R mutation (12) (Table 1).

Table 1. Primary hyperparathyroidism (HPT) and pheochromocytoma (pheo) in MEN2 (Endocrine Practice, Heidelberg) in a series of 150 patients with *RET* mutations.

RET-Mutation Codon	Number of HPT	Number of kindred	Number of carriers	% HPT of carriers	Number of pheo	Number of kindred	% pheo of carriers	Number HPT + Pheo
C618R					2	2	66	
C618S					1	1	33	
C620F					1	1	100	
C630R	1	1	4	25	1	4	25	1
C634Y	2	8	32	6	11	6	34	1
C634R	5	5	9	56	6	6	100	4
C634W	1	1	7	14	4	1	57	1
C634S	1	1	2	50	1	2	50	1
C634G	2	5	20	10	5	5	25	1
791S					1	2	50	
918					6	10	60	

Pheochromocytoma are associated with 634 and 918 mutations in approximately 50% of patients, and are rarely associated with mutations in exon 10 (codon 609,611,618, 620) or exon 15 (codon 791, 804) (Fig.2, Table 2) (13,14).

Fig. 2. Pheochromocytoma (pheo) in MEN2. Number of patients with pheo and different *RET* mutations (endocrine practice, Heidelberg) in a series of 130 patients



The association between disease phenotype and *RET* mutation genotype may have important implications for the clinical management of MEN2 patients and their families. If the genotype can be fully correlated with certain phenotypic features, then a clinician could use a patient's genotype to decide if intense screening for pheochromocytoma or hyperparathyroidism is necessary in those patients with mutations associated with a higher risk of disease.

All cases of MEN2A with Hirschsprung's diseases have mutations in exon 10 (codon 609, 611, 618, 620), and MEN2A with cutaneous lichen amyloides is associated with mutations in codon 634. More than 95% of MEN2B patients have mutations in codon 918 (exon 16, tyrosine kinase domain), but mutations are rarely identified at codon 883 exon 15. The 918 mutation resulted in an ATG (methionine) to ACG (threonine) alteration, which is significant because Met 918 is a critical component of the substrate recognition pocket in the tyrosine kinase catalytic core of the RET protein.

In FMTC germline mutations are distributed throughout the *RET* gene with an accumulation in exon 13 (codon 768, 790, 791), and exon 14 (codon 804,844); some of these mutations have also been identified in families with MEN2A. Because FMTC shares a common genetic defect with MEN2A, it can be difficult to distinguish a family that initially appears to be FMTC from one with MEN2A, as the manifestation of pheochromocytoma and/or hyperparathyroidism occurs later in the course of the disease.

Extensive reports in the literature show a correlation between the specific germline *RET* mutation and the age of onset and aggressiveness of MTC development and the presence of nodal metastases (9). Patients with codon 918 mutation and MEN2B have a high risk of aggressive MTC occurring at a young age. In contrast, patients with codon 791 mutations have a relative low risk of aggressive disease, and develop slow-growing tumours as a late manifestation (15). This genotype-phenotype correlation between mutation and age of onset and tumour aggressiveness is the basis for decision making in the clinical management of MEN2 patients. This is particularly true in pre-symptomatic *RET* mutation carriers, as prophylactic thyroidectomy has to be performed prior to the development of cancer. This strategy for prevention of familial MTC should be tailored according to the specific mutation carried by each patient.

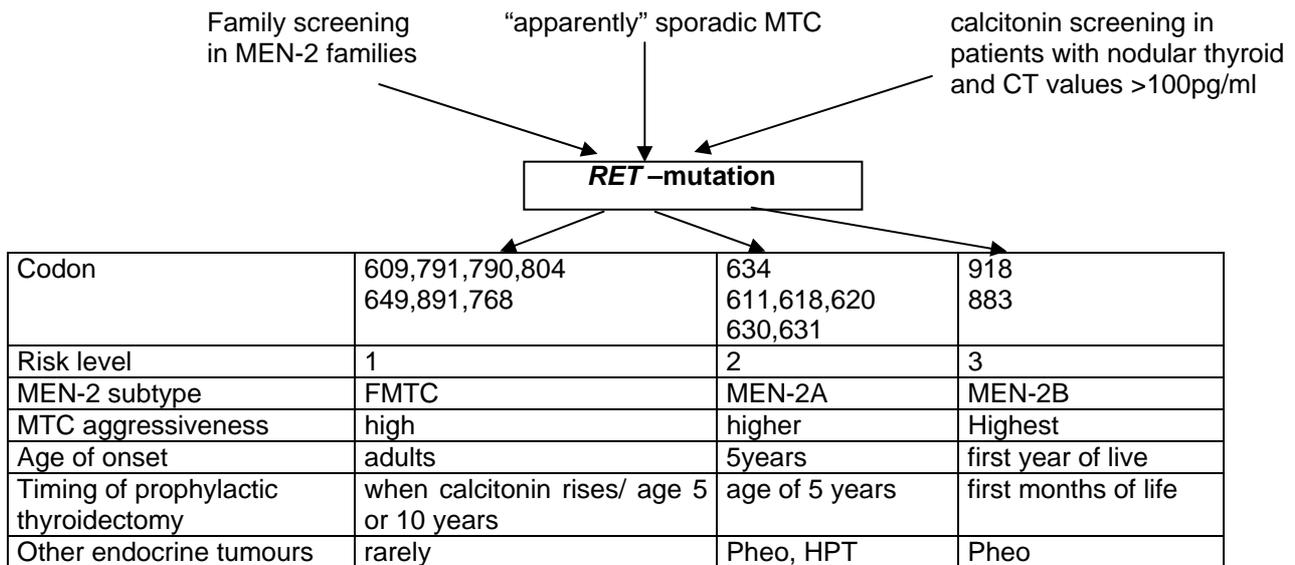
Recommendations for the timing of prophylactic thyroidectomy and the extent of surgical resection are based upon a model that utilizes these genotype-phenotype correlations to stratify mutations into three risk levels (16,17). Patients with level 1 mutations (codons 609, 768, 790, 791, 804, and 891) have a high risk for MTC development and growth, patients with level 2 mutations (codons 611, 618, 620, and 634) are at a higher risk, and patients with level 3 mutations (codons 883 and 918) are at the highest risk for early development and growth of MTC (Table 2).

Table 2. Codon based genotype phenotype correlation, strength of data

Phenotype - Risk of MTC development	Genotype – codon of <i>RET</i> Mutation		
	Very good evidence, Large number of families	Sufficient evidence	Single cases, less clinical experience
high (level 1)		609,791,790,804	649,891,768
higher (level 2)	634	611,618,620	630,631
highest (level 3)	918		883

Codon-specific prognosis would facilitate individual risk stratification for each patient. Furthermore, good evidence exists for a significant age-related progression from C-cell hyperplasia (CCH) to MTC that correlates with the transforming potential of level 2 (codon 634) and level 3 mutations (codon 918) (9). In the cases of these higher risk mutations, a thyroidectomy is recommended at the age of five years with level 2 mutations, and as early as possible, preferably in the first year after birth, for patients with level 3 mutations (16). For patients with level 1 mutations there are three alternatives concerning recommended age at prophylactic surgery: some authors suggest thyroidectomy at age 5, others at age 10, while others, including the current authors (15), suggest that surgery may be postponed until an abnormal C cell stimulation test result is observed (i.e., an abnormal Calcitonin response to pentagastrin or calcium stimulation). Further studies, particularly regarding rare mutations (18), are necessary before common recommendations can be made.

Table 3. Management of patients with different *RET* Mutations



MTC, medullary thyroid carcinoma; HPT, primary hyperparathyroidism; Pheo, pheochromocytoma.

The primary gene sequence is not the sole determining factor of clinical disease

- Identical genotypes can cause different phenotypes

It is of considerable interest to note that identical germline mutations have been reported in families with MEN2A and FMTC. In the Netherlands two large families with mutations of cysteine codon 618 exon 10 were investigated. Pheochromocytomas were found in only two of 60 patients in one family and in one of 20 patients in the other (19). The investigators concluded that FMTC associated with a *RET* gene exon 10 mutation constitutes a subtype of MEN2A with a low frequency of pheochromocytoma rather than a separate clinical entity. The same is true for mutations in exon 13 codons 768, 790, and 791, and in exon 14 (Val 804 Leu) (20), which was thought to be specific for FMTC. However, some rare families with MTC and pheochromocytoma were also identified (3).

- *Different amino acid exchanges at the same codon cause different phenotypes*

Specific amino acid exchanges at a particular codon might have a direct impact on tumour aggressiveness in MEN-2A syndromes. This is demonstrated by individuals with the C634R substitution, who presented with an earlier appearance of lymph node metastases and significantly more distant metastases than individuals carrying C634Y (21).

- *SNPs may play a role*

In recent years a spectrum in the clinical presentation among genetically related individuals was observed. This intrafamilial phenotype variability regarding the age of onset and disease extent at diagnosis (tumour size, presence of lymph node or distant metastases) is a matter of current research. It has been speculated that interacting genetic alterations may be responsible for this phenotypic heterogeneity. Single nucleotide polymorphisms (SNPs) within the *RET* oncogene have been reported at G691S, L769L, S836S, and S904S, and their modifying roles in the pathogenesis of MEN-2 have been discussed (22,23). Only the *RET* G691S variant was found to modulate the age of onset of familial and sporadic MTC with higher basal calcitonin levels (24).

Associations of drug responsiveness and genome markers

RET seems to be a promising target for molecular therapy of patients with MTC. Different strategies that might obstruct the kinase function of *RET* are in development (25), and competitive inhibitors of ATP binding have been tested (26,27) or are currently in clinical trials. Vandetanib (ZD6474, AstraZeneca), a multikinase inhibitor, inhibits the wild-type enzyme and most of the activated forms of *RET*, but *RET* molecules with mutations in codon Val804 are not inhibited. This might be a mechanism of acquired resistance to vandetanib (25).

Change in the spectrum of detected *RET* mutations over the last two decades

Our analysis of the *RET* proto-oncogene in patients with hereditary MTC provided evidence for a change in the spectrum of detected mutations: in 39% of families level 1 mutations, so called "rare mutations", were diagnosed, and in 54% and 6% level 2 and 3 mutations, respectively, were detected (Fig 1)(5). This change in the frequency of diagnosed mutations in classical MEN2A families from a rate of 85% level 2 mutations (primarily exon 11 codon 634), to a dominance of the so called rare mutations localized at codons (10),13,14,and 15 (level 1), may impact the overall prognosis of hereditary MTC, i.e. the overall prognosis of hereditary MTC might improve. Reasons for this change in mutation detection may be due to routine *RET* diagnostics in all patients diagnosed with MTC, the occurrence of hereditary cases in apparently sporadic (4-7%), and extension of analysis to involve mutations other than known "hot spots." (4). Additionally, there is a distinct distribution of *RET* mutations in different parts of the world depending on the genetic background of the local population, which may also affect detection rates of specific *RET* mutation detection.

Future

The biological behaviour of MTC can now be stratified based on mutations of the *RET* proto-oncogene, which guides the timing of prophylactic thyroidectomy and the extent of surgical resection. The appropriate treatment remains to be established for rare mutations. Advances in our understanding of the molecular pathways underlying the different MEN-2 phenotypes may aid in the development of individualized therapeutic modalities based on codon-specific inhibition of tumour growth.

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CLINICAL UTILITY OF HUMAN TSH-RECEPTOR ANTIBODY DETERMINATION – IS THERE STILL DOUBT?

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Abstract

Clinical thyrotoxicosis in patients with Graves' disease (GD) is caused by thyrotropin receptor autoantibodies (TRAb) that stimulate the thyrotropin receptor. The clinical usefulness of TRAb detection was established half a century ago. Although TRAb assay was originally reserved for the diagnosis of GD, recent studies have extended its use to therapy monitoring and even to the assessment of the severity of Graves' ophthalmopathy. As with many other laboratory parameters, the measurement of TRAb is considered by many to be useful, whilst others believe it to be unnecessary. The reservation to use TRAb is questioned in this article and compared with assays routinely carried out for other clinical specialities. Second-generation TRAb assays with high clinical sensitivity and specificity are the method of choice as they are widely available in Europe and less costly than cell-based bioassays. They have 95–99% sensitivity for the detection of GD, with close to 100% specificity. The use of these assays in clinical routine is helpful in the differential diagnosis of hyperthyroidism, since the presence of autoantibodies confirms GD, whilst their absence indicates a non-autoimmune origin of hyperthyroidism. Elevated TRAb are also helpful in predicting disease outcome during antithyroid drug treatment of patients with GD, since patients with TRAb >10 IU/L are likely to relapse after initial treatment with antithyroid drugs, and patients with TRAb >4 IU/L are likely to relapse after completion of their treatment. Recent work also suggests that TRAb detection offers information for the disease management of patients with Graves' ophthalmopathy.

Key-words: *TSH-receptor antibodies; TRAb; Graves' disease*

1. Introduction

Thyroid hyperfunction in Graves' disease (GD) is caused by autoantibodies that activate the thyrotropin (TSH) receptor (TSH-R). These TSH-R autoantibodies (TRAb) are one of the few examples, where autoantibodies are not simply an epiphenomenon of an underlying disease, but also the very cause of the clinical manifestations of that disease. Whilst GD is characterised by thyroid-stimulating autoantibodies (TSAb) to the TSH-R, some patients with autoimmune thyroid disease have TSH-R autoantibodies that block thyroid activation (TBAbs), leading to hypothyroidism (1). Much work went into solving the riddle of what makes a TRAb a thyroid stimulator or a thyroid blocker, and the definition of the precise location of TSAb or TBAbs epitopes seemed to be the key to understanding the pathophysiology of hyper- or hypo-thyroidism. However, despite recent

progress in modelling the structure of the TSH-R, our understanding of the molecular mechanism is far from complete (1).

2. Assays for TRAb

Our understanding of the clinical utility of TRAb measurement goes hand in hand with the development of appropriate methods for their detection. Although a detailed review of TRAb assay technology cannot be the focus of this hot topic article (see instead (2,3)), it is impossible to understand the clinical relevance (and controversy) of TRAb determination without a basic understanding of the history and quality of TRAb assays.

2.1. Historical methods

The first approaches in the detection of TRAb were actually a side line of the initial attempts to measure the biological effect of TSH. Adams and Purves (4) fed ^{125}I to guinea pigs, and measured the radioactive thyroid hormones released on a TSH stimulus. By injecting guinea pigs with TSH-containing sera, they observed a belated radioiodine secretion in one animal treated with serum from a patient with GD. To distinguish this unknown factor from TSH, it was later termed long-acting thyroid stimulator (LATS). In a subsequent adaptation of this approach in mice, McKenzie established his famous mouse bioassay for LATS (5).

Over the following years, the McKenzie mouse assay was simplified to a cAMP readout after the stimulation of TSH-R with TSH or antibodies. The use of human thyroid tissue seemed to be optimal; however these assays were subject to significant changes in sensitivity based on the individual differences of thyroid specimens from patients undergoing thyroidectomy. The application in routine laboratories required a standardised source of tissue to overcome this problem. For some time, the Fisher rat thyroid line was used (6), but after the cloning of the TSH-R, recombinant human TSH-R-expressing cells, such as the Chinese hamster ovary (CHO) cells (7), became standard.

2.2. Modern bioassays

Simplified versions of the bioassay with cAMP readout (8) are still in use in some research laboratories. However, 10 years ago, a luciferase reporter gene was introduced into TSH-R-expressing cells under the control of cAMP-responsive elements (9) (10). Adding sera from GD patients to these cells amplifies the TSH-R signal via the signal pathway, resulting in luciferase expression. In the presence of luciferin, the light output can be quantified by a luminometer. This assay was also adaptable for TBAb detection (11). A commercialised version of this assay is now broadly available in the USA.

2.3. TBII assays

Besides bioassays, alternative *in vitro* diagnostics for GD were evaluated. One breakthrough in assay technology was the development of a robust competitive TRAb assay by Rees Smith (12), which led to widespread clinical use of these assays in Europe and Japan. The advantage over bioassays was the standardised large-scale production which allowed assay systems with comparable quality and clinical sensitivity over long periods. The assay could be completed within hours instead of days, and was more cost efficient than bioassays. However, there were two disadvantages. First, the TRAb detected by this assay system are characterized by their competition with TSH as TSH-binding-inhibiting immunoglobulins (TBII) and not according to their biological activity as TSAbs or TBAbs, which is a constant cause of controversy over the clinical usefulness of such measurements. Second, the sensitivity of this assay system for untreated GD was only between 70% and 80%. Whilst the first limitation has not yet been overcome, the matter of low sensitivity was solved by a second-generation TBII assay with 99% sensitivity for GD (13). This high sensitivity was subsequently confirmed by many independent studies, and the commercialised version of this assay is now generally accepted as the gold standard for TBII detection (14-20). Alternative second-generation TBII assay systems based on porcine TSH-R are also available (21).

2.4. Searching for the third generation of TRAb detection

Since even the second-generation TBII assay cannot distinguish between TSAb and TBAb, the search for a third-generation assay, which combines high technical precision and high clinical sensitivity with the ability to discriminate the bioactivity of the antibodies, is still ongoing. Over the last few years, several alternative assay formats were suggested, using either TSH-R/LH-R chimeras (22), purified human TRAb (23) or monoclonal antibodies (24-26). However, despite some progress, the discrimination of TSAb and TBAb by these methods is still imperfect. The reason for this failure is the relative close proximity of the epitopes for TSAb and TBAb, so that labelled TSAb (or TBAb) used as tracer will always compete with both subtypes of TRAb.

Even the replacement of the TSH tracer in the TBII assay by a labelled stimulating monoclonal antibody (21) did not further improve the already very high sensitivity and specificity of second-generation assays. Although commercially promoted as “third-generation assay”, this assay offers neither clinical nor technical advantages (27) and it also fails to discriminate between TSAb and TBAb.

2.5. What to use – Second-generation TBII assays or bioassay?

With the availability of highly sensitive second-generation TBII assays, bioassays are more and more reserved for limited clinical situations, such as the detection of TSAb or TBAb in pregnancy (28), in newborns with a family history of GD, or the evaluation of patients suspected of carrying an activating mutation of the TSH-R (29). Commercial TBII assays are widely available, but the acceptance of bioassays is still limited to specialised centres using in-house assays. No commercialised bioassays are presently established in Europe. A radioactive bioassay, based on porcine thyroid cells, has managed to survive in Japan and is used for about 10–20% of TRAb determinations in that country. A luciferase-based bioassay has recently gained some market share in the USA, where “TSI” measurement is still dominant and recommended (30), whilst TBII measurement has never reached the acceptance it has in Europe.

There are several reasons for the limited availability of bioassays. One important, albeit rather unscientific, point is related to the costs, which, in a time of limited healthcare resources, gains increasing relevance. Unless an institution has a strong interest in thyroid research, the establishment of a TRAb bioassay is an extravagant investment, since all bioassays are more expensive than commercialised TBII assays. They require a tissue culture facility and trained technicians, whilst the costs for culture medium and cAMP kits may already be as high as a complete TBII kit. The time to obtain a result for bioassays is at least 1–2 working days, but only 4–5 hours for a TBII assay. Another obstacle is legal regulation, since all modern bioassays applying TSH-R-transfected CHO cells require permission to work with genetically altered organisms. Thus, there is no simple “bioassay in a box” available for the clinical thyroidologist, whilst TBII assays are presently taking the next step towards a fast and rapid availability on automated platform systems. It will be interesting to see if the high clinical sensitivity of second-generation TBII assays can be maintained on automated systems, which require on-board stability of the delicate TSH-R for several days.

3. Clinical usefulness of TRAb

The clinical usefulness of TRAb detection was established half a century ago. Originally reserved for the diagnosis of GD, recent studies have extended the use of TRAb to therapy monitoring and even to the assessment of the severity of Graves’ ophthalmopathy (GO). As with other laboratory parameters, the measurement of TRAb is considered to be useful by many, whilst others believe it to be unnecessary.

3.1. Differential diagnosis of hyperthyroidism

The differential diagnosis of hyperthyroidism is essentially the discrimination of GD from non-autoimmune forms of hyperthyroidism, such as toxic multinodular goitre, solitary hyperfunctioning nodule, painless thyroiditis, iodine-induced hyperthyroidism or exogenous ingestion of thyroid hormones (30). The diagnosis of GD is generally based on the clinical presentation of the patient (“Merseburger Triad”). If there is doubt, the confirmation of GD can be easily and cost effectively achieved by the detection of TRAb. It is worth mentioning that costs are

lowest in those countries which have a long tradition of TRAb detection in clinical routine. For instance, costs for a TRAb determination using commercial assays amount to less than 10 Euro for the patient or insurance provider in Germany.

3.1.1. Graves' disease – a clinical diagnosis?

Like virtually all diseases, GD was originally diagnosed on the basis of clinical symptoms. This is often still possible, but only about 50% of patients develop the tell-tale sign of GO, and even in those patients, the ophthalmic symptoms do not always appear at the same time as hyperthyroidism (31). However, it is probably the legacy of the low sensitivity of early assays that GD is not yet perceived as a biochemical diagnosis on the basis of detectable TRAb. Nowadays, few laboratory tests have such high accuracy as second-generation TRAb assays, with specificity close to 100% and sensitivities for GD between 95% and 99%, depending on the study cohort (14-20). But, with the less sensitive assays of the past, the term "TRAb-negative GD" was introduced, which is an obvious oxymoron in the light of GD pathophysiology.

3.1.2. Problems with the "gold standard"

The term "TRAb-negative GD" is still used by thyroidologists, and although there may be rare cases of GD with TRAb levels below the detection limit of modern assays, the reason for this discrepancy is more likely to be based on the common practise that in studies examining the sensitivity and specificity of diagnostic tests the "correct diagnosis" is based on a clinical "gold standard" definition. A patient could be adjudicated by "gold standard diagnosis" to the group of GD, but the patient's blood does not contain detectable TRAb. Now, who is right? The "gold standard physician" or the biomarker? By definition, it is the "gold standard physician". However, often the differential diagnosis is not simple; for example, the difference between the diagnosis of "TRAb-negative uncomplicated GD with spontaneous remission after a few months' treatment with carbimazole" and the more "exotic" diagnosis of painless/silent thyroiditis. The latter disease simply does not exist in Germany outside the circle of a few well-read and internationally active thyroidologists. Thus, a "gold standard physician" in Germany might come to a different diagnosis compared to his Japanese colleagues, who seem to be very familiar with the discrimination of these two diseases (32).

3.1.3. Correcting the clinical diagnosis

With the advent of sensitive second-generation TBII assays the "biochemical" diagnosis of GD by TRAb determination seems to be catching up, and may even be more reliable than a diagnosis based on the clinical presentation in combination with ultrasound. A recent study re-evaluated the clinical diagnoses of those "GD patients with negative TBII" in a first-generation assay, and found that almost all of those patients were either TBII-positive in a second generation TBII assay, or had no GD in the first place, but non-autoimmune hyperthyroidism (20). Thus, a negative TRAb in a patient with the working diagnosis of GD should stress the need for further differential diagnosis.

3.1.4. Looking over the fence

One could argue that the measurement of TRAb to establish or confirm the diagnosis of GD would be absolutely mandatory if GD was a life-threatening disease, or if the treatment of GD was as complex and difficult as the treatment of cancer, diabetes or heart failure. The administration of chemotherapy to a patient with a benign tumour would be considered severe malpractice. But what about giving a standard antithyroid drug regimen of 18 months to a patient, who does not have GD, but silent thyroiditis?

Many physicians in other specialities would pay readily if they had a marker like TRAb, which confirms the diagnosis with such high accuracy at the costs of a few Euro. An almost "biomarker hype" can presently be seen in the field of cardiology, where cardiologists have taken on the endocrine heart, and hormones like brain natriuretic peptide and atrial natriuretic peptide (or their respective precursor fragments), are now increasingly being used for the diagnosis and prognosis assessment of heart failure (33), despite the higher costs, and although sensitivity/specificity figures are about as low as first-generation TRAb assays (34). Are cardiologists no longer able to diagnose heart failure based on clinical symptoms?

In other diseases with potentially fatal outcome, imperfect diagnostic procedures are well established: the Papanicolaou smear (area under the curve [AUC] 0.70) (35), or mammography (AUC 0.85) (36).

Taking the evidence into account and comparing it with other specialities, any thyroidologist who discards the use of TRAb for the diagnosis of GD has a very purist approach to their patient, which they might justify by the relative mildness of the disease, and the fact that a false diagnosis or treatment causes relatively little harm.

3.2. Prediction of relapse of GD after treatment

Whilst the use of TRAb for differential diagnosis of hyperthyroidism has a long tradition and clinical acceptance, a constant matter of discussion is the usefulness of TRAb for the prediction of relapse or remission in the follow-up of patients with GD.

3.2.1. Early results

Especially the use of first-generation porcine TBII was often questioned for this indication (37). Despite several reports on the benefit of TRAb determination for outcome prediction (38-40), a meta-analysis showed that although the absence of TRAb is a significant predictor of no relapse of GD after antithyroid drug treatment, as much as 25% of tested patients may be misclassified (41). Again, it helps to stand back a step and compare these results with prediction data of other diseases, and the usefulness of the corresponding “biomarker”. How useful are cholesterol or triglycerides in predicting cardiac events *in the individual patient*? Would anybody dare to question a cardiologist if they perform regular measurements of those biomarkers in their patients, despite the fact that the association of increased cardiovascular risk and elevated blood lipids is derived from large population studies, has no guaranteed predictive value for the *individual patient* and is still heavily disputed (42)? Unfortunately, we lack comparable studies with sufficient power, in our speciality, which would provide data to calculate the relative risk of relapse of patients with elevated TRAb. It is acknowledged that the severity of a cardiac event may be far more critical than a relapse of GD, but are thyroidologists not being too stringent in the application of laboratory data?

3.2.2. Duration of disease and antibody levels matter

The broad availability of second-generation TBII assays led to several studies that re-examined the predictive value of TRAb measurements. Looking at positive and negative TRAb values at the diagnostic cut-off (1.5 IU/L) showed no improved predictive value for relapse of hyperthyroidism (19). However, subsequent studies considered the severity of the autoimmune process and compared different decision thresholds for their usefulness in predicting remission or relapse. These studies stressed the importance of adjusting decision thresholds considering duration of disease and severity of the autoimmune response. Measurement of TRAb 6 months after initiation of treatment with antithyroid drugs, identified, in a *post hoc* analysis, those patients who subsequently relapsed (positive predictive value 97%) using a cut-off above 10 IU/L (43). Performing the same TRAb measurement after 12 months of treatment, the cut-off could be adjusted to 7.5 IU/L (positive predictive value 97%; odds ratio 24.3) (44). Finally, the same positive predictive value was obtained after 15 months of treatment at a TRAb decision threshold of about 4 IU/L (45).

Translating these results into the above example of cholesterol measurement in cardiology means that a low-density-lipoprotein (LDL) cholesterol value above a certain threshold would predict almost certain heart attack within the observation period of a few years. We all know that LDL cholesterol is far from such a prognostic value, but this does not prevent regular measurements, particularly in patients with risk factors or a history of cardiovascular disease. It is not my intention to stress this comparison too much, since the scientific evidence for the lipid hypothesis is certainly much stronger than the present data on the predictive power of TRAb measurement. Nevertheless, these studies suggest that TRAb measurement during antithyroid drug treatment may identify patients with a chance to benefit from a longer antithyroid drug therapy (those with TRAb <10 IU/L after 6 months), and identify those patients who will most likely not benefit (those with TRAb >10 IU/L), and for whom early definite therapy is a reasonable alternative. Furthermore, at

the termination of antithyroid drug treatment, TRAb measurements above certain cut-off points are useful in identifying those patients with a still active immune process, who will most likely not stay in remission.

3.3. TRAb and the eye – a new clinical indication?

GO is one of the three clinical cardinal symptoms of GD, and is arguably the most severe part of GD. Although severe GO with potential blindness of the affected eye is rare, patients with GD find even moderate GO very disturbing. Clinical decisions on the long-term treatment of patients with GO are sometimes difficult. Despite clinical examination and standard treatment regimens, if in doubt, ophthalmologists have no real GO outcome prediction or therapy stratification marker. Although there is increasing evidence that disturbed thyroid function and “risk factors” like smoking have a negative effect, these parameters have little practical value. In this context, a recent study examined the role of TRAb during the follow-up of patients with GO. In this retrospective study, these patients were followed over 2 years and were later grouped into mild- or severe-course GO (46). Both groups differed from each other significantly with respect to TRAb levels and autoantibody prevalence. This difference was evident over the entire 2-year follow-up period. Also, differences of TRAb levels between patients with mild and severe GO were substantial enough to draw conclusions concerning the disease management. Receiver-operating characteristic plot analysis revealed that prognostic decisions were possible at nearly all time points of the disease for about half of the patients. The calculated AUCs for decision cut-offs at different time points were between 0.82 and 0.87. Patients with GO with TBII values above certain cut-offs had a 8.7–31.1 times higher risk of a severe course of their GO (46). A multiple regression analysis combining several risk factors revealed that TBII, smoking behaviour and age all had significant influence on the course of GO. An increase in TBII levels of 1 IU/L increased the relative risk to develop a severe course of GO by 27% (46). If confirmed by other studies, the measurement of TRAb may be given an additional role in the monitoring of patients with GO.

4. Conclusion

Second-generation TBII assays with high clinical sensitivity and specificity are the method of choice for the detection of TRAb. They are widely available and cost efficient. Using these assays in clinical routine is helpful in the differential diagnosis of hyperthyroidism, since the presence of autoantibodies confirms GD, while their absence indicates a non-autoimmune origin of hyperthyroidism. Elevated TRAb are also helpful in the prediction of disease outcome during antithyroid drug treatment of patients with GD, since patients with TRAb >10 IU/L are likely to relapse after initial treatment with antithyroid drugs, and patients with TRAb >4 IU/L after completion of treatment. Recent work suggests that TRAb detection offers information for the disease management of patients with GO.

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NONGENOMIC ACTIONS OF THYROID HORMONE INITIATED AT THE PLASMA MEMBRANE OR IN CYTOPLASM

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ABSTRACT

Genomic actions of thyroid hormone are initiated with intranuclear binding of the hormone by heterodimeric nuclear thyroid hormone receptor (TR) proteins that are transcription factors. There is increasing recent experimental appreciation of the existence of nongenomic actions of the hormone that are initiated in cytoplasm or at the plasma membrane. Those that begin in cytoplasm may involve nuclear TRs now recognized to reside in cytoplasm and may alter the state of the actin cytoskeleton or foster insertion into the plasma membrane of the Na,K-ATPase; cytoplasmic action of the hormone via TR may also culminate downstream in the nucleus in the transcription of specific genes, such as *hypoxia-inducible factor-1 α* . A plasma membrane receptor for L-thyroxine (T₄) and 3, 5, 3'-triiodo-L-thyronine (T₃) has been described on integrin α v β 3. Among the complex mitogen-activated protein kinase (MAPK; ERK1/2)-mediated activities initiated at the integrin receptor are tumor cell proliferation and angiogenesis. Shuttling of TRs between cytoplasm and nucleus may be promoted by thyroid hormone via the integrin. The hormonal actions attributable to integrin-liganded T₄ or T₃ can be reproduced by hormone analogues tethered to agarose or to nanoparticles that prevent cell entry of the hormone. Tetraiodothyroacetic acid (tetrac) is an inhibitor of the actions of T₄ and T₃ at the integrin. Tetrac has anti-proliferative and antiangiogenic activities that are currently under exploration.

Key-words: integrin α v β 3; actin; tetraiodothyroacetic acid; phosphatidylinositol 3-kinase; mitogen-activated protein kinase; nanoparticles

Introduction

Mediated by intranuclear complexing of 3, 5, 3'-triiodo-L-thyronine (T_3) with a heterodimeric receptor from the nuclear superfamily of such receptors, genomic actions of thyroid hormone have been well-characterized (1, 2). Formation in the cell nucleus of receptor-ligand-accessory protein transcriptional complexes leads to changes in rates of expression of a number of specific genes (3, 4). The relatively constant thyroid hormone levels in tissues of intact euthyroid organisms means that thyroid hormone, particularly T_3 , is an important contributor via nuclear thyroid hormone receptors (TRs) to the basal rate of transcription of specific genes. That TRs need not be involved in certain actions of thyroid hormone was suggested by studies of the actions of thyroid hormone on enucleate cells (5, 6) and on nucleated cells that lack TR (7, 8). The actions of thyroid hormone on mitochondria are complex and may involve transcription, but certain of these effects are direct and do not require prior effects in the nucleus (9-11).

Nongenomic actions of thyroid hormone are those that do not require intranuclear interactions of TRs and thyroid hormone. They may be initiated in cytoplasm or at the plasma membrane. A number of laboratories have contributed to our understanding of such nongenomic effects. Nongenomic actions of the hormone include intracellular shuttling of TRs resident in cytoplasm to the nucleus (12-15), regulation of the state of the actin cytoskeleton (16), insertion of Na, K-ATPase into the plasma membrane (17) and modulation of its activity (18, 19) and regulation of specific gene expression, such as *hypoxia-inducible factor-1 α* (*HIF-1 α*) (20) and *ZAK1-4 α* (21). The foregoing actions are seen to be initiated by thyroid hormone in cytoplasm. They are largely T_3 -initiated, except for the hormonal effect on actin polymerization which is not sensitive to T_3 , but is regulated by L-thyroxine (T_4) and 3, 3', 5-triiodothyronine (reverse T_3 , rT_3) (16, 22). The fact that an event can be initiated in cytoplasm, such as activation of phosphatidylinositol 3-kinase (PI 3-K) by T_3 , and culminate in specific gene transcription (23) is an example of an interface between nongenomic and genomic hormone actions. There is increasing appreciation of such interfaces, as will be seen in the subsequent discussion.

The identification of a cell surface receptor for T_4 and T_3 has led to recognition of a number of complex cellular events that are under the control of iodothyronines. The cell surface receptor is at the Arg-Gly-Asp (RGD) recognition site on integrin $\alpha v \beta 3$ and the hormone signal (T_4 or T_3) at the integrin is transduced by mitogen-activated protein kinase (extracellular-regulated kinase 1/2; ERK1/2) into a proliferative response in certain tumor cells (24, 25) and into angiogenesis (8, 26, 27). $TR\beta 1$ and $TR\alpha$ shuttling from cytoplasm-to-nucleus can also be affected by the binding of T_4 or T_3 to the integrin receptor (28). There is also evidence that regulation of the activity of the Na^+/H^+ antiporter by T_3 may be directed from the plasma membrane integrin receptor (29). Very recently, the integrin receptor for thyroid hormone has been implicated by Yonkers and Ribera (30) in the regulation of the Na^+ current (I_{Na}) in excitable cells (sensory neurons). The full importance of this observation will not be clear until the action is confirmed in other nerve cells, glia and

myocardocytes. In the case of the latter, the hormonal effect on I_{Na} may be speculated to contribute of itself, or with attendant changes in intracellular K^+ metabolism, to myocardocyte irritability. Certain of these nongenomic actions of the hormone are described in **Table 1**.

Table 1. Selected non-genomic models of thyroid hormone action

Action	Model	Signal transduction pathways	Action of hormone analogues		Refs.	
			T ₄	T ₃		T ₄ /T ₃ + Tetrac
Sodium current	Zebrafish sensory neurons	ERK1/2	↑	0	Inhibition	28
Ca ²⁺ -ATPase activity	Human, animal erythrocytes, muscle cells	PKC	↑	↑	Inhibition	5, 6
Actin polymerization	Rat glial cells, neurons		↑	0 *		14, 29
Na ⁺ /H ⁺ antiporter	Rat myoblasts	ERK1/2	↑	↑ **		27, 35
Na, K-ATPase activity	Rat alveolar cells	ERK1/2, PI 3-K		↑		15, 16, 37
Protein trafficking cytoplasm to nucleus	Human, animal cells	ERK1/2	↑	↑	Inhibition	10, 11, 26, 31, 43
Angiogenesis	CAM, HDMEC, tumor xenografts	ERK1/2	↑	↑	Inhibition	8, 24, 25
Cancer cell proliferation	Human, animal tumor cells <i>in vitro</i> , xenografts	ERK1/2	↑	↑	Inhibition	22, 23, 43, 58
Specific gene transcription, e.g., <i>HIF-1α</i> , <i>ZAKI-4</i>	Human fibroblasts	PI 3-K		↑		18, 21

* Actions of T₄ that depend upon actin polymerization are inhibited by anti-integrin $\alpha v \beta 3$ and RGD peptide, consistent with involvement of the integrin receptor for thyroid hormone in such actions.

** Triac (triiodothyroacetic acid) was shown in these studies to inhibit the action of T₃ on the antiporter; tetrac was not tested.

Abbreviations and symbols. ERK1/2, extracellular-regulated kinase 1/2 (mitogen-activated protein kinase); PKC, protein kinase C; PI 3-K, phosphatidylinositol 3-kinase;

CAM, chick chorioallantoic membrane angiogenesis model; HDMEC, human dermal microvascular endothelial cell angiogenesis model

In Action of hormone analogues column, 0 = no effect, blank = effect not known, Inhib. = tetrac inhibits the action of T₄ or T₃

Farwell and Leonard and collaborators have also shown that the mobility of brain cells during brain development is regulated by T_4 and rT_3 (16, 31) and that this is dependent upon the nongenomic conversion of soluble to fibrous (F) actin that the same investigators had previously reported (16, 22). Neurite outgrowth is also stimulated by thyroid hormone. These effects are inhibited by anti-integrin and by RGD peptides that are known to interfere with the binding of thyroid hormone by the $\alpha v \beta 3$ receptor. These observations provide new insights into the critical role of thyroid hormone in brain development and have recently been reviewed (32).

Another interface of nongenomic and genomic effects of thyroid hormone is seen in the regulation of the state of phosphorylation of TR resident in the nucleus. Acting at the cell surface and via ERK1/2, T_4 can promote the phosphorylation of Ser-142 of TR β 1, leading to the shedding of corepressor protein and recruitment of coactivator (33, 34). The functional result is that the transcriptional state of TR is changed from repressed to basal without the entry of the iodothyronine into the cell. Full transcriptional activity of the receptor is seen to require access of T_3 to the nucleus and binding of nuclear T_3 by the phosphorylated TR. It would appear in this case of hormone action that there is not only an interface between nongenomic and genomic effects, but perhaps between T_4 and T_3 , without the conversion of T_4 to T_3 .

As in the case of genomic effects, the nongenomic actions of thyroid hormone are not 'acute' or 'rapid onset' in the intact organism because of the relative constancy of ambient thyroid hormone levels. The nongenomic effects may be described as acute or rapid onset in the experimental context in which they are described, e.g., the thyroprival cell or tissue newly re-exposed to T_3 or T_4 . In the intact organism, however, the actions are seen as modulators of basal activity of a cellular function.

This review will briefly examine certain features of the nongenomic effects of thyroid hormone enumerated above.

Actions of Thyroid Hormone on Na^+ current (I_{Na}) and on the Na^+/H^+ antiporter

Thyroid hormone was shown by Dietzel and co-workers (35, 36) to modulate neuronal excitability via I_{Na} , but it was not clear until the report of Yonkers and Ribera in 2008 (30) that this effect of the hormone was 1) nongenomic in mechanism and 2) mediated by the integrin receptor for thyroid hormone. Among the results in neurons of the I_{Na} effect is an increased firing frequency of action potentials. Total T_4 concentration in these studies was physiologic (10^{-7} M) and a supraphysiologic level of T_3 did not affect I_{Na} . Assuming a similar change occurs in whole myocardiocyte I_{Na} , this effect may contribute to firing rate of the cells. However, a change in intracellular Na^+ induced by iodothyronines will be coupled to alterations in the outward K^+ channel and other ionic functions. The latter may include Na, K-ATPase and Na^+/Ca^{2+} exchange. It will be the algebraic sum of the coupled responses that will determine changes in rate or rhythm and

whether this nongenomic mechanism may be implicated in rhythm or rate changes that are attributed clinically to thyroid hormone.

Incerpi et al. have identified nongenomic effects of T_3 on the Na^+/H^+ antiporter (exchanger) (29, 37). The functional significance of the action of the hormone was shown by an increase in pH in the myoblasts studied and by shortened recovery time after administration of an acid load to the cells (37). The concentration of T_3 used in these experiments approximated physiologic (10^{-11} M). T_3 -agarose does not gain access to the interior of the cells and was as effective as unmodified T_3 in activating the exchanger. Transduction of the T_3 signal is complex in these muscle cells and involves ERK1/2, as well as other kinases, and intracellular Ca^{2+} release. That the hormonal effect may be initiated at integrin $\alpha v \beta 3$ was suggested by the inhibition of T_3 action by triiodothyroacetic acid (triac) (29). Tetraiodothyroacetic acid (tetrac) is an inhibitor of binding of T_4 to the integrin receptor for thyroid hormone (8), as is triac assumed to be (29). The setpoint of the activity of the antiporter is important to the ability of muscle cells to maintain intracellular pH in the face of acid-loading that accompanies exercise or ischemia. It is assumed that T_3 is a contributor to this setpoint.

Thyroid Hormone and Na, K-ATPase

The genomic regulation of *Na, K-ATPase* expression by T_3 was described by Chaudhury et al. (38). Secure support for nongenomic stimulation by T_3 of plasma membrane Na,K-ATPase activity and for nongenomic regulation by the hormone of the insertion of sodium pump subunits into the plasma membrane of rat pulmonary alveolar cells has been provided by Lei et al. (17, 18, 39). T_3 was effective in these studies at 10^{-9} M or higher concentrations. Because 20% of the basal energy consumption of muscle at rest is dedicated to Na^+ and K^+ transport (40), the contribution of thyroid hormone to the setpoint of Na, K-ATPase activity is metabolically important. Further, intracellular Na^+ concentration ($[Na^+]_i$) is a predictor of ventricular fibrillation in the hypoxic myocardium (41) and, through its actions on Na^+ current and Na, K-ATPase activity, thyroid hormone is a regulator of $[Na^+]_i$.

The molecular basis of control by T_3 of Na, K-ATPase activity has been shown to involve Src kinase and transient activation of ERK1/2 (18), then, downstream of ERKs, the activation of PI 3-K.

Thyroid Hormone and Intracellular Protein Shuttling

When studied by confocal microscopy, a green fluorescent protein (GFP)-nuclear thyroid hormone receptor TR β 1 chimera (12, 13) has been shown to reside in cytoplasm, as well as in the nuclear compartment. Shuttling of GFP-TR β 1 from cytoplasm to nucleus is promoted nongenomically by T_3 at 10^{-6} M (13) or 10^{-7} M (12) concentrations. It has also been shown in cell fractionation studies that the thyroid hormone-directed transfer of TR β 1 from cytoplasm into the

nucleus is associated with serine phosphorylation of the receptor (32, 33). TR β may also reside in cytoplasm and may translocate to the nucleus in T₄-treated cells (28, 42). It is now clear that nuclear thyroid hormone receptors located in cytoplasm are functional. For example, cytoplasmic T₃-liganded TR β 1 is involved in PI 3-K-mediated enhancement of ZAKI-4 α synthesis (21). In this case, the T₃-TR β 1 complex interacts with the p85 regulatory subunit of PI 3-K. A truncated isoform of TR α (TR $\Delta\alpha$ 1) in cytoplasm is involved in the action of T₄ on the actin cytoskeleton (32). Nuclear estrogen receptor (ER)- α in cytoplasm or at the plasma membrane is functional when extranuclear in location (43, 44).

It is also apparent that thyroid hormone causes translocation of cytoplasmic ER α into the cell nucleus (45), as well as cytoplasm-to-nucleus shuttling and retention of the oncogene suppressor protein, p53 (46), and of signal transducer and activator of transcription (STAT)-1 α (47). In each of these three cases the thyroid hormone-driven translocation is associated with activated ERK1/2-mediated specific serine phosphorylation of the proteins and attendant changes in function. Chen and co-workers (48) have shown that T₃ promotes the transfer of Trip230, a TR coactivator, from the Golgi apparatus to the cell nucleus. Thus, ambient levels of thyroid hormone in the intact organism appear to facilitate by intracellular protein trafficking the actions of diverse factors, including thyroid hormone, itself, estrogen, interferon- γ (via STAT1 α [47]) and p53.

Thyroid Hormone and *HIF-1 α* Gene Expression

Moeller et al. have shown that T₃ (10⁻⁹ M) acting via PI 3-K, nongenomically induces *HIF-1 α* expression (20). The gene product is a transcription factor important to the hypoxic stress response of cells, but under normoxic conditions can be induced by growth factors and cytokines whose actions are receptor-mediated, as well as by oncogenes (49). HIF-1 α transcribes as many as 40 genes. Included among the latter are genes relevant to carbohydrate metabolism, such as *glucose transporter-1 (GLUT-1)* and *phosphofructokinase*, and to angiogenesis, cell proliferation and cell migration (50). The factor is regarded as a mediator of apoptosis-resistance (51). Thus, the action of an iodothyronine on *HIF-1 α* gene expression is an attractive target in cancer cells. STAT-3 is a signal transducing factor that is necessary for *HIF-1 α* expression in cancer cells (52) and that been shown, like STAT1 α , to be activated by thyroid hormone (47). Whether thyroid hormone-directed activation of STAT3 does contribute to *HIF-1 α* expression is not yet known.

It should also be noted that T₃ can cause *HIF-1 α* gene expression by a genomic mechanism. That is, the intranuclear complex of T₃ with the nuclear receptor heterodimer of TR β and retinoid x receptor (RXR) leads to *hepatic leukemia factor* expression and consequent transcription of *HIF-1 α* (53). The T₃-TR β -RXR complex apparently does not directly transcribe *HIF-1 α* .

Thyroid Hormone and Angiogenesis

That thyroid hormone is pro-angiogenic has been reported by several laboratories (26, 54, 55). It is now appreciated that the molecular basis for the initiation of the angiogenic response to iodothyronines is nongenomic. That is, acting via the thyroid hormone receptor on integrin $\alpha v \beta 3$, T_4 and T_3 can induce angiogenesis experimentally in the chick chorioallantoic membrane (CAM) assay (26, 56) and in the human dermal microvascular endothelial (HDMEC) sprouting system (57). Integrin $\alpha v \beta 3$ is regularly expressed on the surface of endothelial cells and vascular smooth muscle cells (58). In studies of the contribution of T_4 to angiogenesis via the integrin receptor, the concentration of free T_4 is 10^{-10} M (8).

The angiogenic response to thyroid hormone requires ERK1/2 activation (26) and appears to involve release of cellular basic fibroblast growth factor (bFGF) (26) and perhaps vascular endothelial growth factor (VEGF) (SA Mousa, FB Davis, PJ Davis: unpublished observations) that act in an autocrine fashion to promote neovascularization. Added to the medium in which the CAM is based, anti-bFGF reduces the effect of thyroid hormone on vascular buds (26). This supports an application for certain thyroid hormone agonist analogues at the integrin receptor, e.g., diiodothyropropionic acid (DITPA), as angiogenic agents in ischemic tissues. T_4 and T_3 are proangiogenic, but T_4 aggregates platelets (59) and T_3 may have proarrhythmic actions which are undesirable if coronary artery angiogenesis is to be promoted by a thyroid hormone analogue. DITPA lacks both of these undesirable qualities of T_4 and T_3 , but is pro-angiogenic (27).

Tetrac interferes with iodothyronine-binding to the integrin receptor (8) and blocks the pro-angiogenic action of the hormone (8, 26, 27). It was somewhat surprising to find that tetrac also inhibited the angiogenic effects of VEGF and bFGF in the absence of thyroid hormone (60). This action is proposed to involve interruption by tetrac of pro-angiogenic crosstalk between the RGD recognition site of the integrin and the adjacent (clustered) vascular growth factor receptors. In studies of human cancer cell xenografts in immunocompromised mice, the vascular supply of the xenografts has been reduced by systemic administration of tetrac (61).

Thyroid Hormone and Tumor Cell Proliferation

A number of human and animal cancer cell lines have been shown to proliferate *in vitro* in response to physiologic concentrations of thyroid hormone and to do so via the cell surface receptor for the hormone and downstream ERK1/2 activation. Among the tumor cells susceptible to the proliferative effect of thyroid hormone are breast cancer cells (45), glioma cells (25) and thyroid cancer (24). Integrin $\alpha v \beta 3$ is regularly expressed in tumor cells and mediates the cancer cell proliferation activity of iodothyronines. Tetrac inhibits integrin receptor-initiated effects of thyroid hormone on tumor cells. Growth of xenografts of human breast cancer and thyroid cancer are thyroid hormone-dependent, as inferred by the effectiveness of tetrac as an anti-proliferative—and, as noted above, anti-angiogenic—agent in this mouse model (SA Mousa, FB Davis, PJ Davis:

unpublished observations). Tetrac may also increase the intracellular residence time in cancer cells of conventional chemotherapeutic agents (62).

Thyrotropin (TSH)-dependence of thyroid cancers is a more important feature of these cancers than is a putative contribution of thyroid hormone; this is an important issue because of the routine use of exogenous T₄ to suppress endogenous TSH in thyroid cancer patients. Thyroid cancers may recur as apparently TSH-independent lesions and in this setting it is possible that the proliferative action of thyroid hormone is expressed. Recent clinical observations suggest that concurrent hypothyroidism retards the growth of certain tumors, e.g., breast cancer (63), glioblastoma (64) and head and neck squamous cell cancer (65). However, it is not clear that this effects reported reflect actions of the hormone that are nongenomic, genomic or both.

Thyroid hormone has been shown to be anti-apoptotic in several cancer cell lines in which the pro-apoptotic action of a stilbene, resveratrol, has been demonstrated (24, 66). The molecular basis of the anti-apoptotic effect of the hormone is interference with the complexing of activated nuclear ERK1/2 with other nucleoproteins involved in p53-dependent apoptosis that is induced by resveratrol (66).

There are several other mechanisms by which thyroid hormone may support tumor growth. One of these mechanisms is tumor-related angiogenesis, as discussed above. A second mechanism may be the action of the hormone on the Na⁺/H⁺ antiporter. This effect of the hormone protects the cell against acidosis, one effect of which is activation of endonucleases that degrade DNA and participate in apoptosis (67). Such an action can be viewed to contribute to the anti-apoptotic effect of iodothyronines. The activity of multi-drug resistance (MDR) pumps in the cancer cell plasma membrane is associated with alkaline cytosolic pH and the pumps contribute to chemoresistance of cancer cells. However, it is not clear that the Na⁺/H⁺ exchanger—and thus thyroid hormone—contributes to this MDR pump-associated intracellular pH elevation (68). A third mechanism by which thyroid hormone may support the tumor cell is stimulation by the hormone of cell migration. Boyden chamber studies by SA Mousa et al. (unpublished observations) have shown that migration of glioma cells toward a vitronectin cue is enhanced by thyroid hormone.

Conclusions

Nongenomic effects of thyroid hormone are now understood to underlie a number of complex cellular behaviors that involve transcriptional events, as in the cases of hormone-stimulated cell division or transcription of specific genes. A number of plasma membrane ion pump or channel activities—such as I_{Na}, Na⁺/H⁺ antiporter and Na, K-ATPase—are modulated via nongenomic mechanisms by thyroid hormone. In the case of the plasma membrane receptor for thyroid hormone, it has been possible to design thyroid hormone analogues that act exclusively at this site and do not enter the cell. The receptor is predominantly expressed on cancer cells and endothelial and vascular smooth muscle cells. This conceivably permits the clinical manipulation

of hormone actions on angiogenesis and tumor cell division, without affecting the metabolic effects of the hormone or its principally genomic actions. Medicinal chemistry may also permit modification of the hormone to create analogues that act within the cell, but do not gain access to the nuclear compartment. Specific actions of the hormone on the cytoskeleton or on Na, K-ATPase, for example, may be achievable.

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