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International Federation of Clinical Chemistry and Laboratory Medicine

## The 9th EFCC Continuous Postgraduate Course in Clinical Chemistry

# NEW TRENDS IN CLASSIFICATION, DIAGNOSIS AND MANAGEMENT OF THYROID DISEASES

**Dubrovnik, October 24-25, 2009, Croatia**

European Federation of Clinical Chemistry and Laboratory Medicine

Croatian Society of Medical Biochemists

Slovenian Association for Clinical Chemistry

**For all participants registration desk will be open in Inter-University Centre Dubrovnik, Don Frana Bulića 4 Saturday, October 24, from 8:15 to 9:00**

Inter-University Centre Dubrovnik

*The 9th EFCC Continuous Postgraduate Course in Clinical Chemistry*

**Under the Auspices of IFCC**

# NEW TRENDS IN CLASSIFICATION, DIAGNOSIS AND MANAGEMENT OF THYROID DISEASES

Handbook

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*Croatian Society of Medical Biochemists*
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**The Eight EFCC Continuous Postgraduate Course in Clinical Chemistry:**
**New Trends in Classification, Diagnosis and Management of Thyroid Diseases**

The Croatian Society of Medical Biochemists and Slovenian Association for Clinical Chemistry, together with the European Federation of Clinical Chemistry and Laboratory Medicine (EFCC), have again organized this ninth postgraduate weekend course under the auspices of IFCC. The Course entitled "New Trends in Classification, Diagnosis and Management of Thyroid Diseases" promotes continuing postgraduate education of professionals in clinical chemistry and laboratory medicine, and ensures the laboratory knowledge harmonization.

In this Course the state-of-the-art on thyroid physiology, pathophysiology as well as new approach to diagnosis and management will be presented by well-known experts. The integrated knowledge of the authors and the material prepared by these experts especially for this course, is intended to provide updated information of supreme quality to the reader. Renowned experts in different fields have tried to cover the clinical and laboratory aspects of thyroid diseases.

We hope that all those attending the Course, will have an excellent opportunity to acquire new knowledge and exchange experience in the field.

*Ana-Maria Šimundić*

*Zagreb, October 2009*

# 1. THYROID HORMONE SYNTHESIS, STORAGE AND RELEASE

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## 1.1 Thyroid gland

The thyroid gland consists of two lobes (left and right) connected by a thin, median isthmus forming a "butterfly" shape. It is located in the neck, in front of the trachea, just below the larynx, weighs 15-20g in the adult. This is highly vascularized organ with blood flow about 5mL/g/min of tissue. Although the thyroid represents about 0.4% of body weight it accounts for 2% of total blood flow. The gland receives fibers from both sympathetic and parasympathetic divisions of the autonomic nervous system. The sympathetic fibers are derived from the cervical ganglia and enter the gland along the blood vessels. The parasympathetic fibers are derived from the vagus and reach the gland by branches of the laryngeal nerves.

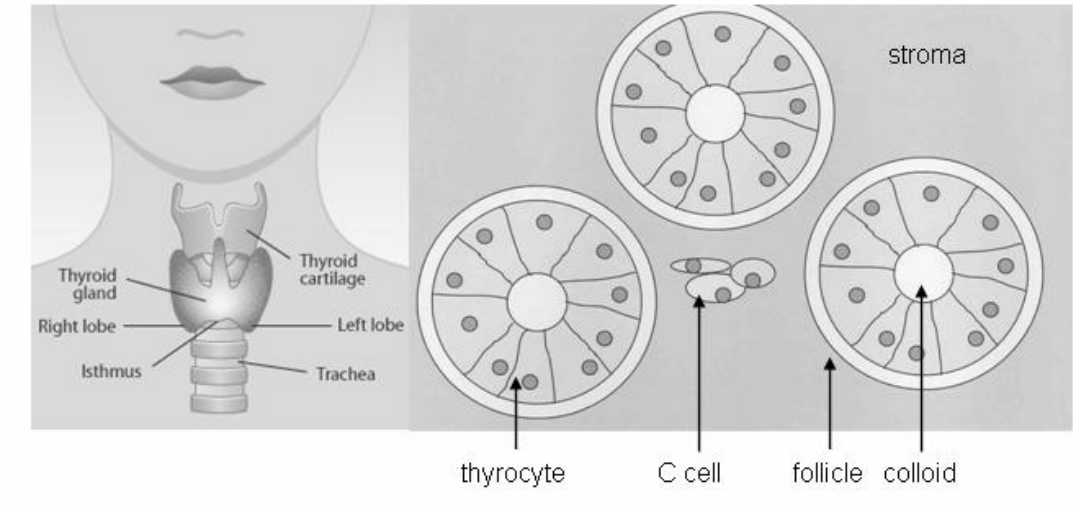


***Figure 1.1.*** *Structure of the thyroid gland.*

The gland consists of thousands of follicles, each a spheroidal sac of epithelial cells (thyrocytes) surrounding a lumen containing colloid, a depot of thyroid hormone precursor, thyroglobulin. The average diameter of follicle is 300 microns. The epithelium of the normal gland is usually described as cuboidal, with the apical plasma membrane (facing the follicle lumen) and the basolateral plasma membrane on opposite site. The most conspicuous structural difference between the apical and basolateral cell surface is that only former is furnished with microvili (pseudopods). Cytochemical studies show differences in enzyme components between the two domains, e.g. presence of peroxidase, aminopeptidase and $H_2O_2$-genereting activity only at the apical surface and $Na^+/K^+$ ATPase only at the basolateral surface.

The thyroid gland secretes three hormones: thyroxine (T4) and triiodothyronine (T3), both of which are iodinated derivatives of tyrosine, and calcitonin, a polypeptide hormone. T4 and T3 are produced by the follicular cells but calcitonin is secreted by the C (parafolicular) cells, which are of separate embryological origin. Calcitonin is functionally unrelated to the other thyroid hormones. It has a minor role in calcium homeostasis.

## 1.2 Biological actions of thyroid hormones

Thyroid hormones (THs) are essential for normal growth and development and stimulate metabolism in most tissues (adult brain is a conspicuous exception).

THs increase mitochondrial oxidative phosphorylation and maintain amino acid and electrolyte transport into cells. They increase calorigenesis and oxygen consumption in most tissues. THs stimulate the synthesis of proteins that can be structural proteins or enzymes. They regulate carbohydrate metabolism, accelerating insulin degradation and increasing gluconeogenesis. Stimulation of lipid metabolism leads to a fall cholesterol concentration in plasma. THs also increase the sensitivity of the cardiovascular and nervous system to catecholamines, the former leading to increases in heart rate and cardiac output, and the latter to increased arousal.

Most of the actions of THs are exerted through modulation of gene expression. They enter into the cells and act by binding to specific receptors in the nuclei, where they stimulate the synthesis of a variety of species of mRNA, thus stimulating the synthesis of proteins, including enzymes and hormones. T3 exerts its effects through interaction with nuclear thyroid receptors (TR) that have a high affinity and high specificity for T3. These receptors belong to the family of ligand-regulated transcription factors that are associated with chromatin. The formation of the T3-TR/DNA complex and subsequent recruitment of a variety of transcriptional coactivators leads to activation of the target genes, giving increased mRNA and protein production.

## 1.3 Synthesis, storage and release of thyroid hormones

The thyroid gland produces two related hormones, 3,5,3',5'-tetraiodothyronine (thyroxine; T4) and 3,5,3'-triiodothyronine (T3). The major product of the thyroid gland is T4 (approximately 90%). Most T3 (more than 80%) is derived from T4 by deiodination in peripheral tissues (liver, kidneys, muscle). In target cells, most of the effect of T4 results from this conversion to T3. Deiodination can also produce 3,3',5'-triiodothyronine (reverse T3; rT3) which is physiologically inactive.

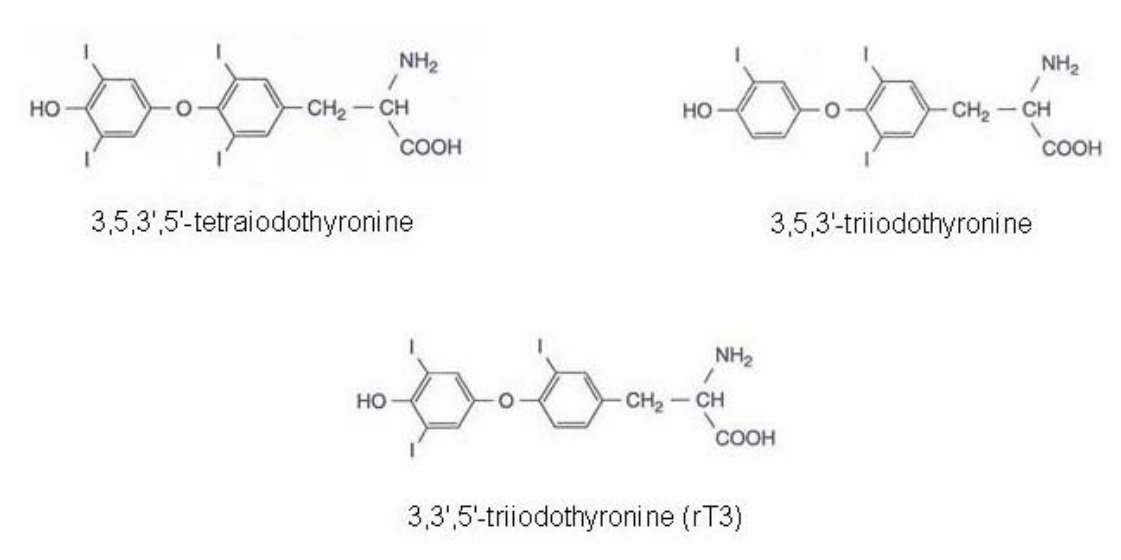


***Figure 1.2.*** *Chemical structure of the thyroid hormones*

The synthesis of these hormones requires the amino acid thyrosine and the trace mineral iodine. The production of these iodinated amino acids begins with the synthesis of thyroglobulin that is posttranslationally modified in a series of biochemically unique reactions. Within thyrocytes, iodide is oxidized to iodine. This reaction is catalyzed by enzyme thyroperoxidase (TPO) in the presence of hydrogen peroxide ($H_2O_2$). Iodine then binds to 3' position in the tyrosyl ring, a reaction yielding 3-monoiodotyrosine (MIT). A subsequent addition of another iodine to 5' position of the tyrosyl residue on MIT creates 3,5-diiodotyrosine (DIT). T4 is created by the condensation or coupling of two DIT molecules. Smaller amounts of DIT within the thyroid can also condense with MIT to form either T3 or rT3. All these biosynthetic processes within the thyroid gland are controlled by feedback mechanism within the hypothalamic-pituitary-thyroid axis.

### 1.3.1 Iodine metabolism in thyroid gland

Iodine plays a central role in thyroid physiology, being both a major constituent of thyroid hormones and a regulator of thyroid gland function. Thyroid gland concentrates iodide ($I^-$) against an electrochemical gradient by a carrier-mediated mechanism driven by ATP and is under the control of thyroid-stimulating hormone (TSH; thyrotropin). All of the subsequent steps in biosynthesis of thyroid hormones, from oxidation and organification of iodide to the secretion of T4 and T3 into the circulation, are stimulated by TSH and inhibited by excess iodine.

TH biosynthesis requires iodide uptake into the thyrocytes and efflux into the follicular lumen, where it is organified. Uptake of iodide into the thyrocytes is mediated by an intrinsic membrane glycoprotein, the sodium-iodide symporter (NIS), which cotransports two sodium cations per each iodide anion. NIS-mediated transport of iodide is driven by the electrochemical sodium gradient generated by the Na+/K+ - ATPase. TSH and iodide regulate iodide accumulation by modulating NIS activity.

In order to attain normal levels of TH synthesis, an adequate supply of iodine is essential. The recommended intake of iodine is 50 mg a year or 1 mg a week or 150 μg a day.

In thyroid gland iodine pump on basolateral plasma membrane can concentrate ions of iodine 30 times more than in blood. During the maximum activity of gland the concentration rises up to 250 times more than in blood. Mild increase of iodine concentration in circulation leads to higher concentration of ions in thyroid gland and increases production of THs. High increase of iodine concentration in blood, uptake of iodine higher than 2 mg/day, leads to much higher concentration of ions in thyroid gland and decrease the biosynthetic processes within the gland. This autoregulatory phenomenon is known as Wolf-Chaikoff effect which inhibits formation of thyroid hormones inside of the thyroid follicle.

At the apical membrane, pendrin, a protein involved in anion transport and apical iodide transporter (AIT), mediates iodide efflux from thyrocyte to colloid.

### 1.3.2 Thyroglobulin

The thyroid hormones, T4 and T3 are relatively simple molecules that are formed in a giant prohormone molecule, thyroglobulin (Tg). In its major form, Tg is a 660 kDa dimeric glycoprotein, composed of two identical subunits. A molecule of Tg contains 140 tyrosines, although only around 20% of these are actually used to synthesize T4 and T3. Tg is produced by thyrocytes and follows the usual biosynthetic pathway. It is synthesized and initially processed in the endoplasmatic reticulum with the formation of dimers and with addition of N-linked glycoside residues. Further is processed in the Golgi apparatus, especially by modification of carbohydrate residues. Tg is transported via vesicles from the trans-Golgi network to the apical surface of thyrocytes and released into the lumen of thyroid follicles where it is stored in colloid as the major component (>95%). Heterogeneity of Tg within the colloid is not restricted to its iodine and carbohydrate content. Thus, although the predominant form of Tg is the 660 kDa, free 330 kDa monomers can be found in minimal amounts. Reduction or degradation of both can lead to the formation of smaller polypeptides usually in trace amounts in the colloid. Tg is posttranslationally modified in a series of biochemically unique reactions.

### 1.3.3 Formatting of thyroid hormones in molecule of thyroglobulin

Formation of THs within the Tg molecule occurs at the cell-colloid interface by coupling of tyrosyl residues of Tg with iodide. Iodination of tyrosines on Tg, also known as "organification of iodide" is carried out by TPO. This reaction results in either MIT or DIT being incorporated into thyroglobulin. The other synthetic reaction is a coupling reaction where iodotyrosine molecules are coupled together. If two DIT molecules couple together, the result is the formation of T4. If MIT molecule and DIT molecule are coupled together, the result is the formation either T3 or rT3. THs accumulate in colloid, on the surface of the thyroid epithelial cells, still tied up in molecules of thyroglobulin. THs have to be liberated from the Tg and secreted as a free hormones into the blood.

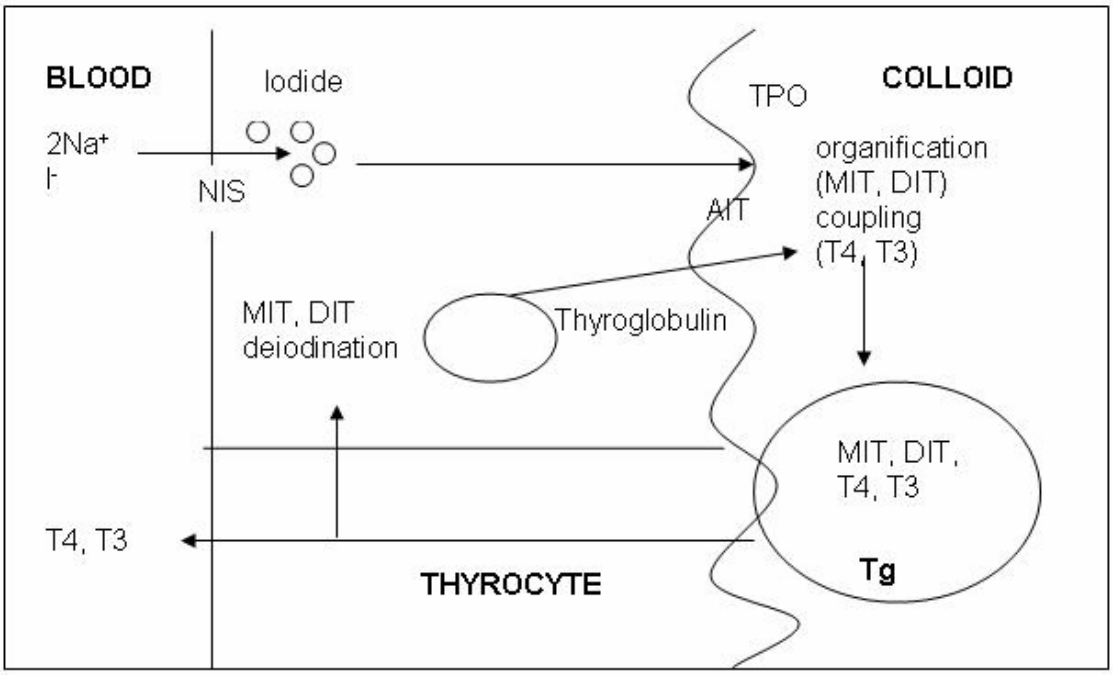


***Figure 1.3.*** *Biosynthesis of the thyroid hormones. Uptake of iodide into the thyrocyte is mediated by the sodium-iodide symporter (NIS). Apical iodide transporter (AIT) mediates iodide efflux to colloid. Organification of iodide is carried out by thyroperoxidase (TPO). This reaction results in formatting MIT or DIT into thyroglobulin. Coupling two molecules of MIT gives T4 and coupling MIT and DIT molecules gives T3.*

There are several steps at the end of hormone synthesis. Thyroid epithelial cells ingest colloid that contains thyroglobulin molecules. Uptake of Tg by thyrocytes occurs by micropinocytosis, which can be either nonspecific (fluid phase) or receptor mediated. Both forms of micropinocytosis, also called endocytosis or vascular internalization, involve the formation of small vesicles at the apical membrane, which invaginate to form intracellular vesicles that fuse with lysosomes. Nonspecific endocytosis is a constitutive process. In contrast, receptor-mediated endocytosis involves specific binding of certain substances to cell surface receptors, with high or low-affinity. Several receptors have been proposed on the apical surface, where they could mediate endocytosis, or on intracellular membranes, where they might influence intracellular trafficking. Megalin is a 600 kDa cell surface protein expressed on the apical surface of a restricted group of absorptive epithelial cells of the human body including the renal proximal tubule cells, epididymal cells, type II pneumocytes and thyroid epithelial cells. Tg binds to megalin in solid-phase assay, with characteristics of high-affinity receptor-ligand interactions. This receptor probably has function in a process of endocytosis and transcytosis. Under physiological conditions megalin expression on thyrocites is relatively low but under conditions of intense TSH stimulation there is increase of megalin expression on thyrocytes. A thyroid asialoglycoprotein receptor may internalize and recycle immature forms of Tg back to the colloid. There is also evidence of low-affinity receptors on thyrocytes, but their role in Tg uptake is not finally established. Finally, there are several principal intracellular pathways of Tg after endocytosis by thyrocytes: a) Tg is internalized by fluid-phase nonspecific micropinocytosis and transported to lysosomes, where Tg is degraded and THs are released; b) Tg is internalized by an unidentified low-affinity receptor and possibly transported to lysosomes; c) Tg is internalized by a receptor (possible the asialoglycoprotein receptor) and recycled back into the colloid; d) Tg is internalized by megalin and transported by transcytosis at the basolateral surface where Tg is released by exocytosis into blood.

### 1.3.4 Release of T4 and T3 from thyroid gland

Thyroid epithelial cells ingest colloid by endocytosis from their apical membrane. Colloid contains thyroglobulin molecules that consist of MIT, DIT, T3 and T4. Colloid-laden endosomes fuse with lysosomes, which contain proteolytic enzymes that digest Tg, thereby liberating THs. Free THs apparently diffuse out of lysosomes, through the basal plasma membrane of the cell into blood where they bind to carrier proteins for transport to target cells. Proteolysis also results in the liberation of MIT and DIT that are usually degraded within thyroid follicular cells and their iodine is retained and re-utilized. A small amount of thyroglobulin also reaches the bloodstream.

The major product of the thyroid gland is T4. T3 is produced 10 times less but most T3 is derived from T4 by deiodination in peripheral tissues, liver, kidneys and muscle, catalysed by deiodinases. T3 is 3-4 times more potent than T4. In tissues, most of the effect of T4 results from this conversation to T3, so that T4 is a prohormone. Deiodination can also produce rT3, which is physiologically inactive. The majority of the activation of the prohormone T4 to the T3 occurs through non-thyroidal deiodination. Three deiodinase families are recognized and are termed as isoforms type I, II and III. Type I deiodinase is the major enzyme in the liver and kidneys. Type II enzyme is found in the heart, skeletal muscle, central nervous system, fat and thyroid. Type III deiodinase isoform is found in fetal tissue and placenta. Further degradation of rT3 and T3 results in the formation of several distinct diiodothyroxines (T2). The metabolic role of the T2 isomers is poorly understood and is unclear in humans.

When T4 is released from the thyroid, it is primarily in a bound form with thyroxine-binding globulin (TBG), with lesser amounts bound to thyroxine-binding prealbumin (TBPA) and albumin. Only 0.03-0.05% of T4 within the circulatory system is in a free (unbound) form (fT4). In peripheral tissues, T4 is either converted to T3 or rT3, or eliminated. The half-life of T4 is 5-7 days.

T3 is considered to be the most metabolically active thyroid hormone. Although some T3 is produced in the thyroid, approximately 80% is generated outside the gland, primarily by conversion of T4 in the liver and kidneys. The nervous system is capable of converting T4 to T3. Majority of circulating T3 is in a bound form. TBPA and albumin, not TBG, are the binding proteins with high affinity for T3. Approximately 0.2% of T3 is in unbound or free form (fT3) in normal subjects. The half-life of T3 is 1-2 days.

The normal plasma concentrations of T4 and T3 are 60-150nmol/L and 1.0-2.9nmol/L, respectively. Both hormones are extensively protein bound, some 99.98% of T4 and 99.66% of T3 are bound principally to a specific TBG and, to a lesser extent, to prealbumin and albumin.

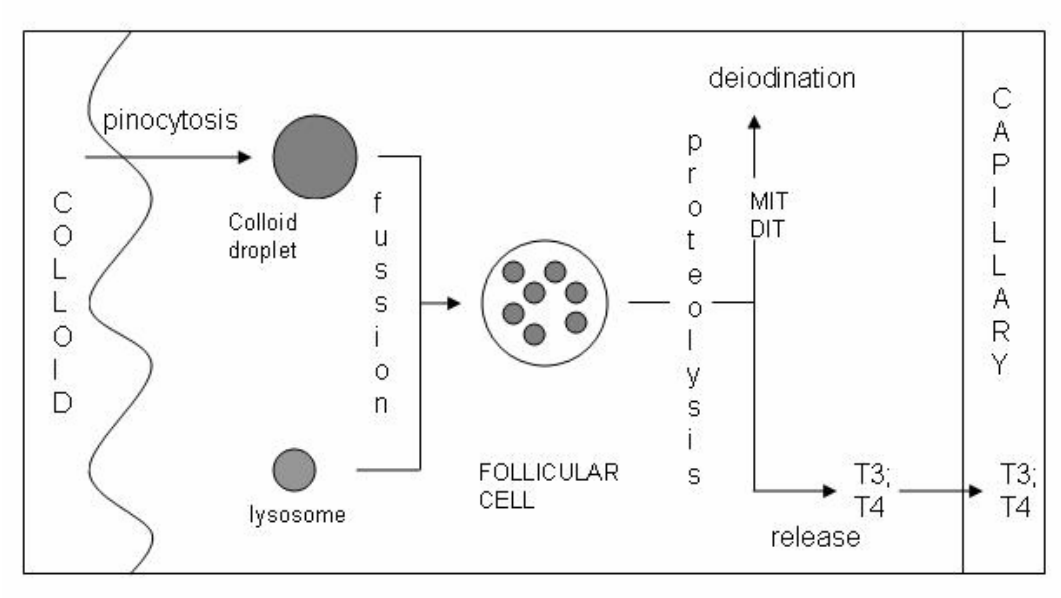


***Figure 1.4.*** *Release of T4 and T3 from thyrocyte. Tg molecule in colloid is taken up into thyrocyte by pynocytosis. Colloid droplet fuses with lysosome and undergoes lysosomal proteolysis, resulting in the release of T4 and T3 and deiodination of MIT and DIT.*

## 1.4 Control of THs synthesis and secretion

The most important regulator of thyroid homeostasis is thyroid-stimulating hormone (thyrotrophin; TSH). The secretion of TSH is controlled by negative feedback by the THs hormones, which modulate the response of the pituitary to the hypothalamic hormone, thyrotrophin-releasing hormone (TRH, thyroliberin). The feedback mechanisms result in maintain of steady plasma concentrations of THs.

TSH is glycoprotein hormone composed of alpha and beta subunits which are non-covalently bound to one another. Free alpha and beta subunits have essentially no biological activity. TSH is secreted from cells called thyrotrophs in the anterior pituitary gland. TRH is major controller of TSH secretion. TRH is secreted by hypothalamic neurons into hypothalamic-hypophyseal portal blood and through its receptors on thyrotrophs stimulates secretion of TSH. Secretion either TRH or TSH hormones is inhibited by high blood concentrations of free THs in a classical negative feedback loop. The basic mechanisms for control in this system are: hypothalamus neurons secrete TRH which stimulates pituitary gland to secrete TSH; TSH stimulates thyroid gland to secrete THs; when blood levels of THs increase above a certain threshold TRH secretion is inhibited; inhibition of TRH secretion leads to inhibition of TSH secretion; inhibition of TSH secretion leads to inhibition of THs secretion in blood.

Binding of TSH to receptors on thyroid epithelial cells (TSHR) seems to enhance all of the processes necessary for synthesis of THs, including synthesis of iodide transporter, thyroid peroxidase and thyroglobulin. High concentrations of TSH lead to faster rates of endocytosis and THs release into the blood. Conversely, when TSH levels are low, rates of thyroid hormone synthesis and release are diminished.

TSH modifies THs synthesis by binding to a specific TSHR on the basal membrane of the thyrocyte. TSHR is a single protein with a large extracellular domain involved in the binding

of TSH, seven transmembrane domains and a short intracellular domain involved in activation of G-protein modulators of the adenylate cyclase-protein kinase-A system. Binding of TSH results in activation of adenylate cyclase and accumulation of cAMP. The calcium and phosphoinositol signalling pathways may also be activated by TSH. TSH increases release of THs, synthesis of iodinated thyroglobulin and also causes a general increase in the metabolism, size and activity of the follicular cells.

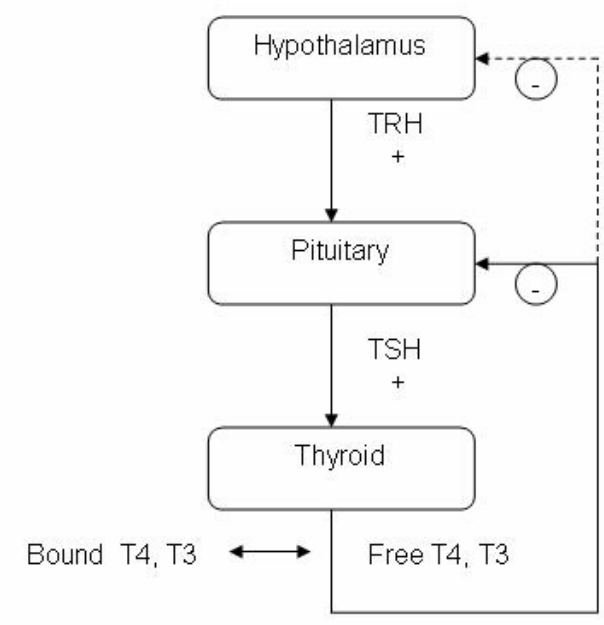


***Figure 1.5.*** *The hypothalamo-pituitary-thyroid axis. TSH production is controlled by stimulatory effect of TRH and by a negative feedback from circulating free T3 and T4 (fT3; fT4).*

## 1.5 Summary

The process of THs synthesis, storage and secretion requires a series of highly regulated steps:

- Uptake of iodide: iodide from plasma is actively transported by a sodium-iodine symporter on basal membrane of thyrocytes.
- Oxidation of iodide to iodine: this occurs on the luminal side of the apical membrane and requires thyroid peroxidase (TPO) and hydrogen peroxide, which is generated by a calcium-dependent flavoprotein enzyme system situated at the apical membrane.
- Organification: incorporation of iodine into tyrosyl residues on thyroglobulin. MIT and DIT are formed through action of TPO.
- Coupling of MIT and DIT: If two DIT molecules couple together, the result is the formation of T4; If a MIT and a DIT are coupled together, the result is the formation either T3 or rT3. T4, T3 and rT3 remain linked to thyroglobulin.
- Internalization: when there is demand for THs, Tg is internalized by pinocytosis and appears as colloid droplets that fuse with lysosomes and undergo proteolytic degradation to release: T4, T3, MIT and DIT; any MIT and DIT is deiodinated and the iodine conserved.
- Delivery of T4 and T3 into the circulation.
- TSH appears to stimulate each of the above processes.

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# 2. THYROID DISEASES: EPIDEMIOLOGY, PATHOPHYSIOLOGY AND CLASSIFICATION

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## 2.1 Epidemiology

The most common cause of thyroid disorders in the world is iodine deficiency that leads to goiter development and other consequences caused by lack of iodine. It is estimated that over 30% of school-aged children (over 250 million) have insufficient iodine intake and in the general population, 2 billion people have insufficient iodine intake. The greatest proportion of children with inadequate iodine intake live in Europe (over 50%), where it is found that 19 countries have insufficient iodine intake.

Croatia has crossed a path from severe iodine deficiency detected in the 1950' when along with the cretinism, goiter was detected in 50-90% of schoolchildren, to the period of mild to moderate iodine deficiency during the 1990' when proportion of goiter was reduced to 10-30% of schoolchildren, and finally, nowadays, iodine sufficiency has been reached. After the first regulation of obligatory salt iodination requiring 10 mg of potassium iodide (KI) per kg of salt in 1953, and still persisting mild to moderate iodine deficiency, the new one, requiring 25 mg of KI/kg of salt, was established in Croatia in 1996. In 2002 Croatia has finally reached iodine sufficiency according to the WHO (World Health Organization) and ICCIDD (International Council for the Control of Iodine Deficiency Disorders) reference values. In iodine sufficient countries the most common disorder is the appearance of thyroid nodules.

### 2.1.1 Hyperthyroidism

The prevalence of hyperthyroidism ranges from 0,5 to 2% in women while it is ten times lower in men. The most common causes of hyperthyroidism are Graves' disease (M. Basedow) and multinodular toxic goiter. Rare causes are toxic adenoma, thyroiditis and overdose with thyroid hormones.

Subclinical thyrotoxicosis is characterized with low TSH and normal values of thyroid hormones. The frequency of the subclinical thyrotoxicosis ranges from 0,5 to 6,3%, and the highest prevalence is among women and men over 65 years of age of which half of them take thyroid hormones. Subclinical thyrotoxicosis is more often seen in the areas with iodine deficiency.

### 2.1.2 Chronic autoimmune thyroiditis

The presence of thyroid antibodies (anti - thyroglobuline and anti - peroxidase) indicates chronic autoimmune thyroiditis. Among women there is progressive rise of the antibodies with age. This disorder is present in 5 % of the population.

### 2.1.3 Hypothyroidism

The prevalence of the spontaneous hypothyroidism ranges from 1 to 2%. It is more common in older women and ten times more frequent in women than in men.

In areas with iodine sufficiency the most common causes of hypothyroidism are: chronic autoimmune thyroiditis or destructive therapy of hyperthyroidism.

After the radioiodine treatment of hyperthyroidism, the development of hypothyroidism takes place almost in every patient, especially during the first year. The similar thing happens after the surgical treatment of hyperthyroidism. If TSH is on the upper limit and if the thyroid antibodies are positive, it is more probable for the spontaneous hypothyroidism to develop.

### 2.1.4 Subclinical hypothyroidism

Even 10% of women and 3% of men over 55 years have this disorder. In the USA it is less frequent among Afro Americans than among Caucasians. This frequency is higher if there is higher iodine intake.

### 2.1.5 Sporadic goiter

One of the common thyroid diseases is diffuse goiter. The highest prevalence is among premenopausal women and the ratio women/men ratio is 4:1. With age there is a fall of the diffuse goiter prevalence in contrast to the rise of nodules and antibodies. It seems that the ultrasound is too sensitive test and that it detects too many nodules that have no clinical value. In iodine deficient areas there is higher prevalence of multinodular goiter.

### 2.1.6 Thyroid nodule

In the past twenty to thirty years by introducing ultrasound in the everyday practice it has been established that over 50% of the population has a thyroid nodule.

Thyroid nodules are the most common thyroid disease. The prevalence of palpable thyroid nodules in iodine sufficient areas is about 5% in women and 1% in men. Much higher prevalence of thyroid nodules is detected by ultrasound, or in autopsy findings (over 50%). The prevalence of thyroid nodules detected by ultrasound or at autopsy linearly increases with age from 0% at the age of 15 years, 30% at the age of 50 years, and even up to 50% at the age of 60 to 65 years. The prevalence is about 5 times higher in women. Furthermore, the prevalence of thyroid nodules is higher in persons previously exposed to ionizing radiation and in those living in iodine deficient areas.

Incidentally discovered thyroid nodules are called “incidentalomas”.

### 2.1.7 Thyroid cancer

About 5% of thyroid nodules are malignant, regardless of whether the gland contains single nodule or multiple nodules, and the risk of malignancy is the same in palpable nodules and small nonpalpable nodules detected by ultrasound. Therefore, guidelines for management of patients with thyroid nodules are very important due to successful confrontation with appearing epidemic of multinodular goiter and in the same manner, the epidemic of thyroid cancer.

During the past decades, multifold increase in the incidence of thyroid cancer was recorded worldwide, and also in Croatia. However, mortality from thyroid cancer has remained low or even declined. During the time period from 1968 to 2004, age standardized incidence rate of thyroid cancer has increased in Croatia 8,6 times in women and 3,6 times in men. However, mortality from thyroid cancer in Croatia has remained low in both females and males with mild declining trend in females during the last 20 years. In 2004, age standardized mortality rate from thyroid cancer in Croatia was 0,4 per 100 000 of population in both females and males. Croatia is among countries with high incidence and low mortality rate from thyroid cancer, like Italy, France, Finland, USA and Australia.

Recently, occult papillary thyroid carcinomas (papillary thyroid microcarcinomas) are frequently discovered due to improved diagnostics. According to the autopsy findings, their prevalence in the population is 5-35%. World Health Organization defines papillary thyroid microcarcinoma as papillary thyroid carcinoma less or equaling 1 cm in diameter. It is generally believed that the increase in the incidence of thyroid cancer worldwide is mainly due to improved diagnostics (wide use of ultrasound and fine needle aspiration biopsy). It is presumed that if the entire pool of occult thyroid carcinomas were identified ante mortem, the result would be almost 50-fold increase in the apparent incidence of thyroid cancer.

## 2.2 Pathophysiology

### 2.2.1 Iodine deficiency disorders (IDD)

Iodine is an essential component of thyroid hormones and it plays an important role in a normal thyroid functioning. According to the ICCIDD and the WHO, the ideal daily iodine intake for normal, healthy adults should be 150 μg. A median urinary iodine concentration (UIC) is used as an indicator of an adequate iodine intake and an optimal status of iodine nutrition, because 90% of ingested iodine is excreted in 24-h urine. When iodine intake is reduced in the organism below a specific minimum it causes a number of functional and developmental abnormalities, called iodine deficiency disorders (IDD). In order to prevent iodine deficiency disorders, most countries have introduced public health programs that are based on iodized salt as the preferred strategy in order to supply iodine to the population. There are three different degrees of IDD severity: severe, moderate and mild.

***Table 1.1.*** *Iodine deficiency disorders*

| SEVERE Iodine deficiency | MILD to MODERATE iodine deficiency |
|---|---|
| - endemic goiter/cretinism (irreversible brain damage and mental retardation)<br>- decreased fertility rate<br>- increased perinatal death<br>- increased infant mortality | - goiter<br>- neuropsychointellectual deficits (low psychomotor and mental development, low IQ, perception, motor and attentive functions)<br>- high serum TSH and risk of transient neonatal hypothyroidism<br>- increased susceptibility of the thyroid to nuclear radiation |

During pregnancy, the requirement of iodine increases. The WHO, United Nations Children's Fund (UNICEF) and ICCIDD recommend the increase of iodine intake during gestation to 200-300 μg/day to compensate augmented requirements of T4 in pregnant women in order to prevent IDD. In the areas with mild to moderate iodine deficiency and even in the iodine sufficient areas it has been shown that pregnant women or a portion of pregnant women have inadequate iodine intake. Therefore, it is recommended that pregnant women, and women who are planning pregnancy should use iodine supplementation in the form of mineral/vitamin tablets.

### 2.2.2 Euthyroid goiter

There are several factors that act at the same time or consecutively in the goiter development. It is considered that following factors take part: 1) the factors that contribute to the development of the new follicles: a) TSH (the disorders of iodine metabolism and thyroid hormone synthesis, iodine deficiency), b) local tissue growth factors (EGF, IGF), autoimmune growth stimulators, 2) the factors that cause functional diversity in thyroid tissue: a) division of cells with higher autoimmunity, b) the development of cells and follicles with higher division ability, c) secondary degenerative changes. It is also considered that during goiter development higher thyreocyte sensibility on the TSH stimulation is present and that nodular changes develop in diffuse goiter.

### 2.2.3 Hyperthyroidism

Diffuse hyperthyroidism (Graves' disease, M. Basedow) arises in persons with genetic susceptibility along with environmental factors. Auto reactive helper T lymphocytes are not being eliminated because of the defected mechanism of the immunological control and they stimulate auto reactive B lymphocyte in generating organ specific antibodies on one or more antigens. The main role play TSab (Thyroid stimulating antibodies) that stimulate thyroid by linking to the TSH receptors on the thyrocytes. There are other antibodies that stimulate goiter development or are blocking linking TSH to the receptors. The course of the disease depends on the relationship between the stimulating and blocking antibodies for the TSH receptors and because of that the disease can be in the euthyroid, hyperthyroid or hypothyroid phase. The thyroid cells do not recognize TSab from TSH and so their effect lasts longer. In that way, there is higher excretion of thyroid hormones. Ophtalmopathy develops because of the immunological stimulation on the preadipocyte fibroblasts in the orbit. Depending on which of the T lymphocyte clones are affected, M. Basedow, Hashimoto's thyroiditis and ophtalmopathy can exist individually, by two or all three together. This kind of patients can also have other autoimmune diseases (M. Addison, Diabetes).

Toxic adenoma is highly differentiated tumor tissue with autonomous secretion of thyroid hormones. The pathophisiologic basis of thyroid autonomy is consistent activation of TSH receptors, mainly due to somatic TSH receptor mutation. On the same basis autonomous areas often develop in the multinodular goiter. The development of the hyperthyroidism in sensitive people (autoimmune disease, autonomous areas in goiter) can be caused by the iodine excess (amiodarone, iodine contrast agents). In the subacute and silent thyreoiditis, thyrotoxicosis develops because of the thyrocytes destruction.

The thyroid hormone excess during hyperthyroidism leads to the acceleration of all processes in the organism and enhanced calorigenesis. That causes weight loss, muscular weakness, myocardiopathy. The rise in the number of adrenergic receptors leads to the expressed signs of the sympaticotony.

### 2.2.4 Hypothyroidism

People with genetic susceptibility for autoimmune disorders (HLA-DR 3, 4, 5, 9) have a high risk of failure of immunological tolerance towards their own thyroid gland. It, than, results in autoimmune inflammation that leads to the functional thyroid tissue deterioration or in the production of the antibodies for TSH receptors that are preventing the TSH effects. At the same time other autoimmune diseases can be developed (pernicious anemia, vitiligo, diabetes, rheumatoid arthritis, etc.). Autoimmune disorders are more common in women. Hypothyroidism is a systematic disease which slows down the metabolism of all cells in the body leading to the loss of balance between them.

### 2.2.5 Chronic autoimmune thyroiditis

Chronic autoimmune thyroiditis is another thyroid disease present among people with genetic susceptibility, characterized by the autoimmune thyroid tissue destruction. It is 10-20 times more prevalent in women than in men. 20% of people with autoimmune thyreoiditis have hypothyroidism. Autoimmune thyroid diseases are often caused by iodine excess. Smoking can also contribute to their development.

### 2.2.6 Subacute thyroiditis

In the etiology of the subacute thyroiditis most often there is a presence of virus and this is probably result of several virus inflammations. Cell damage usually causes thyreotoxicosis after which transient hypothyroidism follows.

### 2.2.7 Tumors

For the development of differentiated thyroid tumors the most important step is the activation or excessive expression of the oncogenes Ras, RET, TRK and others. The loss of the tumor suppressing gene P53 function is significant for the anaplastic carcinoma. Most of the mutations are acquired. The inheritance of the family medullary carcinoma within MEN 2A and 2B as well as non MEN is auto somatic-dominant and it is the consequence of the RET mutation on the $10^{th}$ chromosome that causes constitutive activation of the tyrosine kinesis receptors in the C cells. Benign tumors are follicular adenomas of which some are autonomous (toxic adenoma). Malignant tumors originate from follicular epithelium (papillary, follicular and anaplastic), parafollicular C cells (medullary) and lymphatic tissue (lymphomas). Papillary carcinoma is the most common one (up to 95%) and it develops in iodine sufficient areas. In the differentiated carcinomas the production of the hormones is disrupted, and in the presence of the normal thyroid tissue they rarely accumulate 131-I. The differentiated tumors secrete thyroglobulin which is used as a tumor marker while medullary carcinoma secretes calcitonine. The differentiated carcinomas depend on the TSH. In patients with medullary carcinoma it is possible to discover endangered relatives by RET gene mutation detection, which is nowadays possible with the DNA analysis.

## 2.3 Classification

In the last few decades there have been significant discoveries about the thyroid disease pathophisiology and, consequential, classification changes. Now we can distinguish thyroid dysfunction on the targeted tissue level and also, we can better understand clinical evolution of the diseases that changes, for example, from hyperfunction to hypofunction. The

Hashimotos' disease can cross from euthyroidism into hypothyroidism. Present classification takes into consideration new disease entities and new discoveries about molecular and immunological mechanisms that are responsible for the disorder and disease evolution. Subclinical hyperthyroidism and hypothyroidism are considered as mild forms of the disease and not as disease for itself. Subacute thyreoiditis can have all three functional stages: euthyroidism, hyperthyroidism and hypothyroidism.

The thyroid function is the base of the classification, that is, normal, excessive or too low production of the hormones, and that is also the base for diagnostic and therapy. The term thyreotoxicosis is used to determine hormone excess and it doesn't indicate increased thyroid hormone production.

***Table 1.2.*** *Classification of thyroid disorders*

| | | | |
|---|---|---|---|
| **1. EUTHYROIDISM** | Euthyroid goiter<br>- diffuse<br>- nodular | Tumors<br>- benign<br>- malignant<br>- differentiated (papillary, follicular)<br>- non-differentiated (anaplastic)<br>- medullary | Thyroiditis<br>- acute<br>- subacute (De Quervain) (in the euthyroid phase)<br>- chronic autoimmune thyroiditis (Hashimotos' disease) (in the euthyroid phase)<br>- postpartum and silent thyroiditis (in the euthyroid phase) |
| **2. THYROTOXICOSIS** | Associated with hyperthyroidism:<br>- Graves' disease<br>- toxic adenoma and toxic multinodular goiter<br>- production of thyroid-stimulating hormones (TSH hypersecretion, , etc.) | Not associated with hyperthyroidism:<br>- silent, subacute and postpartum thyroiditis<br>- excess intake of thyroid hormones<br>- iodine induced thyroiditis (amiodarone, radiographic contrast agents, etc.) | Other (uncommon causes):<br>- pituitary resistance on thyroid hormones<br>- thyroid carcinoma functional metastases<br>- radiation thyroiditis<br>- struma ovarii |
| **3. HYPOTHYROIDISM** | Primary hypothyroidism<br>- chronic autoimmune thyroidits<br>- postoperative or after 131-I therapy<br>- iodine deficiency<br>- late stage of the Graves' disease<br>- neonatal hypothyroidism (ectopic, agenesis, dishormonogenesis) | Secondary hypothyroidism (disorders of the pituitary gland or hypothalamus) | Resistance to thyroid hormones<br><br>Transient hypothyroidism |

| | | | |
|---|---|---|---|
| **4. OPHTALMOPATHY (caused by thyroid disease)** | - only signs<br>- soft tissue involvement with signs and symptoms<br>- exophthalmus<br>- extraocular mucle involvement<br>- corneal involvement<br>- sight loss | | |
| **5. ABNORMAL THYROID PARAMETERS (those who are not caused by thyroid disease)** | Nonthyroidal illness<br><br>Deficit of TBG | | |

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# 3. HYPERTHYROIDISM

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## 3.1 Abstract

Hyperthyroidism is a syndrome that occurs when increased levels of serum free thyroid hormones target the capable thyroid hormone receptors in different tissues. The gravity of clinical picture is different for different types of hyperthyroidism. Various factors are involved in the development of hyperthyroidism. Besides genetic predisposition, iodine supply in a population seems to be the most important environmental factor influencing the type of hyperthyroidism. Before treatment, the type of hyperthyroidism has to be established. Laboratory diagnostics is usually combined with thyroid ultrasound and, if necessary, with the scintigraphy of the thyroid gland. Considering the cause of hyperthyroidism, the following therapetic options are at hand: wait-and-see, antithyroid drugs, perchlorate, glucocorticoids, radioactive iodine and surgery. Whichever the cause of hyperthyroidism, a correct diagnosis and a proper treatment enable a good quality of life for the majority of patients.

## 3.2 Introduction

Hyperthyroidism develops when an increased level of serum free thyroid hormones affects the working receptors for thyroid hormones in different tissues. The gravity of clinical picture depends upon the concentration of thyroid hormones, the dynamics of the development of hyperthyroidism or the duration of hyperthyroidism, the age of patient, the accompanying diseases, the current medication and other factors. In subclinical hyperthyroidism, the level of TSH is decreased, while the levels of free thyroxine ($fT_4$) and free triiodothyronine ($fT_3$) are within normal range. In overt hyperthyroidism, the level of TSH is decreased while the levels of $fT_4$ and $fT_3$ are increased. In diagnostic procedure, the cause of the disease must be established, since treatment modality differs with respect to the type of the disease. First, a short review of physiology and pathophysiology of thyroid hormones will be described in order to better understand thyroid hormones action in hyperthyroidism.

## 3.3 Physiology and pathophysiology of thyroid hormones

The thyroid gland synthesises mostly $T_4$ and only a smaller part of $T_3$. Approximately 80% of $T_3$ is produced by extrathyroidal deiodination of the outer or phenolic ring of $T_4$ (1). In humans, deiodinase type 2 (D2) is mainly responsible for that, however, also deiodinase type 1 (D1) is able to deiodinate $fT_4$ to $fT_3$. In hyperthyroidism, the contribution of D1 is higher (about 50%) because of an increase in D1 activity. $fT_4$ and $fT_3$ can be innactivated by deiodination of the inner or tyrosyl ring by D1 or type 3 deiodinase (D3). So, deiodinases influence the thyroid hormone action. The half life of $T_3$ is approximately 1 day, while the half life of $T_4$ is about 7 days. It binds to serum proteins and represents a large extrathyroidal pool of $T_4$ (1 micromol) in adults. Free $T_4$ and $T_3$ enter the cells in different tissues by specific energy-dependent transporters and by diffusion. The saturation of nuclear receptors for $T_3$ with $T_3$ is about 40 to 50%. In hyperthyroidism, the increase in serum $fT_3$ concentration is

reflected in the occupancy of the nuclear receptor in tissue. Some tissues (pituitary gland, brain, brown adipose tissues) contain D2 and convert $T_4$ to $T_3$. Therefore, the occupancy of receptors in these tissues is higher (70 to 90%). Thyroid hormones regulate growth, development and metabolism. They are especially important during gestation and early childhood. Metabolic effects of thyroid hormones are expressed in oxygen comsumption, protein, carbohydrate, lipid and vitamin metabolism. Thyroid hormones have genomic and nongenomic actions. $T_3$ is biologically more active than $T_4$, which is considered as a prohormone. Thyroid hormone enters the nucleus and binds to the nuclear receptor for thyroid hormones. $T_3$ binds to its receptor with a 10- to 15-times higher affinity than $T_4$. Thyroid receptors are connected with chromatine, act as transcription factors, binding both ligand and thyroid hormone response elements located in the promoters of target genes. This way, they regulate gene expression. Thyroid hormones have also nongenomic effects, such as an effect on plasma membrane transport systems for glucose, on pyruvate kinase, on cell structure proteins, on mitochondria, on kinase activities (2). In continuation, different causes of hyperthyroidism will be presented.

## 3.4 Types of hyperthyroidism

The types of hyperthyroidism differ between various regions in the world. Most likely, this is due to different iodine supply, not only in the present time, but also in the past. In a way, the thyroid gland »remembers« the past iodine supply. In areas with a normal iodine intake, a higher incidence of Graves' disease has been observed - in contrast with an iodine-deficient area, where thyroid autonomy was more frequent (3). Certainly, other reasons are also responsible for the different incidence of thyroid disorders. Graves' disease is the most frequent cause of hyperthyroidism, it is responsible for 60 to 90% of hyperthyroid cases. Less frequent is hyperthyroidism due to thyroid autonomy, which is depicted in the literature also as a toxic multinodular goiter or toxic adenoma (solitary hyperfunctioning nodule). Rare causes of increased level of serum thyroid hormones are subacute thyroiditis, hyperthyroid phase of Hashimoto's thyroiditis, hyperthyroid phase of postpartum thyroiditis, TSH hypersecretion (TSH adenoma), trophoblastic tumor, gestational thyrotoxicosis, struma ovarii, iodine-induced hyperthyroidism, radiation thyroiditis. In continuation, clinicaly most relevant causes of hyperthyroidism will be discussed.

### 3.4.1 Graves' disease

Graves' disease is an autoimmune disease characterized by the presence of thyrotropin receptor (TSH-R) stimulating antibodies, sometimes also depicted as thyroid stimulating immunoglobulins (TSI). 60 do 80% of patients with Graves' disease have also an increased level of thyroid peroxidase antibodies (TPOAb) while 20 to 40% of the patients have thyroglobulin antibodies (TgAb). Most likely, TPOAb and TgAb have no significant role in Graves' disease, while the role of TSI in the pathogenesis of the disease is doubtless. Graves' disease appears because of interplay of different factors. A strong genetic susceptibility has been proven. It seems, that the genetic factors contribute around 80%. Therefore, the disease is more frequent in relatives, especially in females. External factors influencing the occurrence of the disease are iodine intake, stress, smoking, infection, trauma, interferon α. Sometimes, the disease appears for the first time in the postpartum period. The patients with Graves' disease are usually younger than the patients with thyroid autonomy. Women get ill 5 to 10-times more frequently than man, commonly at around 40 years of age. In Graves' disease, hyperthyroidism develops rather quickly. Therefore, the symptoms are more dramatic than in the thyroid autonomy where hyperthyroidism develops gradually. Without treatment,

the levels of $fT_4$ and $fT_3$ reach very high values - amongst the highest in all types of hyperthyroidism. The level of $fT_3$ follows that of $fT_4$. So, $fT_4$ and $fT_3$ increase simultaneously. It is known that deiodination of $fT_4$ is increased because of the higher activity of thyroidal deiodinase D2 in Graves' disease. Therefore, the high concentration of $fT_3$ maintains the hyperthyroid state. Usually, the symptoms and signs last for several weeks before recognized. The patients are restless, tired, have palpitations, tremor, lose weight, have warm and moist skin, muscular weakness, and often an enlarged thyroid gland. Around 50% of patients have also mild, moderate or severe signs of *thyroid orbitopathy* (thyroid-associated ophthalmopathy, Graves' ophthalmopathy, thyroid eye disease). Rarely, the patients have *localized myxedema,* limited to the pretibial area. The disorder often occurs concomitantly with thyroid orbitopathy. Mild symptoms and signs of thyroid orbitopathy are lachrymation, mild exophthalmus, mild retraction of the upper lid, photophobia. The moderate form of thyroid orbitopathy is characterized by the edema of the eyelids, chemosis, swollen caruncle, proptosis. The patients may also experience pain and a more severe exophthalmus. In severe orbitopathy, the patients may have double vision because of impaired eye movements, corneal defects or even a pressure on optical nerv associated with a decrease in visual acuity.

In patients with subclinical Graves' disease, the excess of iodine may deteriorate hyperthyroidism to the severe stage. In the initial phase, the iodine load may ameliorate the Graves' disease because of inhibition of thyroid hormone secretion. After all, in the past iodine was used for treatment of hyperthyroidism. Unfortunately, patients with Graves' disease and with overexpression of NIS (sodium-iodide symporter, a transport protein for iodide into thyroid follicular cell), may escape from the inhibitory effect of iodine and from autoregulation. Another element that has to be considered in patients with Graves' disease is a low intrathyroidal iodine concentration when compared with healthy thyroids. Namely, low intrathyroidal iodine content presents an additional risk for deleterious effects of iodine.

### 3.4.2 Thyroid autonomy

Thyroid autonomy is a broader term for toxic adenoma and other forms of autonomous tissue. Thyroid cells in thyroid autonomy sintesize and secrete thyroid hormones irrespective of TSH concentration, which is usually decreased. Patients with thyroid autonomy are negative for TSI. Mutation of TSH-R and rarely, mutation of subunits of G proteins, causes a permanent activation of thyroid cell, and therefore, the hyperthyroidism. Patients with thyroid autonomy are on average older than those with Graves' disease. Prevalence of thyroid autonomy is higher in iodine-deficient or previously-iodine-deficient areas. It increases with age and is more frequent in lasting nodular goiters. It seems, that in iodine-deficient areas, hyperplasia of the thyroid gland occurs already in youth. In hyperplastic glands, the rate of mutations is increased, resulting in thyroid autonomy or in dedifferentiated cold thyroid nodules. Thyroid autonomy develops slowly. Often it takes many years, before hyperthyroidism aggravates from subclinical to overt. It has been reported that hyperthyroidism develops when the product between the weight of autonomous tissue, the efficiency per gram of tissue, and iodide supply goes above a treshold. Hence it follows that an increase in iodine intake represents a risk factor for aggravation of hyperthyroidism in these patients. Iodine excess often deteriorates subclinical or overt hyperthyroidism to the severe stage. Mutation of TSH receptor enables a continuous iodide transport via overexpressed NIS and therefore preserves the hyperthyroidism. Low intrathyroidal iodine concentration, as observed in patients with toxic adenomas when compared with healthy thyroids, presents an additional risk for deleterious effects of iodine load.

Patients with thyroid autonomy may be euthyroid, subclinical or overt hyperthyroid. In the hyperthyroidism due to thyroid autonomy, the patients are often oligosymptomatic. They may have an enlarged thyroid gland, palpitations, arrythmias, restlessness or weight loss. Because hyperthyroidism develops slowly, the patients get used to the increasingly higher levels of thyroid hormones, probably by down-regulation of thyroid receptors. In that type of hyperthyroidism, the laboratory tests usually show mild hyperthyroidism but rarely severely increased thyroid hormones. $FT_3$ is often relatively more increased than $fT_4$, therefore the ratio between $fT_4$ and $fT_3$ is similar or lower than in Graves' disease. This is probably due to the low iodine content in the thyroid cells in autonomous tissue, while cells in the healthy thyroid gland secrete more $fT_4$ than $fT_3$.

### 3.4.3 Iodine-induced hyperthyroidism

Iodine-induced hyperthyroidism develops when iodine intake increases in patients with an enlarged thyroid gland, in patients with thyroid autonomy or with latent Graves' disease. However, even in subjects with healthy thyroid gland, hyperthyroidism may developed after iodine load. How much iodine is too much for the development of iodine-induced hyperthyroidism? This depends on the previous iodine supply, on the source and amount of iodine intake and on previous thyroid pathology.

In healthy thyroid with normal function, iodine load usually causes no change in TSH concentration, a normal or slight increase of $fT_4$ values and a normal or slight decrease of $fT_3$ values. Autoregulation is a mechanism that enables a normal thyroid function in spite of iodine excess. Autoregulation decreases the intrathyroidal iodine content after an initial increase following the iodine load. High levels of intracellular iodide decrease the NIS mRNA and protein levels, partly by decreasing the transcription and partly by increasing the NIS protein turnover.

The main sources of iodine excess are amiodarone, contrast media in radiology, topical antiseptics and iodine containing vitamins. Among them, the most important are amiodarone - because of its high iodine content, radiology contrast agents - because of their broad usage, the same holds true for iodine containing vitamins.

The most important iodine source is amiodarone, a fat-soluble agent with a prolonged half-life of 100 days. One 200 milligram tablet contains 75 milligrams of iodide, about 10% is deiodized daily. So, amiodarone provides the organism with several miligrams of iodide daily.

Water soluble contrast media contain around 400 milligrams of iodide per mL, but only a small amount as a free iodide, which is rapidly cleared from the plasma through kidneys. They usually do not have lasting effects on thyroid gland. During recent years, multivitamins which, beside other vitamins, usually also contain 100 to 200 micrograms of iodine, have become increasingly popular in different age groups, especially in older people and in pregnant women. Individuals often combine different vitamins. This way, they can increase the iodine intake above recommended or even above safe values.

Besides large amounts of iodine, amiodarone influences the thyroid status also by inhibition of deiodinase D1, which decreases serum concentrations of $fT_3$, by inhibiting transcription of $T_3$ receptor and by a cytotoxic action on thyroid follicular cells. The latter effect can lead to abnormal thyroid function even in normal, healthy thyroid glands and not only in individuals

with preexisting thyroid disease, who are usually more susceptible to deleterious effects of amiodarone.

The incidence of thyroid function abnormalities under the influence of amiodarone lies between 14 and 18%. In areas with adequate iodine intake, amiodarone provokes hypothyroidism more often than hyperthyroidism. This is due to the large amount of iodine, the higher incidence of thyroid autoimmunity and due to a sort of resistance of thyroids, probably caused by the changed autoregulatory mechanism. In iodine-deficient areas, hyperthyroidism is more frequent than hypothyroidism because of higher incidence of autonomy in goiters. The patients with iodine-induced hyperthyroidism are often older, suffering also from other diseases, especially cardiovascular. Therefore, hyperthyroidism worsens their condition and may even end fatally. Iodine-induced hyperthyroidism is a very serious condition to treat and it often takes several months to restore the normal thyroid function. Laboratory tests in this type of hyperthyroidism usually show a higher ratio between $fT_4$ and $fT_3$ than in Graves' disease. This is due to a faster synthesis of $T_4$ in the thyroid gland due to a very high iodine supply. At the initial stage of the development of iodine-induced hyperthyroidism, we can observe increased $fT_4$ level and only slightly increased $fT_3$ level, which later follows $fT_4$ level more adequatly, when hyperthyroidism begins to increase deiodase D1 activity.

### 3.4.4 Thyroiditis

Thyroiditis in a broader sense represents every inflammatory condition in the thyroid gland. Thyroiditis causes follicular disruption and release of thyroid hormones, stored in the thyroid cells, into the blood, resulting in hyperthyoidism. Most frequent is subacute thyroiditis, probably caused by a viral infection. Patients suffer from fever, severe pain that extends to the ear, and they may have symptoms of hyperthyroidism. The gland is often enlarged and firm. Biochemical hyperthyroidism is mild. The ratio between $fT_4$ and $fT_3$ is higher than in Graves' disease. This is probably due to the predominant release of $fT_4$ from the intrathyroid stores. Postpartum thyroiditis occurs in 5 to 7% of women post partum. Women with Hashimoto's thyroiditis, with type 1 diabetes, with previous episode of postpartum thyroiditis, with increased level of TPO antibodies are more prone to develop postpartum thyroiditis. Hyperthyroidism is often mild and transient, followed by euthyroidism and hypothyroidism, which can be transient or permanent in 30%. However, in the first year postpartum, Graves' disease may occur for the first time in life, so determination of TSI is needed for the correct diagnosis. The ratio between $fT_4$ and $fT_3$ is similar as in subacute thyroiditis and therefore higher than in Graves' disease, indicating a release of thyroid hormones from intrathyroid stores.

## 3.5 Symptoms and signs of hyperthyroidism

Clinical symptoms and signs considerably differ among the patients. Some patients have only several symptoms, while others develop a whole spectrum of symptoms. Most frequent clinical manifestations of hyperthyroidism are nervousness, fatigue, weakness, heat intolerance, tremor, hyperactivity, palpitations, weight loss, rarely weight gain, hyperactivity, tachycardia or atrial fibrillation, systolic hypertension, warm and moist skin. In continuation, clinically most relevant symptoms and signs of hyperthyroidism will be listed.

### 3.5.1 Cardiovascular system

The patients may have palpitations, intolerance for exercise, difficulties with breathing, symptoms of angina pectoris, increased heart beat (tachycardia) or irregular heart beat (atrial fibrillation), cardiac flow murmurs, oedema. Thyroid hormones decrease the systemic vascular resistance and the diastolic blood pressure, and increase the cardiac output, the nitric oxide, the systolic blood pressure, the heart rate, the cardiac contractility, the cardiac mass and the blood volume.

### 3.5.2 Skin

A direct action of thyroid hormones on skin results in a smooth, thin, warm skin, fine hair, hair loss, shiny, soft, triable nails, increased sweating, hyperpigmentation, erythema.

### 3.5.3 Kidneys and electrolyte metabolism

Hyperthyroidism increases the plasma renin activity, slightly lowers the serum aldosterone, increases the glomerular filtration, the renal blood flow, and the uric acid excretion.

### 3.5.4 Blood

Erytropoiesis is increased to satisfy the increased oxygene consumption in tissues. Patients may have anemia due to ineffective erytropoiesis, iron deficiency, vitamin $B_{12}$ deficiency and folate deficiency. Soma patients with Graves' disease have trombocytopenia and/or granulocytopenia of the unknown cause.

### 3.5.5 Neuromuscular system and brain

A high percentage of patients has a proximal muscular weakness. They have difficulty arising from a sitting or supine position and raising arms over the head. The degree of muscular weakness is more connected with the duration of hyperthyroidism than with the biochemical severity. Brain symptoms are anxiety, irritability, emotional lability. Rarely, patients present with psychosis. In untreated Graves' disease, patients with high level of anxiety have increased glucose metablism in specific brain regions on FDG-PET scanning, particularly in the limbic system (4).

## 3.6 Subclinical hyperthyroidism

Subclinical hyperthyroidism causes atrial fibrillation in people over 60 years and a bone loss in postmenopausal women. Even TSH levels between 0.1 and 0.5 mU/L have been associated with atrial fibrillation (5). Meta analysis showed a 41% increase in all-cause mortality in case of subclinical hyperthyroidism, the risk seems to be dependent on the age at diagnosis, with a significant increase beginning at the age of 60 years, especially in men (6). These data indicate that even a very mild hyperthyroidism should be treated, even in asymptomatic older patients.

## 3.7 Diagnostics of hyperthyroidism

Diagnostics of hyperthyroidism depends upon the suspected cause of hyperthyroidism. Often, the patient's anamnesis, symptoms and signs indicate the type of disease that caused the

hyperthyroidism. Besides clinical data, diagnostics of hyperthyroidism include also laboratoy findings, ultrasound of the thyroid gland, and if necessary, also scintigraphy of the thyroid gland.

### 3.7.1 Non-specific changes in laboratory tests

Some patients have pathological liver tests. 10% of patients has hypercalcemia due to increased bone turnover and decreased parathyroid hormone in serum. Some patients also have decreased levels of total cholesterol, LDL and HDL cholesterol.

### 3.7.2 Thyroid laboratory tests

The majority of patients with overt hyperthyroidism has a decreased level of TSH and increased levels of $fT_4$ and $fT_3$. For the determination of thyroid status, free thyroid hormones should be measured. Approximately 1% of patients has a normal $fT_4$ and an increased $fT_3$ level (tri-iodothyronine toxicocis). Such a finding is more common in patients with thyroid autonomy. If one only relied on the determined $fT_4$ levels, these patients would be misdiagnosed. If the TSH level does not appropriately follow the increase of fT4 and fT3, the patient mas have a syndrom of inappropriate thyrotropin, due to a TSH-secreting pituitary tumour or due to thyroid hormone resistance. In such a case, the mutation of β-nuclear receptor causes a resistance to thyroid hormone. If pituitary is more affected by mutation than the periphery, the patients have symptoms and signs of hyperthyroidism. There are several other factors that decrease the TSH level. Therefore, a low TSH is not always a sign of hyperthyroidism. These factors are mainly drugs, such as glucocorticoids and dopamine. Similarly, in the first trimester of pregnancy, the increased level of human chorionic gonadotropin mimics the effects of TSH on the thyroid gland and, therefore, lowers its concentration. The patients with iodine-induced hyperthyroidism have significantly higher $fT_4$ than $fT_3$ values. A normal TSH value, an increased $fT_4$ and a normal or a decreased $FT_3$ value are not signs of hyperthyrodism but represent a normal adaptation of healthy thyroid gland to iodine excess.

### 3.7.3 Thyroid ultrasound

Thyroid ultrasound, especially when combined with colour flow Doppler sonography, is a very useful method for the diagnostics of the type of hyperthyroidism. In Graves' disease, the thyroid gland appears enlarged, darker (hypoechoic) than the healthy thyroid, irregular, with very increased blood flow, detected with colour flow Doppler sonography. Thyroid autonomy presents with one or more thyroid nodules. Colour flow Doppler sonography seems not to be useful for the distinction between cold and hot nodules. In iodine-induced hyperthyroidism, the thyroid gland may be enlarged or not, with thyroid nodules or not, while the colour flow Doppler sonography usually revelas a decreased blood flow. A similar picture can be observed in various forms of thyroiditis.

### 3.7.4 Thyroid scintigraphy

Thyroid scintigraphy is usually performed with technetium-99m-pertechnetate that enters thyroid cell via NIS. In Graves' disease, thyroid scintigraphy reveals diffuse and intensive uptake of radioisotope in the thyroid gland. In thyroid autonomy, this method is essential for the diagnosis and shows an increased uptake in solitary autonomous nodule (hot nodule) or in several nodules. In iodine-induced hyperthyroidism, the thyroid uptake in usually very low, a

similar picture is seen in various forms of thyroiditis. Except in the thyroid autonomy, thyroid scintigraphy is not essential for the diagnosis of hyperthyroidism.

## 3.8 Treatment of hyperthyroidism

There are three main therapeutic options for the treatment of hyperthyroidism: antithyroid drugs, radioactive iodine ($^{131}$I) and surgery (7). The selection of treatment depends upon the cause of hyperthyroidism. Therefore, a correct diagnosis is crucial for the treatment choice. Less frequent therapeutic options are wait-and-see in women with the hyperthyroid phase of postpartum thyroiditis, or glucocorticoids in the case of subacute thyroiditis or in the case of a certain form of amiodarone-induced hyperthyroidism.

*Antithyroid drugs* are non-invasive, low-cost and represent a low risk for permanent hypothyroidism. However, they have a low cure rate (30 to 80%), may cause adverse reactions and may be a subject of questionable compliance (8). Methimazole, the antithyroid drug, is the preferred first-line treatment of Graves' disease in Europe (8). Propylthiouracil is a second-line therapy due to the higher rate of side effects and lower efficacy in patients with severe hyperthyroidism (9). Propylthiouracil is preferred in pregnant women because of some reports about teratogenic effects of methimazole. When treatment of Graves' disease with antithyroid drugs failes, in the case of adverse drug reaction, or in the case of thyroid orbitopathy, we seek for the definitive treatment of the disease. The most frequent option in such a case is radioactive iodine, rarely thyroidectomy. In iodine-induced hyperthyroidism we can use, besides antithyroid drugs, also perchlorate, a drug that inhibits the iodine transport via NIS. This way, the level of iodine in the thyroid cell gradually falls. Together with antithyroid drugs, perchlorate usually proves successful in the treatment of such type of hyperthyroidism.

*Radioactive iodine ($^{131}$I)* has a potential to cure hyperthyroidism, is cost effective, but causes permanent hypothyroidism in most cases, might transiently worsen orbitopathy, pregnancy has to be postponed for 6 to 12 months, and there is a small potential risk of exacerbation of hyperthyroidism. Radioactive iodine has been used for more than 60 years and has proven a safe treatment with no evidence for infertility, birth defects or cancer. Pretreatment of thyroid autonomy with antithyroid drugs may reduce the success of subsequent radioiodine therapy, a phenomenon that is more pronounced in pretreatment with propylthiouracil. Radioactive iodine cannot be used for the treatment of iodine-induced hyperthyroidism, because the thyroid gland is already saturated with iodine, and the transport via NIS is decreased.

*Surgery* is a rapid and effective treatment, especially in patients with large goiters. The procedure is most invasive and costly, has potential complications (transient or permanent hypoparathyroidism, transient or permanent recurrent laryngeal nerve damage, shown as hoarseness), causes permanent hypothyroidism, patients have a scar. In Graves' disease, the procedure may be useful in pregnancy in the case of major side-effects of antithyroid drugs. It is also useful in the case of suspicious thyroid nodules and in patients with thyroid autonomy, who refuse radioactive iodine. Additionally, surgery can be used in severe hyperthyroidism, usually iodine-induced hyperthyroidism, when no amelioration of hyperthyroidism with antithyroid drugs could be achieved.

## 3.9 Prognosis

Most patients with Graves' disease experience recurrence of the disease. After treatment with radioactive iodine, they became hypothyroid - as soon as in the first year after therapy. Differently, patients with hyperthyroidism due to thyroid autonomy usually become hypothyroid several years after radioiodine treatment or never. In iodine-induced hyperthyroidism, especially in amiodarone-induced disease, the normal thyroid function is established not earlier than several months after the beginning of treatment. Afterwards, preventive treatment with radioiodine is a good option for these patients. Various forms of thyroiditis usually cure without serious consequences.

In conclusion, when the correct cause of hyperthyroidism is established and a proper treatment is implemented, the patients with hyperthyroidism have a good prognosis and mostly a good quality of life.

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# 4. HYPOTHYROIDISM

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## 4.1 Definition

There are many definitions of hypothyroidism, as hypothyroidism is a condition in which thyroid gland does not produce enough thyroid hormones. The most simple and I think most accurate is that hypothyroidism is thyroid hormone deficiency. However, we must always keep in mind that hypothyroidism is a condition in tissues, and tissues are able to adapt to different concentrations of thyroid hormones.

## 4.2 Epidemiology

Hypothyroidism occurs at any age but is particularly common in the elderly. It occurs in close to 10% of women and 6% of men over 65 years in regions with appropriate iodine intake. In iodine deficiency countries, the incidence is even greater.

## 4.3 Classification

Hypothyroidism is often classified as primary, when thyroid gland is incapable to produce desired amount of thyroid hormones, as secondary, when pituitary gland does not create appropriate amount of thyroid stimulation hormone (TSH) and as tertiary, when hypothalamus fails to produce sufficient thyrotropin-releasing hormone (TRH).

## 4.4 Causes

### 4.4.1 Primary hypothyroidism

There are three principal causes of primary hypothyroidism: destruction of thyroid gland, underdevelopment of the gland and blocking of signalling pathway.

#### *4.4.1.1 Destruction of thyroid gland*

In areas of adequate iodine intake, autoimmune thyroid disease is most common reason for hypothyroidism. The prevalence of antibodies is higher in women, and increases with age. The most frequent cause of acquired hypothyroidism is autoimmune (Hashimoto) thyroiditis. The body recognizes the thyroid antigens as foreign, and a chronic immune reaction ensues, resulting in lymphocytic infiltration of the gland and progressive destruction of functional thyroid tissue.

Destruction of thyroid gland can result from postpartum thyroiditis, a condition that affects about 5% of all women within a year after giving birth. The frequency may be as high as 25% in women with type 1 diabetes mellitus. The course of the disease could be different, but of those women who experience hypothyroidism associated with postpartum thyroiditis, one in five will develop permanent hypothyroidism requiring life-long treatment.

Inflammatory conditions or viral syndromes may be associated with transient hyperthyroidism followed by transient hypothyroidism (subacute thyroiditis de Quervain). In some patients, subacute thyroiditis could provoke autoimmune thyroiditis and permanent hypothyroidism could develop.

The second major cause is the broad category of "medical treatments." The treatment of many thyroid conditions warrants surgical removal of a portion or all of the thyroid gland. Use of radioactive iodine for treatment of Graves' disease generally results in permanent hypothyroidism within 1 year after therapy. The frequency is much lower in patients with toxic nodular goiters and those with autonomously functioning thyroid nodules. External neck irradiation (for head and neck neoplasm, breast cancer, or Hodgkin disease) may result in hypothyroidism.

#### *4.4.1.2 Underdevelopment of the gland*

Some babies are born with a thyroid gland that is not fully developed or does not function properly. The incidence of congenital hypothyroidism is one in 3 to 4 000 babies and is related to iodine intake. If untreated, congenital hypothyroidism can lead to mental retardation and growth failure. Often, infants with congenital hypothyroidism appear normal at birth. That is one reason why most states now require newborn thyroid screening.

#### *4.4.1.3 Blocking of signalling pathway*

Thyroid function is controlled by pituitary gland. Decreased production of thyroid hormones causes an increase in the secretion of Thyroid Stimulating Hormone (TSH) by the pituitary gland. TSH stimulates production and depletion of thyroid hormones. If the receptor for TSH on thyroid cell is occupied by antibody, the production of hormone is blocked. This kind of hypothyroidism is in most cases transient.

### 4.4.2 Secondary hypothyroidism

There are several other rare causes of hypothyroidism. One of them being a completely "normal" thyroid gland that is not making enough hormones because of a problem in the pituitary gland. If the pituitary does not produce enough (TSH) then the thyroid simply does not have the "signal" to make hormone. Main cause for such situation is tumour of pituitary gland.

### 4.4.3 Tertiary hypothyroidism

We have to think on tertiary hypothyroidism when we have patient with tumour of hypothalamus or this region was irradiated. The consequence is impaired hypothalamo-pituitary axis with the influence on other endocrine glands.

## 4.5 Pathophysiology

Normally, the thyroid releases thyroxine ($T_4$ – about 90%) and only small amounts of triiodothyronine ($T_3$ about 10%). The half-life of T4 is approximately 7-10 days. Up to 80% of the $T_4$ is converted to $T_3$ by peripheral organs such as the liver, kidney and spleen by 5'-deiodination. $T_4$, function as prohormone. $T_3$ is about ten times more active than $T_4$.

Decreased production of $T_4$ causes an increase in the secretion of TSH by the pituitary gland. TSH stimulates hypertrophy and hyperplasia of the thyroid gland and thyroid $T_4$-5'-deiodinase activity. This, in turn, causes the thyroid to release more $T_3$. This is a compensatory mechanism to maintain $T_3$ levels.

Receptors for thyroid hormones are intracellular DNA-binding proteins that function as hormone-responsive transcription factors. Thyroid hormones enter cells through membrane transporter proteins. In nucleus, the hormone binds its receptor. All metabolic active cells in the body are targets for thyroid hormones. Thyroid hormones stimulate diverse metabolic activities most tissues, leading to an increase in basal metabolic rate. They influence lipid and carbohydrate metabolism and are clearly necessary for normal growth in children.

## 4.6 Symptoms of hypothyroidism

Because all metabolically active cells require thyroid hormone, deficiency of the hormone has a wide range of effects, which can vary from person to person. Symptoms and signs of primary hypothyroidism are often subtle and insidious. We have to differentiate between the onsets of light hypothyroidism and fully expressed clinical picture.

Some common symptoms are:

- fatigue
- weakness
- weight gain or increased difficulty losing weight
- coarse, dry hair
- dry, rough pale skin
- hair loss
- cold intolerance (you can't tolerate cold temperatures like those around you)
- muscle cramps and frequent muscle aches
- constipation
- depression
- irritability
- memory loss
- abnormal menstrual cycles
- decreased libido

Elderly patients have significantly fewer symptoms than younger adults do, and complaints are often subtle and vague. Many elderly patients with hypothyroidism present with nonspecific geriatric syndromes - confusion, anorexia, weight loss, falling, incontinence, and decreased mobility.

You may have one of these symptoms as your main complaint, while another will not have that problem at all and will be suffering from an entirely different symptom. Most people will have a combination of these symptoms. Occasionally, some patients with hypothyroidism have no symptoms at all, or they are just so subtle that they go unnoticed.

The main problem is the onset of symptoms. As it was stated before, the main cause of hypothyroidism nowadays is Hashimoto thyroiditis. The typical course of this disease is long latent periods interrupted with short active periods. During active periods further deterioration of thyroid gland occurs. Clinical picture develops slowly and some times, it is very difficult to

register the changes. Consequently, the diagnosis of hypothyroidism is based on clinical suspicion and confirmed by laboratory testing.

Although secondary hypothyroidism is uncommon, its causes often affect other endocrine organs controlled by the hypothalamic-pituitary axis. In a woman with hypothyroidism, indications of secondary hypothyroidism are a history of amenorrhea rather than menorrhagia and some suggestive differences on physical examination. Secondary hypothyroidism is characterized by skin and hair that are dry but not very coarse, skin depigmentation, only minimal macroglossia and atrophic breasts.

Left untreated, the symptoms of hypothyroidism will usually progress. Rarely, complications can result in severe life-threatening depression, heart failure, or coma.

Untreated hypothyroidism is associated with an increased risk of heart disease. Beyond consequences of high levels of low-density lipoprotein (LDL) cholesterol, decreased contractility, cardiac enlargement, pericardial effusion, decreased pulse, and decreased cardiac output could be a consequence of an underactive thyroid. Depression and slowed mental functioning may occur early in hypothyroidism especially in elderly and may become more severe over time. In the GI tract, achlorhydria and decreased intestinal transit with gastric stasis can occur. Delayed puberty, anovulation, menstrual irregularities, and infertility are common. Babies born to women with untreated thyroid disease have a higher risk of birth defects than do babies born to healthy mothers. These children are more prone to serious intellectual and developmental problems. Paresthesias of the hands and feet are common, often due to carpal-tarsal tunnel syndrome caused by deposition of proteinaceous ground substance in the ligaments around the wrist and ankle. In addition, hypothyroidism may result in an increase in insulin resistance.

In patients with fully expressed clinical picture of hypothyroidism, the facial expression is dull; the voice is hoarse and speech is slow; facial puffiness and periorbital swelling occur due to infiltration with the mucopolysaccharides hyaluronic acid. Rarely, untreated may lead to myxedema coma, an extreme form of hypothyroidism in which the body slows to the point that it becomes life threatening.

*Myxedema coma*
This rare, life-threatening condition is the result of undiagnosed hypothyroidism. Its symptoms include intense cold intolerance and drowsiness followed by profound lethargy and unconsciousness. A myxedema coma may be triggered by sedatives, infection or other stress on your body. This is not only extreme hypothyroidism, but also the failure of homeostasis. The mortality rate is up to 70%.

## 4.7 Diagnostic testing

To diagnose primary hypothyroidism, many doctors simply measure the amount of TSH being produced by the pituitary gland (TSH test). High levels of TSH indicate that the thyroid is not producing sufficient levels of thyroid hormones. However, measuring just TSH fails to diagnose secondary and tertiary forms of hypothyroidism.

Evaluation of TSH determination is not simple. We have to take into account the physiology of pulsatile TSH release and daily rhythm of TSH. This is the reason, why TSH can vary in short period of time across all normal range. Definitely, general practitioner willing to

exclude hypothyroidism can use TSH alone. If TSH is in the middle of the normal range, we can exclude hypothyroidism with high probability. On the other hand, borderline TSH determination demands additionally determination of free triiodothyronine ($fT_3$) and of free thyroxine ($fT_4$). In hypothyroid patients, we expect elevated TSH, low $fT_4$ and low or normal $fT_3$. Arbitrarily hypothyroidism is defined as TSH levels >4.5 mIU/L.

Many patients with primary hypothyroidism have normal circulating levels of $fT_3$, resulting in preferential synthesis and secretion of the biologically active hormone $fT_3$. Therefore, serum $fT_3$ is not sensitive for hypothyroidism.

Subclinical hypothyroidism is elevated serum TSH in patients with absent or minimal symptoms of hypothyroidism and normal serum levels of fT4. Subclinical thyroid dysfunction is relatively common; it occurs in more than 15% of elderly women and 10% of elderly men, particularly in those with underlying Hashimoto's thyroiditis.

To confirm secondary or tertiary hypothyroidism TRH test is used. The TRH test is different. A baseline TSH test is done. Then the injection of TRH is given, which stimulates the pituitary to release TSH. A second blood sample is drawn 20 to 30 minutes later, and the TSH level is retested.

Kellman believes that this test is the "best way to detect subtle thyroid problems..." and that it overcomes limitations of the TSH test. According to Kellman: The TSH test is a picture in time of circulating levels of thyroid hormone, but by challenging the thyroid, the TRH test evaluates the thyroid's actual ability to function in real life.

Expected results of TRH test

| **Type of hypothyroidism** | **TSH test** | **TSH after stimulation** | **$fT_4$** | **$fT_3$** |
|---|---|---|---|---|
| Primary hypothyroidism | N or ↑ | > 30 mmol/l | ↓ or N | N or ↓ |
| Secondary hypothyroidism | N or ↓ | < 10 mmol/l | ↓ | N or ↓ |
| Tertiary hypothyroidism | N | 20 – 30 mmol/l | ↓ | N or ↓ |

Thinking about hypothyroidism we should always have in mind, that hypothyroidism is a functional disturbance and a consequence of different diseases. If we do not find a disease capable to cause a hypothyroidism, than we should reconsider our decision and probably repeat the tests.

To find the diagnosis we have to do some other tests. In every patient with suspicious symptoms or history of hypothyroidism, the autoimmune thyroid disease should be excluded. Therefore, we have to determine possible presence of thyroid auto antibodies (anti thyroglobulin antibodies – antiTg and anti Thyroid Peroxidase antibodies – antiTPO) and possibly antibodies against TSH receptor. Ultrasound examination of thyroid gland is a part of basic examination in Outpatient department for thyroid diseases. In this case, the typical hypoechogenic ultrasound pattern confirms the presence of autoimmune thyroid disease.

We can conclude that before we are discussing therapy and prognosis we must make a decision about diagnosis of the disease and degree of thyroid failure.

## 4.8 Therapy

Supplementation of thyroid hormones is generally considered treatment of choice for patients with hypothyroidism. Synthetic levorotatory forms of thyroxine (l – thyroxine) is used. Very rarely for patients with decreased conversion of $fT_4$ to $fT_3$ a combination of l – thyroxine and triiodothyronine is indicated. Usually we start with lower dose (50 µg/daily) and in a month, which is usually about 100 µg/daily. In patients with cardiac involvement or with very low thyroid hormone concentrations starting dose is lower and time used to achieve the maintenance dose is much longer.

However, there are some controversies about starting point. Should we treat all forms of hypothyroidism?

In patients with serum TSH > 10 mu/l, there is a high likelihood of progression to overt hypothyroidism with low serum levels of fT4 in the next 10 years. These patients are also more likely to have hypercholesterolemia and atherosclerosis. They should be treated with l - thyroxine, even if they are asymptomatic. For patients with TSH levels between 4.5 and 10 mu/l, a trial of l-thyroxine is reasonable if symptoms of early hypothyroidism (e.g., fatigue, depression) are present. In this group of patients, l-thyroxine therapy is also indicated in pregnant women and in women who plan to become pregnant to avoid deleterious effects of hypothyroidism on the pregnancy and foetal development. Patients should have annual measurement of serum TSH and fT4 to assess progress of the condition if untreated or to adjust the l-thyroxine dosage.

*Pediatric doses are as follows:*

- Neonate to 6 months: 25-50 µg/d
- 6-12 months: 50-75 µg/d
- 1-6 years: 75-100 µg/d
- 6-12 years: 100-150 mcg/d PO
- >12 years: 150 mcg/d PO

Infants with untreated hypothyroidism present at birth are also at risk of serious problems with both physical and mental development. However, if the condition is diagnosed within the first few months of life, the chances of normal development are excellent.

The treatment goals for hypothyroidism are the reversal of clinical progression and the corrections of metabolic derangements as evidenced by normal blood levels of TSH and free T4. Thyroid hormone is administered to supplement or replace endogenous production. In general, hypothyroidism can be adequately treated with a constant daily dose of levothyroxine (LT4).

Achieving a TSH level within the reference range may be slowed because of delay of hypothalamic-pituitary axis readaptation and may take several months. After dose stabilization, patients can be monitored with annual clinical evaluations and TSH monitoring. Patients should be monitored for symptoms and signs of overtreatment, which include tachycardia, palpitations, nervousness, tiredness, headache, increased excitability, sleeplessness, tremors, and possible angina.

A meta-analysis of randomized controlled trials of thyroxine-triiodothyronine combination therapy (T4 + T3) versus thyroxine monotherapy (T4) for treatment of clinical

hypothyroidism found no difference in the effectiveness of the combination vs monotherapy in bodily pain, depression, fatigue, body weight, anxiety, quality of life, total cholesterol, LDL-C, HDL-C and triglyceride levels. Hence, T4 monotherapy remains the treatment of choice.

*Pregnancy*
Hypothyroidism in pregnancy is associated with preeclampsia, anemia, postpartum hemorrhage, cardiac ventricular dysfunction, spontaneous abortion, low birth weight, impaired cognitive development, and fetal mortality. Even mild disease may be associated with adverse affects for offspring.

Increased dosage requirements should be anticipated during pregnancy, especially in the first and second trimesters. Studies have suggested that patients with hypothyroidism should augment the LT4 dose by 30% at the confirmation of pregnancy, followed by adjustments according to TSH levels. For previously diagnosed women, serum TSH should be measured every 3-4 weeks during the first half of pregnancy and every 6 weeks thereafter. LT4 dose should be adjusted to maintain a serum TSH less than 2.5 mIU/L. TSH and free T4 levels should be measured every 3-4 weeks after every dosage adjustment.

Autoimmune thyroid disease without overt hypothyroidism has been associated with a higher miscarriage rate. Negro et al showed that euthyroid Caucasian women with positive anti-TPO antibodies treated with l-thyroxine during the first trimester had lower miscarriage rates when compared with those who were not treated. They also had lower incidence of premature delivery, comparable to women without thyroid antibodies. This will need to be confirmed by other studies, and, if confirmed, there will be an indication to treat euthyroid pregnant women who have thyroid antibodies. And an independent expert panelfound inconclusive evidence to recommend aggressive treatment of patients with TSH levels of 4.5-10 mIU/L. The Endocrine Society recommends thyroxine replacement in pregnant women with subclinical hypothyroidism; the American College of Obstetricians and Gynecologists does not recommend it as a routine measure.

I think that we should not forget our goal. Thyroid hormone is administered to supplement or replace endogenous production. Therefore, following subclinical hypothyroidism and during pregnancy and treating on a case-by-case basis is reasonable.

*Myxedema coma*
An effective approach is to use intravenous $T_4$ at a dose approximately 300-500 µg as a bolus in a single or divided dose, depending on the patient's risk of cardiac disease followed by 100 µg 24 hours later and then 50 µg daily IV or PO. Different strategy of substitution therapy is based on presumption, that initial bolus is used to satisfy enormous hormone deficiency. Corticosteroids are also given. Much more important is good general intensive care. Advanced age, high dose $T_4$ therapy, and cardiac complications had the highest associations with mortality.

As a conclusion I have to say that hypothyroidism is a good example of clinical condition, which could be very good controlled and patients treated according to guidelines have no side effects. On the other hand myxedema coma is a life-threatening disease, which could be avoided with right diagnosis on time. How could we improve the treatment of hypothyroidism? In most countries a good program for screening of neonatal hypothyroidism is established. The results are excellent. What is with screening of elderly people for hypothyroidism?

# 5. THYROID CANCER

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## 5.1 Abstract

Patients with thyroid carcinoma represent only 1% of patients with malignant diseases. Four main types of thyroid carcinoma are: papillary, follicular, medullary, and anaplastic carcinoma. Papillary carcinoma is the most common of all thyroid carcinomas (>80%). Generally, the overall prognosis of patients with papillary carcinoma is very good: 10-year survival rates are 80% to 95%. The most common finding at presentation is a mass or nodule. Fine needle aspiration biopsy is the most accurate and cost effective method for evaluating thyroid nodules. Another very useful diagnostic procedure is the investigation of thyroid with ultrasound. Preoperative laboratory assessments include thyroid function tests and serum calcium measurement. Hypothyroidism and hyperthyroidism should be ruled out before general anesthesia. Serum calcitonin measurement is indicated in patients with medullary thyroid carcinoma. Patients with increased calcitonin concentration should be screened for pheocromocytoma. Treatment of thyroid carcinoma is multimodal and comprises surgical procedure, radioiodine therapy and hormonal treatment. After surgical procedure and subsequent $^{131}$I treatment, all the patients receive thyroxine hormonal therapy. Patients with papillary or follicular carcinoma and higher risk of recurrence or progression should be on suppressive doses of thyroxine.

## 5.2 Incidence

Patients with thyroid carcinoma represent only 1% of patients with malignant diseases. Consequently, thyroid carcinoma is a rare disease nowadays. However, according to autopsy series a papillary microcarcinoma (i.e. papillary carcinoma with a diameter 10 mm or less) was detected in one third of adult population. The use of ultrasound and ultrasound-guided fine needle aspiration biopsies in the examination of the thyroid is steadily rising; therefore the incidence of papillary thyroid microcarcinoma is also increasing.

A radiation exposure increases the risk of malignancy from 30 to 50%. After Chernobyl nuclear disaster the incidence of childhood thyroid carcinoma increased in Ukraine and Belarus for at least 200-fold.

Females are more likely to have thyroid carcinoma than males. The majority of cases (about 80%) occur in women. Thyroid carcinoma can occur in any age group, although it is most common after age 30.

## 5.3 Symptoms

Most patients with thyroid carcinoma have no specific symptoms. The most common finding at presentation is a mass or nodule. Less than 1% of all thyroid nodules are malignant.

Symptoms of advanced thyroid carcinoma are: hoarseness, neck pain, and enlarged lymph nodes.

## 5.4 Diagnosis

The ultimate goal is to avoid operating on benign lesions whenever possible. The cornerstone in the assessment of a solitary thyroid nodule is fine needle aspiration biopsy (FNAB). FNAB is the most accurate and cost effective method for evaluating thyroid nodules. It helps to distinguish if a nodule is benign or malignant. This is often the only test needed to exclude thyroid carcinoma. Thyroid FNAB is generally considered safe, and almost never results in any complications. Ultrasound guided FNAB has higher sensitivity in comparison to free hand FNAB, especially for smaller nodules. FNAB and cytological examination of the specimen is reliable for the diagnosis of papillary, medullary and anaplastic carcinoma. But, a cytologist can not distinguish follicular benign tumor from follicular carcinoma. In follicular tumors the diagnosis can be obtained only by histopathology, therefore a surgical procedure should be performed.

Another very useful diagnostic procedure is the investigation of thyroid with ultrasound. This test is quick, accurate, cheap, painless, and completely safe. Ultrasound is an accurate method for determining the character of thyroid nodule (solid, cystic) and lymph nodes. This method is very useful in the long-term follow-up of patients.

Other diagnostic imaging studies are seldom used in the initial evaluation of a patient with thyroid malignancy.

## 5.5 Laboratory tests

Preoperative laboratory assessments include thyroid function tests and serum calcium measurement. Hypothyroidism and hyperthyroidism should be ruled out before general anesthesia. According to American and European Thyroid Association, measurement of thyroglobulin is indicated only for follow-up studies after initial treatment of carcinoma. However, thyroid antibody test should be performed before surgical procedure because of thyroid carcinoma. Also parathyroid adenoma should be ruled out before thyroid surgery.

Serum calcitonin measurement is indicated in patients with medullary thyroid carcinoma. Patients with increased calcitonin concentration should be screened for pheocromocytoma with a 24-urine collection for vanillylmandelic acid, metanephrine, and free catecholamines and for hereditary medullary thyroid carcinoma with RET proto-oncogene mutation testing.

## 5.6 Histopathology

Thyroid carcinoma is not a single disease. Four types account for more than 90% of thyroid malignancies. These four main types of carcinoma are: papillary, follicular, medullary, and anaplastic carcinoma.

*Papillary thyroid carcinoma* is the most common of all thyroid carcinomas (>80%). Papillary carcinoma typically arises as an irregular, solid or cystic mass that arises from otherwise normal thyroid tissue. This cancer has a high cure rate with ten-year survival rates for all patients with papillary thyroid cancer estimated at 80-90%. Lymph node metastases are

present in over 75% of cases. The presence of lymph node metastasis in these cervical areas causes a higher recurrence rate but not a higher mortality rate. Distant metastasis (spread) is uncommon, but lung and bone are the most common sites.

*Follicular thyroid carcinoma* is the second most common thyroid malignancy (~15 %). It occurs in a slightly older age group and is also less common in children than papillary carcinoma. Follicular carcinoma is more aggressive than papillary carcinoma. Lung and bone are potential sites of distant spread. Lymph node involvement is far less common than in papillary carcinoma (only in 8-13%).

*Medullary thyroid carcinoma* is the third most common of all thyroid carcinomas (about 5 to 8 percent). Unlike papillary and follicular thyroid carcinomas which arise from thyroid hormone producing cells, a medullary cancer of the thyroid originates from the parafollicular cells (also called C-cells) of the thyroid. These C-cells produce hormone calcitonin. Calcitonin is a tumor marker for medullary carcinoma. The other tumor marker is CEA.

Sporadic form of medullary carcinoma accounts for 80% of all cases, while 20% of cases have familiar form. Patient with familiar form may have multiple endocrine neoplasia (MEN) syndrome. MEN syndrome is a group of endocrine disorders which occur together in the same patient and are typically found in their families because they are inherited. MEN syndromes are caused by RET proto-oncogene germline mutations which are inherited in an autosomal dominant fashion. Patients with MEN-2A syndrome may have bilateral medullary carcinoma, *pheochromocytoma and hyperparathyroidism*. Patients with MEN-2B syndrome may have medullary carcinoma, pheochromocytoma, mucosal ganglioneuromas (tumors in the mouth) and a Marfanoid habitus.

Pheochromocytoma must be detected prior to any operation. The pheochromocytoma should be removed as soon as possible because of the risk of severe hypertensive episodes while the thyroid or parathyroid is being operated on.

*Anaplastic (=undifferentiated) thyroid carcinoma* is one of the most malignant tumors. Fortunately this tumor is not common. It represent about 1.5-5% of all thyroid carcinomas. Rapid tumor growth (doubling time one week!), invasion into surrounding structures and early distant metastases are characteristic for this neoplasm.

## 5.7 Treatment

Goals of initial therapy of differentiated thyroid cancer are: to remove the primary tumor, disease that has extended beyond the thyroid capsule, and involved cervical lymph nodes. Other goals of surgical procedure are to permit accurate staging of the disease, to facilitate postoperative treatment with radioactive iodine, to permit accurate long-term surveillance for disease recurrence and to minimize the risk of disease recurrence and metastatic spread.

Treatment of thyroid carcinoma is multimodal and comprises surgical procedure, radioiodine therapy and hormonal treatment. External beam radiotherapy and chemotherapy are indicated only in a minority of patients. External beam radiation therapy is used to palliate a metastatic or locally advanced disease.

### 5.7.1 Surgery

Thyroid lobectomy with isthmectomy is the "smallest" operation performed on the thyroid gland. It is indicated in solitary dominant nodules which are worrisome for cancer or those which are indeterminate according to cytology. This procedure is also appropriate for follicular adenomas, solitary hot or cold nodules, or goiters which are isolated to one lobe, Hürthle cell (=oncocytic) tumors, and some very small and non-aggressive thyroid cancers.

Total thyroidectomy is excision of the whole thyroid gland. Total thyroidectomy is procedure of choice for all thyroid cancers which are not small and non-aggressive. Many surgeons prefer this complete removal of thyroid tissue for all thyroid cancers regardless of the tumor type.

The optimal treatment of papillary microcarcinoma is still debatable. No agreement has been made about the optimal extent of thyroid or lymph node dissection. The most controversial issue is that of prophylactic lateral neck dissection. Because in Japan the use of radioiodine is strictly limited by law, and because lymph node metastases are very common in the lateral neck compartment, there are many proponents of prophylactic lateral node dissection for patients with PTMC among Japanese surgeons. However, in Western countries, a watch-and-wait policy for lateral node dissection in clinically and/or US-negative lymph nodes is widely accepted.

The surgeon must be careful of the recurrent laryngeal nerves which are very close to the back side of the thyroid and are responsible for movement of the vocal cords. Damage to this nerve causes hoarseness of the voice. It is usually temporary, but can be permanent. In experienced hands, this is a rare complication (about 1-2%). Another potential complication of thyroid surgery is hypoparathyroidism, which is due to damage to all four parathyroid glands or their circulation. Hypoparathyroidism never occurs after lobectomy, while it is uncommon complication (in about 1-2%) after total thyroidectomy.

### 5.7.2 Radioiodine

Radioactive iodine ($^{131}$I) is a radioactive isotope that is administered orally, in a liquid or capsule form. The majority of radioactive iodine is taken up by thyroid cells, since the thyroid normally uses iodine to make thyroid hormone. After thyroidectomy, a patient becomes hypothyroid. Because of lack of thyroid hormones the production of TSH by the pituitary is increased. Elevated TSH concentration stimulates uptake of the radioactive iodine into thyroid cells. Patients should be of the thyroid replacement hormones for 4-6 weeks and on a low iodine diet for at least one to two weeks prior to therapy. Radioiodine is usually given 4-6 weeks after surgical procedure and can be repeated after 6 months.

Alternative way for radioactive iodine therapy and thyroid hormone withdrawal is radioactive iodine therapy after recombinant human TSH (rhTSH) injections. With this treatment the patient avoids long lasting hypothyroidism.

### 5.7.3 Hormonal therapy

After surgical procedure and subsequent $^{131}$I treatment, all the patients receive thyroxine hormonal treatment. The dose may vary among patients and is adjusted to reach an appropriate TSH level. Patients with medullary carcinoma, anaplastic carcinoma or low-risk

follicular or papillary carcinoma should be on substitution doses of thyroxine (normal TSH, free-T3 and free-T4 concentration).

Patients with follicular or papillary carcinoma and higher risk of recurrence or progression should be on suppressive doses of thyroxine (TSH below 0.05 mU/L and normal free-T3 concentration; free-T4 concentration may be elevated in 25% of cases). The 3rd generation of TSH tests should be used. Latent hyperthyroidism because of suppressive therapy with thyroxine may cause side effects. The bone and heart are considered the organs at major risk.

## 5.8 Prognosis

Generally, the overall prognosis of patients with papillary carcinoma is very good: 10-year survival rates are 80% to 95%. Aggressiveness increases significantly in older patients and in patients with distant metastases.

Ten-year survival of the patients with follicular thyroid carcinoma is 70-95% and of patients with medullary carcinoma about 65%. In medullary carcinoma, overall 10-year survival rates are 90% when all the disease is confined to the thyroid gland, 70% with spread to cervical lymph nodes, and 20% when spread to distant sites is present.

On the other side, the patients with anaplastic thyroid carcinoma have dismal prognosis. Median survival of all patients is three months and for those patients with distant metastases only one month. Only 10% of patients survive longer than one year.

## 5.9 Surveillance

Follow-up visits typically include clinical examination, and blood tests measuring the serum thyroglobulin, TSH and free-T3 concentrations. The 3rd generation of TSH tests should be used.

Thyroglobulin is a tumor marker of carcinoma recurrence. The patients without disease have thyroglobulin concentration less than 2 ng/mL, if total thyroidectomy followed by therapy with radioiodine was performed. A concentration of thyroglobulin greater than 10 ng/ml is often associated with recurrence. In such cases ultrasound investigation of the neck region, chest X-ray and scintigraphy with $^{131}$I is performed in order to identify the site of recurrence.

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# 6. CELL SIGNALING IN CARCINOMA CELLS – DIFFERENCES BETWEEN HEALTHY CELLS AND TRANSFORMED CELLS

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## 6.1 Signaling networks – basic principle

Cell signaling in carcinoma cells as well as in healthy cells goes through interactive communication between various signaling networks. These networks are organized as cascades and there are points in these networks that are considered as nodes. Nodes may be genes or proteins which control the activity of network. Usually networks are called by node name.

Edges of network are responsible for initiative or executive actions. External stimuli may be initiative action. For example, radiation accident is initiative event, mutation in *RET* gene is node activation and activation of NFκB is executive action. This alteration of signaling pathway may cause thyroid cancer development.

Interconnections between networks are realized through nodes and to a lesser extent edges. Interconnections may be one to one, one to many and many to one relation. These interconnections can form feed-forward and feed-back loops. Activity of network goes through controlled ways and manners which are implicated in preservation of homeostasis.

In a living cell tens-to-hundreds of nodes are linked by hundreds-to-thousands of edges. This makes interpretation difficult to comprehend.

Understanding altered cell signaling pathways allows researchers to tailor new efficient and targeted therapies.

## 6.2 Main stages of carcinogenesis

Cell signaling is extensively involved in all stages of cancer development.

There are three main stages in cancer development:
- initiation
- promotion
- progression

Initiation phase comprises mutation of genetic material. Mutations in carcinoma tissue are diverse and complex. Tumor suppressor genes can be turned off by mutations and oncogenes can be activated by mutations. Tumor suppressor genes have the ability to slow down the cell division or cause cells to die at appropriate time. On the contrary, oncogenes are capable of speeding up the cell division or enable cells to live longer than they should. Most of

malignant tumors are caused by acquired mutations and tumors due to inherited mutations are less often.

During promotion transformed cell is allowed to multiply and creates a clone of transformed cells. In promotion cell growth is increased and in the same time apoptosis is decreased. These two changes are critical for development of cancer.

Additional changes of genetic material establish non-reversible malignant phenotype which is capable of progression. Tumor growth and metastasis are hallmarks of progression. For survival of tumor tissue an adequate supply of oxygen and nutritive substances is essential so angiogenesis is crucial for progression stage.

## 6.3 Signal transduction targeted cancer therapy principle

Cancer is biologically diverse disease. There is substantial evidence that multimodal therapy might be the most useful one. Combination of chemotherapeutic drugs, irradiation, inhibitors of signal transduction and antibodies against various cell surface antigens may be beneficial.

Inhibition of node molecule action in oncogenic signaling networks is goal of targeted cancer therapy. There are mono-target and multi-target drugs. Multi-target drugs are substances that are capable of acting through more than one mechanism. This is possible due to interconnections between signaling molecules. For inhibition of node molecules upstream molecules that are responsible for activation of node molecule may be potential efficient target for drug therapy.

Impaired apoptosis is impediment to cytotoxic therapy. Therefore chemopreventive agents are designed to block anti-apoptotic pathways and potentiate the effect of chemotherapeutic agents. Mechanism of their action is downregulation of cell survival.

## 6.4 Methods applied to research cell signaling pathways

The goal of these methods is to understand cellular mechanisms involved in development of disease. Investigation is aiming towards determination of difference between physiological and pathological phenotype.

### 6.4.1 Micro-array techniques- genome and transcriptome analysis

A deoxyribonucleic acid (DNA) micro-array is a collection of microscopic DNA nucleotide sequences attached to a solid surface, such as a glass, plastic, or silicon chip forming an array. This technique documents expression of thousands of genes on single microscope glass. However, quality of data relays to reliability of image analysis algorithms. There is extensive work in bioinformatics field to analyze micro-array data so that information acquired is reliable for biomedical purposes. This intention is not fully accomplished nowadays.

Instead of cDNA, mRNA can be analyzed so that the difference in transcription activity of certain genes in different phenotypes can be monitored.

### 6.4.2 Functional proteomics

This method is designed to elucidate cellular mechanisms and it is particularly useful in research of signal transduction. Concept of method is to preserve protein complexes in their functional mode. It is well known that distinct phenotypes can be accomplished by the same set of proteins. For this phenomenon cells use different signal transduction modules within the same multiprotein complexes. A large scale of different methodologies is applied in functional proteomics to analyze protein complexes (i.e. electrophoresis, mass spectrometry, immunoblotting, protein ID databases, protein expression, kinase activity, molecular interaction, cell culture models, isolated organ models, transgenic animal models). Functional proteomics offers strategies to employ all of these methodologies in order to elucidate signal transduction module responsible for specific phenotype.

### 6.4.3 DNA transfection assay

Transfection is technique of introducing foreign DNA into intact cells. DNA segment which we want to introduce to cultured cells is mixed in a buffer containing calcium phosphate/chloride and applied to cells in precipitated form. By some yet unknown mechanism cells become receptive to uptake of the foreign DNA. If DNA that is introduced in cultured cells, is an oncogene than cell transformation of cultured cells is expected. In the first transfection assays, DNA from variety of histological types of human tumors was introduced to BALB/ 3T3 cells and they were transformed to malignant phenotype. The results of this test ware variable and sometimes inconsistently reproducible. Advancement in cloning techniques contributed to more effective transfection assay. Variations of this assay are extensively used in screening for potential tumorigenesis initiators.

## 6.5 Signal transduction in carcinogenesis

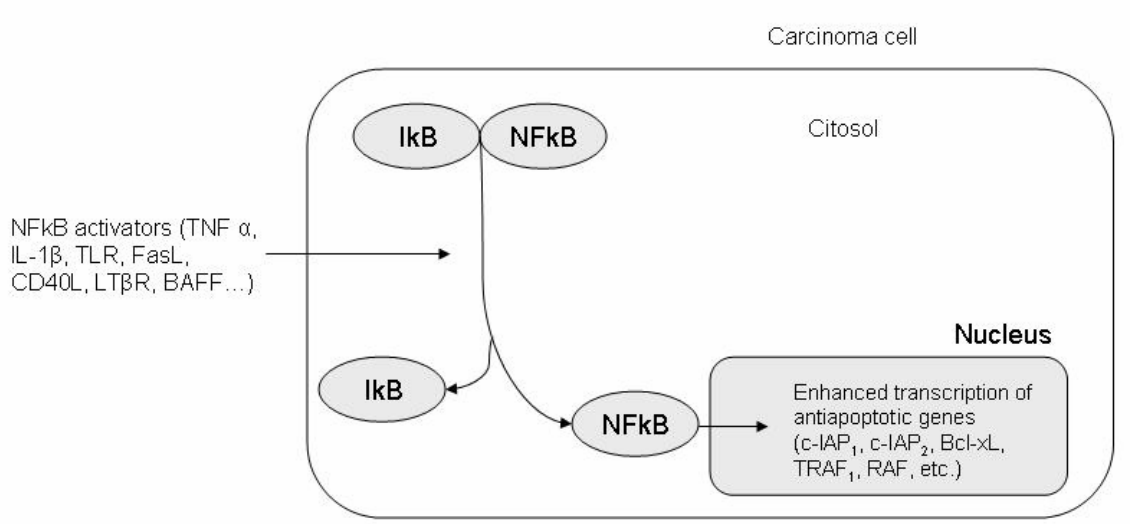
Successful alteration of signal transduction pathways is a key event which allows cells with altered phenotype to survive and establish a clone of neoplastic cells instead of undergoing cell death program. These decisions can be made through various cell signaling pathways of which some are more or less ubiquitous while others are regarded as more specific for tumor type.

## 6.6 Apoptosis

Maintaining of tissue homeostasis is regulated by apoaptosis. Impaired apoptosis is a crucial step in tumorigenesis. Apoptosis is common process of elimination of cells with altered genotype. It requires the activation of genetic suicidal programs which are manifested by caspase activation and subsequent membrane blebbing, DNA fragmentation, shrinking and condensation of the cell and its organelles. Inhibition of apoptosis is proved to cause cancer. There are numerous molecules involved in preventing or inducing apoptosis. One of most important anti-apoptotic pathway is NFκB activation.

## 6.7 Altered NFκB signaling in carcinogenesis enabling cancer cells to escape apoptosis –ubiquitous pathway present in numerous cancer cells as well as in thyroid carcinoma

Activation of the NFκB signaling cascade is constitutively present in human cancers cells and it promotes cancer cell survival.(Figure 6.1.) NFκB is inducible and ubiquitously expressed nuclear transcription factor. NFκB was discovered in 1986. It is governing transcription for genes implicated in some physiological events such as cell survival, cell adhesion, differentiation and cell growth. Also, it is also implicated in pathophysiological events like inflammation, autoimmune diseases and carcinogenesis (cancer promotion, progression and angiogenesis).




*IL-1β, interleukin 1 beta; c-IAP, cellular inhibitors of apoptosis; TRAF, TNF receptor-associated factors; TNF, tumor necrosis factor; FasL, Fas ligand; Bcl, B-cell lymphomas; Bcl-xL, "B-cell lymphomas" x ligand; TLR, toll like receptors; CD 40L, CD 40 ligand; BAFF, B-cell activating factor belonging to the TNF family*

***Figure 6.1.*** *General principle of NFkB signlingl pathway activation.*
*NFκB is transcription factor which is constitutively present in human cancer cells cytosol. It is inhibited by inhibitory proteins IκB. Various stimuli regarded as NFκB activators initiate phosphorylation of IκB proteins. Activated NFκB enters nucleus and initiates transcription of antiapoptotic genes. Antiapoptotic pathways are activated. Instead of undergoing cell death program, cancer cell is enabled to survive and establish a clone of neoplastic cells.*

### 6.7.1 NFκB cascade

In resting cells NFκB is retained in cell cytoplasm bounded to inhibitory IκB proteins.

The NFκB family of proteins consists of five members: NFκB1 (p50/p105), NFκB2 (p52/p100), c-Rel, RelA (p65) and RelB. These proteins share a Rel homology domain (RHD). RHD is responsible for binding to DNA and interactions with inhibitory factors (IκBs). IκBs retain NFκB in cytoplasm inactivated.

There are seven known IκB proteins: IκBα, IκBβ, IκBγ, IκBε, BCL-3 and two precursor proteins p100 and p105. All IκB proteins have in their sequence five to seven ankyrin repeats that are responsible for their interaction with RHD.

Activation of NFκB starts with inflammatory like signals. (Figure 6.1.) Some of those signals are TNF-α, IL-1β, FasL, TLRs, CD40L, LTβR (lymphotoxin β receptor) and BAFF (B cell activating factor belonging to the TNF family). There are several activation pathways of NFκB but the best designated ones are classical and alternative pathways. Different members, of NFκB and IκB proteins, are included in each of these pathways.

Classical pathway is most often started with the one of the well known inflammatory cytokines (TNF-α, IL-1β) or TLRs (Toll-like receptors). IκBα, IκBβ, IκBγ, p50 and p65 molecules are included in classical pathway. Alternative pathway may be started with one of following agents: LTβR, CD40L, BAFF. It comprises IκBα , p100, p52 and Rel B. Activated NFκB proteins induce genes whose products prevent apoptosis. Several Bcl-2 family members act as anti-apoptotic agents. Over activation of NFκB enables cancer cells to escape apoptosis.

Over activation of NFκB has been associated with acute lymphoblastic leukemia, lymphomas, breast cancer, cervical cancer, colorectal cancer, esophageal cancer, fibrosarcoma, head and neck cancers, mammary sarcoma, melanoma, lung carcinoma, ovarian cancer, pancreatic cancer, prostate cancer, squamos-cell carcinoma and thyroid carcinoma.

In research studies the activation of NFκB was confirmed by various methods. Immunohistochemical staining using anti-p65 antibody was positive in papillary, follicular and anaplastic cancer tissue specimens. P65 mRNA and protein expression increase was found in various thyroid cancer cell lines compared to normal thyroid cells. NFκB DNA binding and reporter assays showed the increased transcriptional activities in cultures of thyroid cancer cells.

### 6.7.2 Cancer therapy options through NFκB signaling pathway in thyroid cancer

Current results suggest that inhibition of the NFκB may be a promising strategy for undifferentiated and advanced thyroid cancer. Most of NFκB inhibitors target factors upstream IκB.

Novel NFκB inhibitor dehydroxymethyl-epoxyquinomicin (DHMEQ) enhances anti-tumor activity of taxanes in anaplastic thyroid cancer cells. It is low molecular weight NFκB inhibitor, which was derived from antibiotic epoxyquinomicin C.

The mechanism of DHMEQ action was proved to be the inhibition of NFκB translocation plus decrease of expression level of several downstream anti-apoptotic proteins and prolonged JNK activation. JNK is a member of MAP kinase superfamily which is involved in stress induced apoptosis.

Anaplastic thyroid carcinoma is highly undifferentiated thyroid carcinoma. It shows rapid invasive growth and has a strong metastatic potential to distant organs. Surgery alone is unlikely to cure this carcinoma.

Chemotherapy and radiation in combination are standard therapy for anaplastic carcinoma. Outcome of this malignancy in spite of multimodal therapy is still unsatisfactory. NFκB inhibitors are established as powerful chemo-sensitizers for both, chemotherapy and radiation. Both of these therapies are potent inducers of NFκB signaling pathway. Overactivation of this pathway is known mechanism of resistance to those therapies. Additional usage of NFκB

inhibitors allows reducing the dosage and alleviates side-effects. Taxanes are inhibitors of mitosis and combined with NFκB inhibitor DHMEQ have beneficial effect. This is an example of multimodal therapy as a successful therapy option.

## 6.8 Epidemiologic data for thyroid carcinoma

Annual incidence of thyroid cancer is low (0,5-10 per 100000) but in recent years it is growing faster than in any other type of carcinoma. The increase of incidence is around 6% in United States of America. Therefore management of thyroid carcinoma is becoming more important public health problem.

It is the most common endocrine tumor. Approximately 75% of all cases are women. Eighty percent of all thyroid carcinomas are papillary carcinomas, 15% follicular, 4% medullary and 1% anaplastic.

## 6.9 Cell origin in thyroid cancer

Thyroid cancer can be of follicular or parafollicular cell origin. Cancers that are of follicular cell origin are: papillary, follicular, Hűrthle cell cancer and anaplastic.

Medullary thyroid cell cancer has parafollicular cell origin. Follicular cell thyroid cancer accounts for 95% of all cases of thyroid cancer.

## 6.10 Frequent derangements in cell signaling pathways in thyroid carcinoma

In thyroid cancers four genetic changes are common:

- RET/PTC1, RET/PTC2, RET/PTC3 (papillary carcinomas)
- BRAF (papillary carcinomas)
- RAS oncogene (papillary carcinomas and follicular carcinomas)
- PAX8-PPARγ (follicular carcinomas)

These mutations rarely overlap in the same tumor. The first three of those pathways are related to NFκB pathway through MAPK (mitogen-activated protein kinase) pathway. (Figure 6.2.).

MAP kinase pathways are major signal transduction routes that transfer and amplify messages from the cell surface to the nucleus producing a range of cellular effects. One of those effects is cell proliferation. It has been shown in experimental work that MAP kinase induces NFκB mediated transcriptional activity.

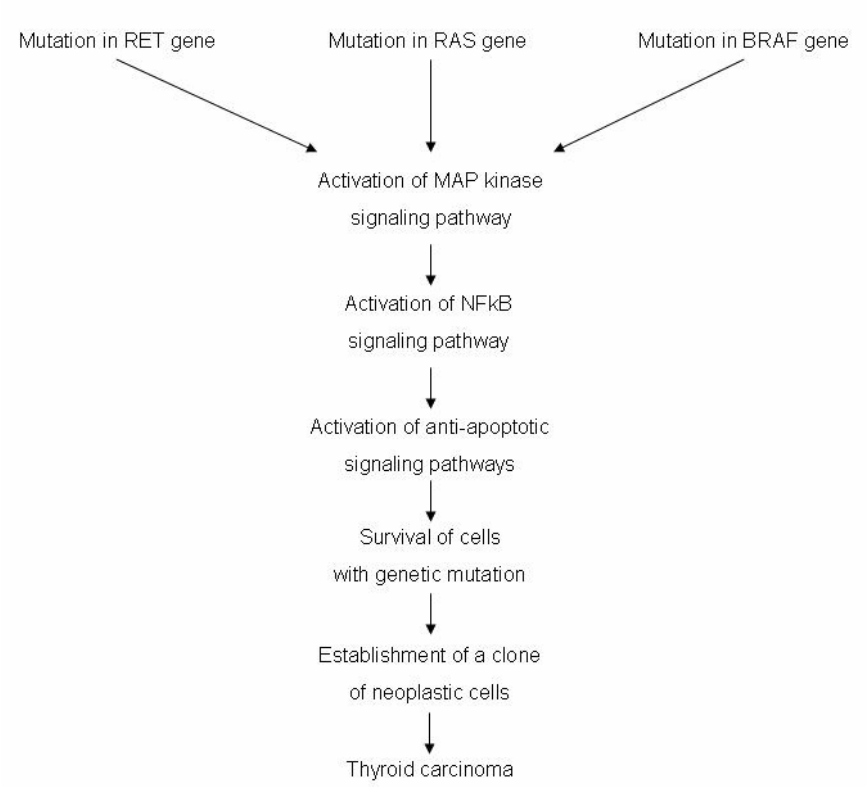



***Figure 6.2.*** *Thyroid cell carcinogenesis due to RET, RAS and BRAF gene mutation (activation of the NFκB signaling cascade)*

## 6.11 RET signaling pathway

The *RET* gene encodes a transmembrane receptor tyrosine kinase. Receptor tyrosine kinases are cell-surface molecules that transduce signals for cell growth and differentiation. The *RET* gene was confirmed as proto-oncogene by transfection assay.

Oncogenic activation is accomplished by cytogenetic rearrangement. DNA sequence of this gene was originally found to be rearranged within a 3T3 fibroblast cell line following its transfection with DNA taken from human lymphoma cells. Therefore, its name comes from expression "REarranged during Transfection".

In the 1988. year, Takahashi et al. cloned a RET proto-oncogene cDNA from a human monocytic leukemia cell line. The altered form of this gene is known as *RET/PTC* oncogene. *RET* gene is located on 10q11.2 chromosome. It is consisted of 21 exons comprising 3415 bp.

This gene codes RET protein consisted of five domains:

- Cadherin-like domain
- Cystein-rich domain
- Transmembrane domain
- Tyrosine kinase 1
- Tyrosine kinase 2

Ligands for RET protein are neutrotrophic factors of the glial-cell line derived neutrotrophic factor (GDNF) family which are GDNF, neurturin, artemin, and persefin. RET activation is mediated via different glycosyl phosphatidylinositol-linked GRF receptors. General structure is similar to other tyrosine kinase receptors but RET differs by the presence of cadherin domain in its extracellular region.

During human embryogenesis RET is expressed in yolk sac, trophoblasts, in developing kidneys and in developing central and peripheral central nervous systems. In an adult, it is expressed in several cell lines: spleen, thymus, lymph nodes, salivary glands, spermatogonia and thyroid tissue.

### 6.11.1 Activation of RET pathway in thyroid carcinoma

RET is normally expressed at a very low levels in thyroid follicular cells.

Chromosomal rearrangements linking unrelated gene and *RET* gene result in the aberrant production of chimeric forms of the receptor (RET/PTC) in thyroid cells that are constitutively active. RET fusion to different partners may be inter or intra-chromosomal. There are 15 types or even more of RET/PTC rearrangements reported. RET/PTC1 is the most common form. The distribution of RET/PTC rearrangement within each tumor is heterogenic. It can be present in almost all tumor cells (clonal RET/PTC) or in a small number of cells (non-clonal RET/PTC)

Germline mutations in the *RET* gene are associated with multiple endocrine neoplasia (MEN) types 2A and 2B, medullary thyroid carcinoma (MTC), Hirschsprung disease (HSCR; aganglionic megacolon), pheochromocytoma and neuroblastoma.

Somatic mutations generated due to radiation, can cause papillary thyroid carcinomas. Due to radiation RET can be rearranged with a number of fusion partners including D10S170 (H4), ELE1 and NTRK1. After Chernobyl radiation incident these mutations occurred more often.

It is found in about 10% to 30% of papillary thyroid carcinoma overall. It is found more often in children with thyroid carcinoma.

Mutations in medullary thyroid carcinoma (MTC) comprise different regions of *RET* gene than mutations present in papillary thyroid carcinoma and they are point mutations. Almost all inherited MTC and one fifth of sporadic MTC have mutation in *RET* gene. Most patients with sporadic MTC have acquired mutations in the *RET* gene. A screening for *RET* mutations is recommended in clinical management for all patients with MTC as soon as the diagnosis is confirmed. The detection of a *RET* oncogene mutation helps to identify family members at risk for developing MTC, which might be cured by prophylactic surgery at an early stage of disease.

### 6.11.2 Cancer therapy targeting RET signaling pathway

Vandetanib (ZD6474) inhibits RET, vascular endothelial growth factor receptor (VEGFR) and epidermal growth factor receptor (EGFR) tyrosine kinases. It was trialed in 30 patients with locally advanced or metastatic hereditary medullary thyroid cancer with germline mutation. Partial response was seen in 20 percent of patients and another 30 percent of patients maintained stable disease during treatment (172 days).

Motesanib is a multikinase inhibitor selectively targeting VEGF, PDGF, Kit and RET. It has antiangiogenic and direct antitumour activity. Early results suggest that motesanib has some effect in patients with advanced $^{131}$I-resistant differentiated thyroid cancer.

## 6.12 BRAF signaling pathway

RAF serine-threonine kinase family comprises three protein isoforms: ARAF, BRAF and CRAF. These isoforms are differentialiy expressed in cells. CRAF is expressed ubiquitously while BRAF is present in hematopoietic cells, neurons and testis. BRAF protein is involved in cell growth.

### 6.12.1 Activation of BRAF pathway in thyroid carcinoma

Constitutively BRAF is in inactive conformation. $BRAF^{V600E}$ protein has a different conformation than BRAF and it keeps protein constantly in catalytically competent conformation. Phosphorylation of MEK (mitogen-activated protein kinase kinase) is a result of its catalytic activity. Phosphorylated MEK starts the MAP kinase pathway.

BRAF mutation is associated with decrease in expression of genes required for thyroid hormone biosynthesis.

Mutations in BRAF gene are mostly point mutations. Almost all of them (95%) involve T-to A transversion in nucleotide 1799 and result in a valine-to-glutamate substitution at residue 600 (V600E). Other rare mutations in BRAF are K600E point mutation and AKAP9-BRAF rearrangement.

Mutations in *BRAF* gene are found in 30%-70% papillary thyroid carcinoma. It is not common in thyroid carcinoma in children, nor in carcinomas caused by exposure to radiation. Changes in *BRAF* gene result with carcinomas that have characteristic of aggressive growth and form distant metastases. In thyroid cancer survival studies $BRAF^{V600E}$ was found to be independent predictor of tumor recurerrence even in patients with stage I-II of the disease.
BRAF mutations are found in some anaplastic and poorly differentiated carcinomas.

### 6.12.2 Cancer therapy targeting BRAF protein

BAY 43-9006 (Sorafenib) is a multi-kinase inhibitor with potent activity against RAF, VEGFR-2, VEGEFR-3, PDGFRβ, FLT-3 and c-KIT kinase. It was tested for several cancer types including thyroid cancer. In progressive papillary carcinoma minimal or partial response was shown in some patients but complete results are yet to be released.

### 6.12.3 RAS signaling pathway

RAS family of proteins is encoded by three proto-oncogenes: *H-RAS, K-RAS and N-RAS.* Functionally, these proteins are involved in cell growth and malignant transformation. Any activation in RAS brings alteration in upstream or downstream signaling components. Activating mutation is found in approximately 30% of human cancers.

### 6.12.4 Activation of RAS pathway in thyroid cancer

RAS proteins are located in cell membrane where they are bound to guanosine diphosphate GDP. During activation of RAS protein GDP is released and guanosine triphosphate is bound. RAS-GTP initiates MAPK signal transduction pathway and PI3K/AKT pathway. Intrinsic GTP-ase activity of RAS protein rapidly deactivates RAS initated signal transduction. Mutation in *RAS* gene enables altered RAS protein to maintain activated formation. Result of impaired RAS function is upregulation of MAPK and PI3K/AKT pathways. PI3K/AKT pathway regulates fundamental cellular processes including glucose metabolism, cell survival, cell cycle progression, adhesion and motility.

RAS mutations are point mutations that result in increased affinity for GTP or decreased intrinsic GTP-ase activity.

These mutations are found in 10-15 % of papillary thyroid cancers and in 40-50 % follicular thyroid carcinomas. There is high prevalence of RAS mutations in undifferentiated thyroid carcinomas. RAS mutation is not exclusive for malignant tumors, it occurs in benign thyroid carcinomas, as well.

## 6.13 PAX8-PPARγ rearrangement

Paired box gene 8 protein is encoded by the *PAX8* gene. This gene is a member of paired box (PAX) family of transcription factors which encodes nuclear protein involved in thyroid follicular cell development and expression of thyroid-specific genes.
Peroxisome proliferators – activated receptors (PPARs) are a group of nuclear receptor proteins that function as transcription factors. PPARs play essential roles in regulation of cellular differentiation, development and metabolism (carbohydrate, lipid, protein) and tumorigenesis.

Paired box 8 gene is fused by interchromosomal translocation with peroxisome proliferator-activated receptor γ (PPAR γ). Function of resulting protein is not known. This mutation is present in 35% of follicular thyroid carcinoma and in a lesser extent in Hurthle-cell carcinoma and follicular adenoma.

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# 7. BASIC CONCEPTS AND MISCONCEPTS IN CLINICAL AND DIAGNOSTIC ENDOCRINOLOGY

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Many of the major principles governing clinical endocrinology taken for granted today were established during the last 50 years. In fact, during this period, twelve individuals received or shared Nobel Prizes for discoveries directly related to endocrinology and hormone assays. This group can be expanded to 20 if we include Nobel Prizes given for discoveries of prostaglandins, growth factors and neurotransmitters. From the tremendous amount of information gathered during half a century, we have selected six topics that had a particular impact on the practice of endocrinology and therefore on hormone measurements. These topics are

- the mediation of hormonal action through extra- and intracellular receptors
- the evolution of the concept of free hormones
- the discovery and identification of brain neuropeptides
- the role of post-translational modifications
- diagnostic significance of fetal mRNS, DNS in maternal blood
- development of laboratory tests
- the role of molecular endocrine diagnostics

In this chapter, meant for post-graduate training, a special emphasis will be given to governing endocrine concepts on the field of thyroid diagnostics.

Laboratory evaluation of thyroid status centers on chemical measurements of thyroid gland secretory products present in the circulation, assessment of the hypothalamic-pituitary-thyroid axis, and measurement of related molecules that affect thyroid gland function, such as thyroid binding proteins and autoantibodies. During the past 10 to 15 years, many advances in thyroid testing have been made including the development of highly sensitive assays for thyroid stimulating hormone (TSH), new approaches to measuring free hormone concentrations, and automation of most routine thyroid assays.

## 7.1 The mediation of hormonal action through extra- and intracellular receptors (TRs):

TRs belong to a large superfamily of nuclear hormone receptors (NRs) that include the steroid hormone, retinoic acid, vitamin D and peroxisomal proliferator receptors (PPARs). TRs are ligand-regulatable transcription factors that bind thyroid hormone (TH) as well as specific DNA-sequences in enhancer elements (thyroid hormone response elements (TREs) located in the promoters of target genes (Figure 7.1.). Like other NRs, they have a central DNA-binding domain containing two “zinc finger” motifs and a carboxy-terminal ligand-binding domain (LBD). The hinge region between these two domains contains a nuclear localization sequence. The carboxy-terminal region also contains multiple contact surfaces that are important for heterodimerization with its partner, retinoid X receptor as well as protein–protein interactions with co-repressors and co-activators. There are two TR two genes, THRA

and THRB, located on human chromosomes 17 and 3, respectively. The THRA gene generates two mature mRNAs by alternative splicing that encode two proteins, TR_-1 and c-erbA_-2, that differ in their carboxytermini. TR_-1 is a bona fide receptor whereas c-erbA_-2 is unable to bind TH and blocks TR-mediated transcription. The THRB gene encodes two TR isoforms, TR_-1 and TR_-2. The major TR isoforms bind T3 with high affinity and mediate thyroid hormone-regulated transcription. They also have highly conserved DNA-binding and ligand-binding domains. TRs bind to distinct thyroid hormone response elements (TREs) typically located in the promoter regions of target genes. TREs usually contain two or more hexamer half-site sequences of AGGT(C/A)A arranged in tandem arrays. TREs vary in the primary nucleotide sequences of half-sites as well as their number, spacing and orientation.

Unlike steroid hormone receptors, TRs regulate transcription both in the absence or presence of ligand. In positively regulated target genes, unliganded TRs bind to TREs and repress basal transcription. So far, two major corepressors have been identified: nuclear receptor co-repressor (NCoR) and silencing mediator for retinoic acid receptor (RAR) and TR (SMRT). These co-repressors preferentially interact with unliganded TRs and RARs and repress the basal transcription of target genes in the absence of their respective hormones (1).

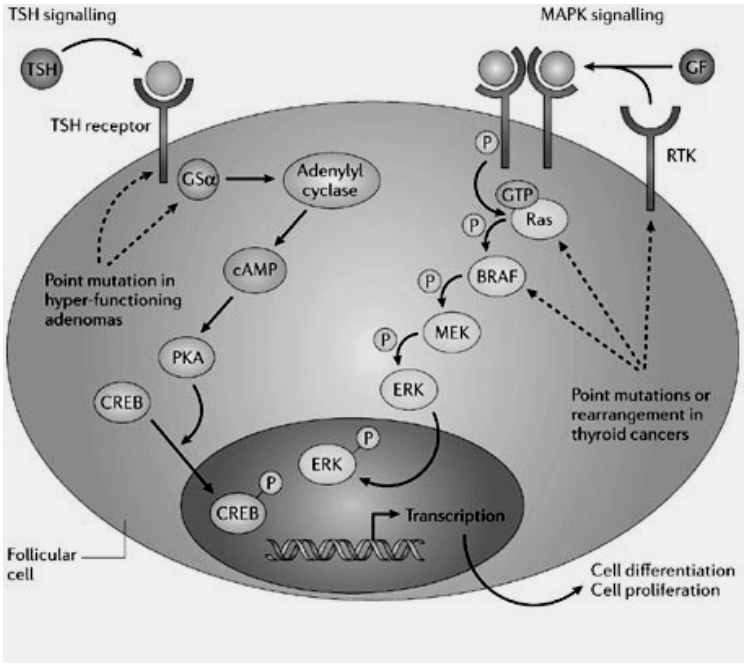


***Figure 7.1.*** *TSH receptor signaling*

## 7.2 The free hormone concept

Assays for determining fT3 and fT4 have been developed for more than 30 years. fT3 or T4 testing in clinical laboratories is usually accomplished by immunoassay (IA) methods or physical separation approaches. Immunoassays are performed in the presence of protein-bound T3 and T4, whereas physical separation methods employ techniques such as equilibrium dialysis, ultra filtration, or gel filtration to separate the free hormone from protein-bound T3 (TT3) and T4 (TT4). Due to the high technique requirements and cost of physical separations, most clinical laboratories routinely use direct analogue immunoassays for fT3 and fT4 measurements, which are typically performed on immunoassay platforms.

Nevertheless, these immunoassays are all binding protein dependent to some extent and thereby susceptible to various interferences and uncertainties. The interferences may involve drug interactions, cross-reactivity of antibodies, sensitivity to unusual binding proteins, influence of free fatty acids (FFA), and endogenous or exogenous binding inhibitors. Accordingly, many researchers have questioned the accuracy, validity, and reliability of direct analogue immunoassays (2).

The thyroid hormones T4 and T3 travel in the serum reversibly bound to transport proteins, which are synthesized in the liver. These proteins bind over 95% of the circulating hormone, the remaining 5% being carried primarily by lipoproteins. This leaves approximately 0.03% of the total thyroxine and 0.3% of the total triiodothyronine which circulates in the free form. The major thyroid binding protein is thyroxine binding globulin (TBG), followed by transthyretin (thyroid binding prealbumin,TBPA) and albumin. TBG binds 65% of the circulating T4 and 75% of T3, TBPA binds 10% of each, and albumin accounts for 20% of the T4 and 10% of T3. Circulating concentrations of TBG can be influenced by hormones, drugs, genetic factors and disease. Changes in carrier protein concentrations can elicit changes in total thyroid hormone concentration in the absence of thyroid disease. These changes in circulating levels of T4 and T3 occur without concomitant changes in fT4 and fT3 concentrations. This occurs because the bound hormone serves as a reservoir for the biologically active free form, and dissociation of hormone from its binding protein occurs rapidly (3).

TBG has a fairly high carbohydrate content (23%), which is increased under the influence of estrogens, as observed in pregnancy and during contraceptive use, and leads to increased clearance of the molecule and higher circulating concentrations of TBG. This is accompanied by increases in total T4 and T3, whereas free T4 and T3 levels do not change. In individuals with TBG deficiency, total T4 and T3 concentrations are decreased but the free hormone (fT4 and fT3) levels do not change, indicating that fT4 and fT3 concentrations are independent of TBG fluctuations. Genetic variants of transthyretin with increased affinity for T4 show increased concentrations of T4 but normal fT4. In the genetic disease familial dysalbuminemic hyperthyroxinemia (FDH), the serum albumin has increased affinity for T4. These individuals are euthyroid and have elevated T4 concentrations but normal fT4. These observations show that total thyroid hormone concentrations are influenced by many factors even in euthyroid individuals, and that measurement of free hormone is almost always preferable (4).

The free hormone hypothesis was already questioned in the early 1960s when it was realized that the dissociation half-time of albumin-bound testosterone was of the order of 1 s compared to 20 s for SHBG-bound testosterone. It was proposed that such a short dissociation time could allow part of the albumin bound fraction to be available for tissue uptake. A few years later, it was shown that the very rapid and intensive uptake of circulating T4 by the liver was hard to explain based on the free hormone hypothesis. A more recent theory seemed to invalidate the free hormone hypothesis (5). This theory was based on the existence of a specific mechanism in the microcirculation of tissues that was able to enhance the dissociation of protein bound hormones and drugs from their plasma binding proteins and therefore increase their availability for tissue uptake. This concept of bioavailability of protein bound hormones was vigorously and sometimes acrimoniously contested. It nevertheless gained credibility and soon started to appear (at least for steroid hormones) in major endocrinology textbooks. It is worth pointing out, however, that even based on the same data, the concept (and measurement) of “bioavailable” thyroid hormones has never been adopted in

clinical practice. The theory of free hormones has been important in the development of assays directly measuring or indirectly evaluating the free fraction of numerous hormones like fT4, fT3, free plasma cortisol or testosterone, etc.

## 7.3 The discovery and identification of brain neuropeptides

The hypothesis of a hypothalamic control of secretion of the anterior pituitary gland dates back to the end of the 1940s when G.W. Harris first postulated the presence of a neurovascular link between the neurohypophysis and the adenohypophysis. Shortly thereafter, C.H. Sawyer demonstrated the involvement of the central nervous system in the control of gonadotropin secretion. Despite strong circumstantial evidence favoring hypothalamic control of the pituitary, this hypothesis remained speculative until one was able to prove the existence of such specific chemical entities capable of controlling the release of pituitary hormones. This lifetime work has been achieved by Roger A. Guillemin and Andrew V. Schally who identified and characterized most of the major hypothalamic neurohormones. Both shared the 1977 Nobel Prize for this important achievement. The identification of the hypothalamic hormones has greatly advanced our understanding of the integration of endocrine functions. This discovery has helped to elucidate diseases (tertiary disorders of peripheral glands) and generate new therapeutic approaches like LHRH treatment of advanced prostate cancer, etc. It has also been at the basis of numerous functional tests of pituitary reserve and as such has significantly influenced the endocrinology laboratory.

Thyroid hormones are key regulatory factors of the normal brain developmental program possibly throughout fetal and extra uterine life. In addition to its role in cellular metabolic activity, TH is critically involved in growth, development, and function of the central nervous system. Environmental factors that interfere with thyroid function or TH action may produce deleterious effects on brain development by interfering with TH action in the developing brain. All degrees of iodine deficiency affect thyroid function of the mother and the neonate as well as the mental development of the child. Experimental studies and clinical research have clarified not only the correlation between nervous system maturation and thyroid function during early development stages but also excess and deficient THs can cause permanent anatomo-functional alterations to the nervous system. If the TH deficiency occurs early in pregnancy, the offspring display problems in visual attention, visual processing and gross motor skills. Also the effect of THs is known on the putative neurotransmitter systems that regulate mood and behavior.

The action of thyroid hormones (THs) in the brain is strictly regulated, since these hormones play a crucial role in the development and physiological functioning of the central nervous system (CNS). Disorders of the thyroid gland are among the most common endocrine maladies. Therefore, the objective of this study was to identify in broad terms the interactions between thyroid hormone states or actions and brain development. THs regulate the neuronal cytoarchitecture, neuronal growth and synaptogenesis, and their receptors are widely distributed in the CNS. Any deficiency or increase of them (hypo- or hyperthyroidism) during these periods may result in an irreversible impairment, morphological and cytoarchitecture abnormalities, disorganization, maldevelopment and physical retardation. This includes abnormal neuronal proliferation, migration, decreased dendritic densities and dendritic arborizations. This drastic effect may be responsible for the loss of neurons vital functions and may lead, in turn, to the biochemical dysfunctions. This could explain the physiological and behavioral changes observed in the animals or human during thyroid dysfunction. It can be hypothesized that the sensitive to the thyroid hormones is not only remarked in the

neonatal period but also prior to birth, and THs change during the development may lead to the brain damage if not corrected shortly after the birth. Thus, the hypothesis that neurodevelopmental abnormalities might be related to the thyroid hormones is plausible. Taken together, the alterations of neurotransmitters and disturbance in the GABA, adenosine and pro/antioxidant systems in CNS due to the thyroid dysfunction may retard the neurogenesis and CNS growth and the reverse is true. In general, THs disorder during early life may lead to distortions rather than synchronized shifts in the relative development of several central transmitter systems that leads to a multitude of irreversible morphological and biochemical abnormalities (pathophysiology). Thus, further studies need to be done to emphasize this concept (6).

## 7.4 The role of post-translational modifications

Most of the proteins that are translated from mRNA undergo chemical modifications before becoming functional in different body cells. The modifications collectively, are known as post-translational modifications. The protein post translational modifications play a crucial role in generating the heterogeneity in proteins and also help in utilizing identical proteins for different cellular functions in different cell types. How a particular protein sequence will act in most of the eukaryotic organisms is regulated by these post translational modifications. Post translational modifications occurring at the peptide terminus of the amino acid chain play an important role in translocating them across biological membranes. These include secretory proteins in prokaryotes and eukaryotes and also proteins that are intended to be incorporated in various cellular and organelle membranes such as lysosomes, chloroplast, mitochondria and plasma membranes. Expression of proteins is important in diseased conditions. Post translational modifications play an important part in modifying the end product of expression and contribute towards biological processes and diseased conditions. The amino terminal sequences are removed by proteolytic cleavage when the proteins cross the membranes. These amino terminal sequences target the proteins for transporting them to their actual point of action in the cell. Protein post translational modifications may happen in several ways, e.g. like glycosylation, acetylation, alkylation, methylation, biotinylation, acylation, glutamylation, glycylation, isoprenylation, lipoylation, phosphopantetheinylation, phosphorylation, sulfation, selenation, C-terminal amidation, etc.

Hyperestrogenemic states, including pregnancy, cause an increase in serum T4-binding globulin (TBG) concentrations and an increase in the proportion of TBG molecules with greater anodal mobility on isoelectric focusing, are indicating greater sialic acid content. The rate of in vivo metabolism of TBG is dependent on its sialic acid content. The increased proportion of TBG molecules with higher sialic acid content contributes to the increase in the serum TBG concentration in hyperestrogenemic states (7).

## 7.5 Diagnostic significance of fetal mRNS, DNS in maternal blood

During pregnancy, fetal and maternal cells of different types are transferred between mother and fetus. This phenomenon of fetal cell transfer to the mother is referred to as fetal microchimerism and has been most readily shown by the unexpected detection of male markers in the maternal circulation and/or maternal tissues. Furthermore, data have shown that fetal cells can persist in the maternal circulation for more than 20 years. This cellular situation has been compared with chronic graft vs. host disease, where donor lymphocytes may react with the host tissues. Indeed, chronic graft vs. host disease shares many clinical and

pathological features of some autoimmune diseases. Because the autoimmune diseases are found characteristically in females, it has been suggested that fetal microchimerism might be involved in their etiology. Of additional interest is the fact that autoimmune diseases are often suppressed during pregnancy and exacerbate postpartum. It was, therefore, hypothesized that fetal cells may be important in influencing autoimmune thyroid disease in pregnancy and postpartum. In fact, it has been demonstrated the expected persisting peripheral blood fetal microchimerism in women as well as excessive persisting intrathyroidal fetal microchimerism in patients with Graves' disease. It has been proposed that this intrathyroidal fetal microchimerism is a candidate mechanism for the modulation of Graves' disease in pregnancy and the postpartum period.

Because papillary thyroid cancer (PTC) is more frequent in women, the role of persisting fetal male cells in this tumor has been investigated. Tumor tissue specimens were obtained from 63 women with PTC who had a male pregnancy before the diagnosis. Male cells, identified by PCR amplification of a male-specific gene, the sex-determining region Y, was detected in 47.5% of women. By fluorescence *in situ* hybridization (FISH) analyses, the total number of microchimeric cells was significantly higher in neoplastic tissue than in controlateral normal sections. In conclusion, fetal microchimerism has been documented in a high proportion of women with PTC. The immuno-FISH studies indicate that $CD45^{+}$/MHC $II^{-}$ male cells found in neoplastic tissues might be committed to destroy tumor cells, whereas $Tg^{+}$/MHC $II^{-}$ cells could have a repair function. Finally, microchimeric cells negative for either CD45 or Tg could have "progenitor-like" properties able to transdifferentiate in different cellular types. Although a pathogenetic mechanism cannot be excluded (Figure 7.2.), the whole of the present results indicates a protective role of microchimerism in thyroid cancer (8).

- Fetal microchimerism has been documented in a high proportion of women with PTC.
- CD45+/MHC II– male cells found in neoplastic tissues might be committed to destroy tumor cells.
- Tg+/MHC II– cells could have a repair function.
- Microchimeric cells negative for either CD45 or Tg could have "progenitor-like" properties able to differentiate in different cellular types.

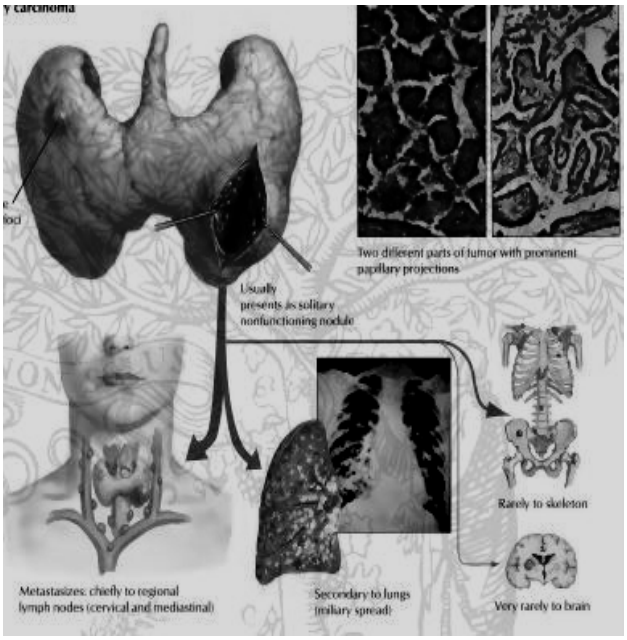


***Figure 7.2.*** *Microchymerism in papillary thyroid cancer.*

## 7.6 Development of laboratory tests

There have been several milestones that have led to the proliferation of modern immunoassays. The development of monoclonal antibodies from mouse hydridoma cells by Millstein and Kohler (Nobel Prize in 1984) enabled the production of high quantities of antibodies with well characterized epitope specificity. The first homogenous immunoassay (no separation step required) was the Enzyme Multiplied Immunoassay Technique (EMIT), which enabled adaptation of this assay onto automated chemistry platforms. EMIT was also one of the first immunoassay that made use of non-isotopic labels. Other non-isotopic labels became available such as chemiluminescence to improve the analytical sensitivity of immunoassays. The advantages of high-sensitivity immunoassays have created expanded diagnostic roles for some existing assays such as thyroid stimulating hormone for hyperthyroidism, C-reactive protein for cardiovascular risk assessment, and other applications. The development of instrumentation capable of automated heterogeneous immunoassays (separation step to improve sensitivity) has enabled movement of this technology from the “special chemistry” sections of a clinical laboratory into the "core" laboratory with other high-volume testing. Today, immunoassays play a prominent role in the analysis of many clinical laboratory analytes such as proteins, hormones, drugs, and nucleic acids. The future involves development of assays with higher sensitivities which will enable the discovery of new biomarkers for disease diagnosis, and technology that will enable simultaneous multimarker analysis of tests whose needs are naturally grouped together (e.g., cytokines and allergens).

Immunoassay technology continues to evolve with new applications and improved analytical platforms and more careful preanalytical conditions (9). The future appears to be headed in two directions: continued improvement in immunodetection methods for very high-sensitivity applications, and multiplex analysis. Improvements in analytical sensitivity will likely lead to the discovery of new analytes for disease detection. One technique enhancement that has promise in this regard is the use of DNA as a label for antibodies in non-competitive immunoassays, as amplification of the signal can be accomplished through polymerase chain reaction (PCR). While this technique has been sparingly used for several years, recent enhancements have the promise of detecting extremely low concentrations in serum (attamoles, $1\times10-18$). The binding of oligonucleotides to nanoparticles (e.g., 10–30 nm) has particular promise.

The “bar-code assay” describes an ultra-sensitive protein detection with gold nanoparticles and single-stranded oligonucleotide barcodes (bio-barcode detection). After bio-barcode release, the bio-barcodes can be hybridized to an oligonucleotide substrate array on one end and a gold nanoparticle probe (with oligonucleotides) on the other end. Silver deposition enhancement on the gold probe can then be used to amplify the optically detected signal. As shown in the right corner above, another path for the detection of the released bio-barcodes is to use fluorescence or chemiluminescence to detect the signal.

In the bead-based flow cytometry for immunoassays, capture antibodies are immobilized on distinct bead populations of micropheres. Different bead sets loaded with specific capture antibodies are pooled together and incubated with the sample and fluorescently labeled detection antibodies. Formation of the different sandwich immunocomplexes happens on the different bead populations are measured with flow cytometry.

Generally, immunoassays employ a hormone labeled with radioactivity, fluorescence, or chemiluminescence for thyroid quantification. In recent years, gas chromatography mass spectrometry (GC-MS), isotope dilution liquid chromatography mass spectrometry (LC-MS), and tandem mass spectrometry (LC-MS/MS) have been used to measure TT3 and TT4 in human serum or plasma (Figure 7.3.). Mass spectrometry with better specificity and less interference provides a more specific method with less interference than immunoassays for thyroid hormone quantification. Ultrafiltration at 25 °C followed by tandem mass spectrometric analysis gives almost identical results for fT4 to those obtained by performing equilibrium dialysis at 37 °C.

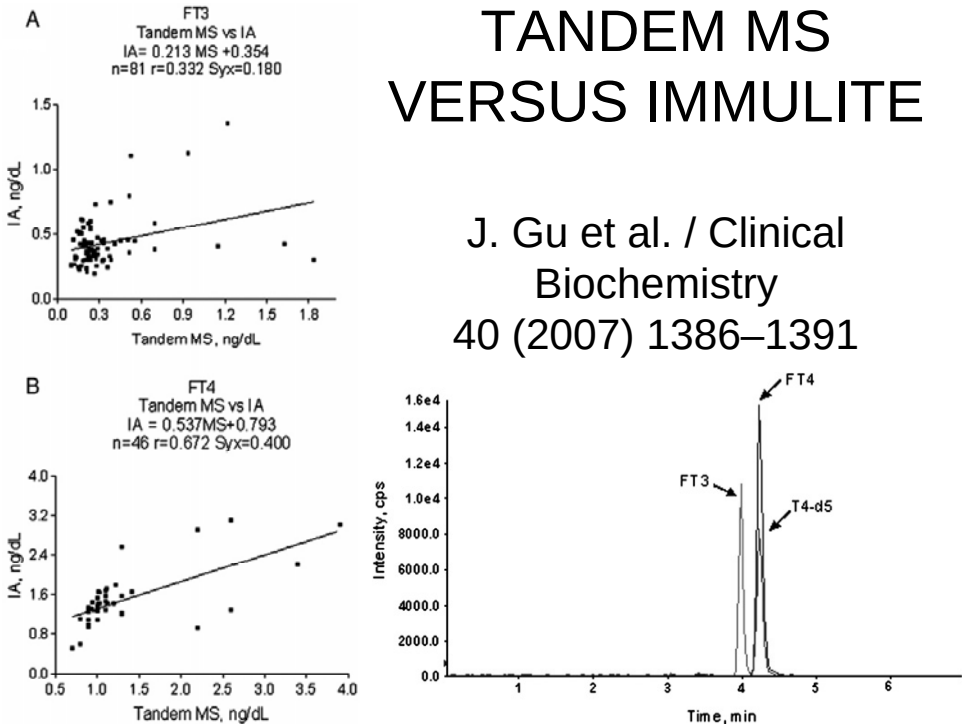


***Figure 7.3.*** *Mass spectrometric analysis of thyroid function*

## 7.7 The role of molecular endocrine diagnostics:

Thyroid carcinoma is the most common endocrine neoplasm and the seventh most frequent human malignancy. It can be distinguished into differentiated and undifferentiated. Differentiated tumors include those arising from thyrocytes, i.e. papillary and follicular carcinoma, while medullary carcinoma originates from parafollicular or C cells. Anaplastic carcinoma comprises undifferentiated tumors. The factors inducing thyroid carcinoma development are not fully understood despite some well-established associations, such as the one between ionizing radiation and papillary carcinoma and that between iodine deficiency and follicular carcinoma. Genetic investigations of differentiated thyroid tumors have documented mutation of genes involved in the regulation of MAP kinase pathway activation in papillary carcinoma, and of genes involved in the regulation of the PI3 kinase pathway in follicular carcinoma.

Analysis of their clinical course and of positivity for mutations has demonstrated that prognosis is greatly affected by the type of mutated gene. Genetic investigations therefore have the potential to direct diagnosis, but especially to tailor therapy and follow-up to the individual patient and even the individual gene. Anaplastic carcinoma, a highly aggressive, undifferentiated form, can arise as such or else be the de-differentiated progression of a papillary or a follicular carcinoma. It displays a mutated tumor suppressor gene (p53), which is crucial in the regulation of cell apoptosis, in addition to the mutations found in papillary and follicular forms. Medullary carcinoma is a malignant neoplasm with an intermediate clinical course between differentiated and undifferentiated forms. It manifests more frequently as a sporadic neoplasm or as a familial MEN. The latter is a high-penetrance, autosomal dominant hereditary disorder. Identification of the gene responsible for medullary carcinoma

has radically changed the diagnostic approach to the familial forms, enabling early neonatal diagnosis of mutation carriers and of the disease, and early surgical approach by prophylactic thyroidectomy. Genetic studies have significantly affected the endocrinologist's diagnostic approach, as in the case of medullary carcinoma; over the next few years they are expected to provide further information to tackle papillary and follicular thyroid carcinoma.

Molecular techniques for the diagnosis of cancer in thyroid nodules are most needed for patients in whom conventional FNAB cytology yields an indeterminate diagnosis of 'follicular neoplasm', which are patients who have either adenomas or low-grade carcinomas. In a recent study, cytological findings of follicular neoplasm on FNAB were confirmed by the histological diagnosis of follicular carcinoma in only 5%, while 81% had follicular adenomas on histological evaluation. In contrast, cytological diagnosis of papillary carcinoma was confirmed by the histological diagnosis in 93%. Additionally, cytological examination may not provide a conclusive diagnosis when biopsy samples are found to be 'unsatisfactory', 'intermediate' or 'suspicious'. A molecular-based diagnostic approach may provide a more objective method to assure reliable diagnosis in these cases.

Postoperative surveillance of patients with thyroid cancer has several limitations. Since the 1970s, serum thyroglobulin has been the established tumor marker of residual and recurrent differentiated thyroid carcinoma. In recent years, recombinant human TSH (rhTSH) has been administered to avoid the necessity for thyroxine withdrawal hypothyroidism, previously necessary to achieve adequate TSH stimulation. However, the use of rhTSH is expensive and may potentially stimulate cancer growth. Furthermore, antithyroglobulin antibodies can be detected in up to 20% of patients with thyroid cancer and may interfere with the serum thyroglobulin immunoassay, although the presence of antithyroglobulin antibodies may perhaps be a sign of residual thyroid tissue itself, leading to appropriate diagnostic and therapeutic consequences. Therefore, a molecular-based, sensitive and convenient screening test to diagnose recurrent thyroid cancer, not requiring TSH stimulation or the absence of thyroglobulin antibodies, would be useful.

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# 8. INAPPROPRIATE TSH SECRETION

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When hypothalamic-pituitary function is normal, a log/linear inverse relationship exists between serum TSH and free $T_4$ concentrations, because thyroid hormone produces a negative feedback inhibition of pituitary TSH secretion. Inappropriate secretion of thyroid stimulating hormone (ISTSH) is defined as elevated free thyroxine ($fT_4$) and non-suppressed thyrotropin (TSH) concentrations (1). It can be caused by a heterogeneous group of disorders and usually means a differential diagnostic problem. It is a rare entity. The prevalence of ISTSH was 85 of 13,286 (0.64%) thyroid samples using the Immulite 2000 assay (2). Causes behind the ISTSH may be preanalytical, analytical problems and organic disorders (Table 8.1.)

***Table 8.1.*** *Differential diagnosis of inappropriate secretion of TSH*

| I. Preanalytical causes | II. Analytical problems | III. Organic disorders |
|---|---|---|
| a. Intermittent L-thyroxine therapy | a. Interfering antibodies to thyroxine and triiodothyronine | a. TSH producing pituitary adenoma |
| b. Initial treatment of primary hypothyroidism | b. Drug interactions | b. Resistance to thyroid hormone |
| c. Amiodarone treatment | | c. Early phase of subacute thyroiditis (thyroid cell destruction) |

Most preanalytic variables and interfering substances have little effect on serum TSH measurement but may influence the binding of thyroid hormones to plasma proteins and thus decrease the diagnostic accuracy of total and free thyroid hormone measurements. An adequate review of the patient's history can usually rule out medications as causes of ISTSH. An increase in thyroxin-binding globulin leading to elevated total $T_4$ levels can be excluded by measuring the free $T_4$ and $T_3$ levels.

*I. Intermittent L-thyroxine therapy or unstable thyroid condition – elevated TSH parallel to high $fT_4$ in patients on L-thyroxine therapy.*

This phenomenon is common in the clinical practice. The patient may forget to take the medicine or decrease the thyroxine dose. Before the next follow-up visit these patients occasionally try to compensate it, increasing the dose. Serum TSH values are misleading during transition periods of unstable thyroid status (early phase of treating hypothyroidism or changing the dose of L-thyroxine). It takes 6-12 weeks for pituitary TSH secretion to re-equilibrate to the new thyroid hormone status. Unstable thyroid function may also occur during an episode of subacute thyroiditis when thyroid hormone is released from destructed thyroid cells (III. c.).

The next problem is when the patient's compliance is good but the TSH value still cannot be normalized by L-thyroxine therapy even with supraphysiologic $fT_4$ value.

**Case 1.** A 20-year-old man suffered from congenital hypothyroidism due to thyroid agenesis. In childhood, he was treated by a combined thyroid hormone product (thyroxine and triiodothyronine). After the withdrawal of the drug from the market, he received thyroxine preparation. His TSH value was not normalized despite the elevated $fT_4$ level. Sella MRI was normal. The addition of triiodothyronine was effective to maintain normal TSH concentration (Table 8.2.).

***Table 8.2.*** *Thyroid function test with different hormone replacement therapy in a case of congenital hypothyroidism*

| Treatment | TSH (mU/l) | FT4 (pmol/l) | $TT_3$ (nmol/l) |
|---|---|---|---|
| $T_3$+$T_4$ | 0,5 | 12,1 | 2,65 |
| $T_4$ 100 ug/d | 7,8 (H) | 16,6 | 1,71 |
| $T_4$ 125 ug/d | 19,4 (H) | 28,9 (H) | 2,55 |
| $T_4$ 150 ug/d | 15,1 (H) | 19,3 | 1,08 (L) |
| $T_4$ 100 ug+$T_3$ 20 ug/d | 2,8 | 16,1 | 3,87 |

*Normal range: TSH: 0,27-4,2 mU/l, $FT_4$: 9,1-24 pmol/l, $TT_3$: 1,5-3,4 nmol/l*

This problem was originally detected in young patients with congenital hypothyroidism, especially due to thyroid agenesis. The prevalence of thyroid hormone resistance was 43% in age <1 year and decreased markedly in the older age group (10%) (3). The authors presume that the hypothyroidism in the intrauterine life changed the set-point of hypothalamus. The decreased sensitivity to thyroid hormone may be transient or permanent. Patients with congenital hypothyroidism in adulthood usually require higher dose of L-thyroxine than those patients whose hypothyroidism developed in adulthood (4). A remarkable abnormality in this group of patients is the low normal or decreased $fT_3$ concentration, which suggests a decreased deiodinase activity behind the thyroid hormone resistance. Both the serum $T_3$ and the intracellularly produced $T_3$ (from $T_4$) participate in the negative feedback regulation of TSH secretion. Deiodinase type 2 (D2) is responsible for the conversion of $T_4$ to $T_3$ in the TSH producing cell. D2 activity is inversely regulated by serum $T_4$ concentration. The decreased D2 activity may result in low intracellular $T_3$ concentration and therefore increased TSH production. The main question for the physician is which parameter reflects the real thyroid hormone supply. The addition of $T_3$ to thyroxine substitution in our case easily normalized TSH and thyroid hormone levels.

*I. c. Amiodarone*

**Case 2.** A 63-year-old woman was referred to the endocrinologist with abnormal thyroid hormone level (TSH: 1,47 mU/l, $fT_4$: 24,49 (H) pmol/l, $fT_3$: 4,1 pmol/l). She was treated by amiodarone for two years because of paroxysmal atrial fibrillation. Ultrasound detected a slightly enlarged thyroid gland and a nodule of 17 mm in diameter in the left lobe. Repeated hormonal evaluation (six months later) confirmed the previous results: TSH: 1,45 mU/l, $fT_4$: 25,45 (H) pmol/l, $fT_3$: 4,32 pmol/l, anti-thyroglobulin antibody: < 10, anti-TPO antibody: < 5,0.

Amiodarone is an antiarrhythmic drug frequently used for atrial fibrillation and ventricular arrhythmias. It contains 75 mg iodine/200 mg active substance. Because of the high iodine content, amiodarone effect on thyroid function was extensively studied. The first parameter influenced by amiodarone administration is the TSH, which is elevated even on the first day of treatment (5). A possible explanation for this event is the inhibition of D2 in TSH producing cells. An increase in the total and free thyroxine level was detected in the next few

days, parallel to the decrease in the total triiodothyronine and a rise in the $rT_3$ level. The inhibitory action of amiodarone on iodothyronine deiodinase activity persists during long-term treatment. Amiodarone also inhibits entry of thyroxine and triiodothyronine into peripheral tissue. After 1 to 4 months of treatment with amiodarone, serum thyroxine levels increase by an average of 40% above pretreatment levels, resulting in a serum thyroxine concentration above the reference range in 40% of all patients. The elevated $fT_4$ itself (without TSH suppression) does not mean thyrotoxicosis and no treatment is necessary. With long-term administration of amiodarone (>3 months), serum levels of TSH often return to normal. The response of TSH to TRH may be reduced.

Moreover, amiodarone induces thyroid dysfunction in many patients. Both hyper- or hypofunctions can develop. The overall incidence of these thyroid dysfunctions is estimated being between 2% to 24% (5). The development of hyperthyroidism is more common in iodine-deficient regions, while the prevalence of hypothyroidism is higher in iodine sufficient areas. Thyrotoxicosis can occur throughout the period during which a patient receives amiodarone; hypothyroidism, however, is rare after the first 18 months of therapy. The laboratory diagnosis of amiodarone-induced hyper- and hypothyroidism is not different from other primary thyroid dysfunctions.

*II. a. Interfering antibodies*

Heterophilic antibodies may be encountered in patient sera. Human anti-mouse antibodies (HAMA) belong to two categories (1). They may be relatively weak, multispecific, polyreactive antibodies that are frequently IgM rheumatoid factor or broadly reactive antibodies induced by infections or exposure to therapies containing monoclonal antibodies. Autoantibodies to thyroid hormones may also occur and can affect the diagnostic accuracy of the test result leading to clinical misinterpretation. These antibodies affect IMA methodology more than competitive immunoassays by forming a bridge between the capture and signal antibodies, thereby creating a false signal, resulting in an inappropriately high value.

*II. b. Drug interactions*

Medications can cause both in vivo and in vitro effects on thyroid tests. Intravenous heparin administration, through in vitro stimulation of lipoprotein lipase can liberate free fatty acids (FFA), which inhibit $T_4$ binding to serum proteins and falsely elevates $fT_4$. Furosemide is also a known inhibitor of thyroid hormone binding in serum and high-dose treatment with furosemide can lower total thyroxine and increase its free fraction in vivo. The drug or a metabolite also can cause false positive thyroid hormone levels in vitro.

**Case 3.** A 42-year-old woman was presented with fatigue, palpitations and headache. Her TSH was normal (0,32 mU/l) but the $fT_4$ and $fT_3$ were markedly elevated, >100 pmol/l and 30,6 pmol/l, respectively. The clinical presentation did not support the severe thyrotoxicosis, so the measurement was repeated and total hormone concentrations were also investigated: TSH: 0,27 mU/l, $fT_4$: >100 (H) pmol/l, $TT_4$: 320 (H) nmol/l, TT3: 10 (H) nmol/l. Ten days later, in another laboratory normal thyroid function was found: TSH: 0,33 mU/l, $fT_4$: 16,1 pmol/l $TT_4$: 151 nmol/l $TT_3$: 2,5 nmol/l. No treatment was initiated. One year later the patient went back to the endocrine department for follow-up visit when the following results were obtained: TSH: 0,22 mU/l, $fT_4$: 74,6 (H) pmol/l, $fT_3$: 39,3 (H) pmol/l. Methimazol therapy was started and because of inefficiency, it was changed to propylthiouracil (PTU). $FT_4$ level was constantly elevated in the next five month with normal TSH. Hypothyroidism developed after 7 months of treatment and thyroxine was added to PTU. TSH and thyroid hormones were in the normal range in the next 18 months with the same treatment. The anti-thyroid

medication was discontinued. Abnormal thyroid function tests recurred in three months time (the laboratory changed the method of free hormone measurements in the meantime) and the PTU treatment was restarted. Radioiodine treatment was planned but the strange hormonal constellation indicated further tests before final decision. Thyroid autoantibodies were negative. Thyroid ultrasound detected a normal thyroid gland, $^{99m}$Tc-pertechnetate scintigraphy showed normal isotope uptake. Sella MRI was normal. TRH test detected a normal TSH response (0 min: 0,28, 30 min: 4,77, 60 min: 3,18 mU/l). The blood sample of the patient was sent to another laboratory (B) which showed a completely normal thyroid function result (Table 8.3.). The diagnostic error resulted in an unnecessary treatment for years.

***Table 8.3.*** *Comparison of thyroid hormone results from two laboratories in case 3*

| | **Lab A** | **Normal range** | **Lab B** | **Normal range** |
|---|---|---|---|---|
| **TSH (mU/l)** | 0,25 | 0,22-4,2 | 0,72 | 0,5-4,6 |
| **FT4 (pmol/l)** | 57,9 (H) | 12,0-22,0 | 9,6 | 9,1-23,8 |
| **FT3 (pmol/l)** | 9,79 (H) | 3,1-6,8 | 4,14 | 3,1-6,8 |

The search for the cause of laboratory misdiagnosis led to an anti-depressive drug, duloxetine which may be responsible for the false elevation of thyroid hormones in lab A.

*III. a. TSH producing pituitary adenoma*

**Case 4.** A 52-year-old woman presented clinical signs of hyperthyreoidism with elevated TSH and thyroid hormone levels. She was treated in another hospital with thiamazol and levothyroxine for one year. A sella MRI was performed to exclude the central hyperthyroidism. No adenoma was found but an adenohypophysis hyperplasia was described (she did not report headaches or visual problems). Her medical and family histories were noncontributory. Physical examination revealed a slightly enlarged, nontender thyroid gland. Laboratory investigations detected a TSH level of 5,87 (H) (normal: 0,27-4,2) mU/l, $fT_4$ 27,9 (H) (normal: 12-22) pmol/l and $fT_3$ 8,88 (H) (normal: 3,1-6,8) pmol/l. Thyroid autoantibodies (anti-TSH receptor, anti-TPO, anti-Tg) were negative. Homogenous, increased isotope accumulation was detected on the $^{99m}$Tc-pertechnetate scintigraphy. The thyroid volume on ultrasound was 26 ml. Anti-thyroid medication was stopped and a TRH-test was performed: TSH 0 min: 6,39, 30 min: 7,56, 60 min: 7,05 mU/l. The thyroid function tests of the patient's children were normal. A repeated sella MRI was done because of the thorough suspicion of TSH secreting pituitary adenoma. An adenoma of 9 mm was diagnosed and removed by transsphenoidal surgery. The immunohistochemical evaluation revealed positivity for TSH, FSH, LH, prolactin and GH secretion (the overproduction of hormones other than TSH was not detected preoperatively). The thyroid and pituitary function tests were normal after the surgery. The serum α-subunit measurement and genetic analysis for the mutation of thyroid hormone receptor β gene were not available.

*III. b. Resistance to thyroid hormone (probable) + amiodarone effect + incidental pituitary adenoma*

**Case 5.** A 50-year-old man was investigated in a cardiology department. In the history, myocardial infarction was recorded 7 years ago. At that time thyroid function tests were abnormal (TSH 17,02 (H) mU/l, $fT_4$ 27,6 (H) pmol/l, $fT_3$ 10,6 (H) pmol/l) but no treatment was initiated. Five years later atrial fibrillation, dilatative cardiomyopathy, decreased left ventricular function indicated a hospitalization when the ISTSH persisted (TSH 7,13 (H) mU/l, $fT_4$ 37,2 (H) pmol/l), and antithyroid and amiodarone medication was started. After one

month of treatment, TSH level was even higher, 42,5 (H) mU/l. The first endocrine investigation detected hypothyroid features, enlarged, non-tender thyroid gland, TSH 79,54 (H) mU/l, $fT_4$ 6,44 (L) pmol/l and negative thyroid autoantibodies. Levothyroxine supplementation resulted in minimal improvement in thyroid status (TSH 58,5 (H) mU/l, $fT_4$ 10,79 (L) pmol/l, $fT_3$ 5,48 pmol/l) but serum lipid levels were markedly elevated. The family history was unremarkable. A small, 3.3 mm in diameter pituitary microadenoma was diagnosed on sella MRI. Pituitary function was normal except thyroid axis. TRH test (without medication) proved a hypothyroid response: 0 min: 38,6, 30 min: 256,8, 60 min: 112,8 mU/l. The markers of tissue thyroid hormone action supported hypo-euthyroidism. Thyroid hormone supplementation resulted in clinical improvement and normalization of TSH level with high $fT_4$ and $fT_3$ (TSH: 3,53 mU/l; $fT_4$: 43,0 (H) pmol/l; $fT_3$: 11,6 (H) pmol/l).

Distinguishing between the TSH producing pituitary adenoma (TSHoma) and resistance to thyroid hormone (RTH) is essential, because delayed diagnosis of TSHomas results in tumour growth and poor surgical cure rates, whereas medical, surgical or radioablative treatments in patients with RTH are usually unnecessary and potentially harmful (6, 7).

TSHomas are rare with a prevalence of 1 in 1 million population. Patients ususally present with symptoms of hyperthyroidism and goiter. Compressive symptoms of pituitary macroadenoma (headache, visual field defect) may occur. RTH is an autosomal dominant inherited syndrome of reduced end-organ responsiveness to thyroid hormone, with an incidence of 1 in 50 000. Mutations in the thyroid hormone receptor β gene are responsible for RTH; 122 different mutations belonging to 300 families have been identified (8). RTH patients have elevated $fT_4$ and $fT_3$ concentrations and normal or slightly elevated serum TSH level. The clinical presentation of RTH is highly variable. The majority of individuals are asymptomatic. Some patients may manifest symptoms suggestive of hypothyroidism such as growth retardation, impaired cognitive ability and hypercholesterolemia, while others show signs of hyperthyroidism such as tachycardia, advanced bone age or hyperactivity. It may happen that a patient has symptoms of both thyroid hormone failure and excess. The most likely explanation for the variable clinical manifestations of this apparently monogenic condition is the genetic heterogeneity of the many cofactors that modulate the action of thyroid hormone.

Patients with ISTSH require additional investigations because of the biochemical similarities between TSHomas and RTH. The presence of a pituitary adenoma on MRI scan and positive pituitary octreotide scintigraphy increase the probability of the diagnosis of a TSHoma. However, because as many as 10-20% of normal people may harbor a small, nonfunctioning pituitary adenoma, patients with RTH may have incidental pituitary adenoma (9), as in our case. It is still a question whether RTH predisposes to pituitary hyperplasia and adenoma development. Peripheral markers of thyroid hormone action, such as sex hormone-binding globulin (SHBG) and angiotensin-converting enzyme (ACE) levels are often elevated in TSHomas but are normal in patients with RTH. The concentration of α-subunit (the common subunit of TSH, FSH and LH) and the α-subunit/TSH molar ratio is usually elevated in TSHomas and low in RTH. The TRH stimulation test is also useful in differentiating between TSHomas and RTH. Patients with the latter condition will have a normal or a hypothyroid response, (>2-fold TSH elevation after administration of TRH), whereas patients with TSHomas generally have a less than 2-fold TRH-induced elevation of TSH level, because of the autonomy of TSH secretion. In a study of 25 patients with TSHomas, an elevated baseline TSH, flat or decreased response to TRH and elevated α-subunit/TSH ratio had the highest sensitivity and specificity for diagnosis of TSHomas (10). The final diagnosis can be

established only after demonstrating positive TSH immunostaining of pituitary tissue obtained by surgery. Detection of ISTSH in asymptomatic family members is highly consistent with the diagnosis of RTH. A mutation in the thyroid hormone receptor β gene provides a definitive diagnosis of RTH, but in about 10% of cases no mutations can be detected (8).

***Table 8.4.*** *Differentiation of TSH-secreting pituitary adenoma and resistance to thyroid hormone*

| TSH-oma | Resistance to thyroid hormone |
|---|---|
| Clinically hyperthyroid | Clinically euthyroid, hypothyroid or hyperthyroid |
| Elevated levels of markers of thyroid hormone action | Normal levels of markers of thyroid hormone action |
| Elevated α-subunit level (α-subunit/TSH ratio) | Normal α-subunit level (α-subunit/TSH ratio) |
| < 2-fold increase in TSH level after administration of TRH | > 2-fold increase in TSH level after administration of TRH |
| Headache, visual field defect | Developmental delay, hearing loss |
| Sella MRI/octreoscan positive | Sella MRI/octreoscan negative |
| Family history negative | Family history positive |
| Mutation in THRB gene negative | Mutation in THRB gene positive |

Surgical treatment is the first line therapy for TSHomas, while the majority of patients with RTH does not need any therapy. Treatment options for TSHomas include transphenoidal adenomectomy, medical treatment with somatostatin analogues and pituitary irradiation in large, invasive tumors (6). Somatostatin analogues may correct the biochemical hyperthyroidism and lead to tumour shrinkage. Treatment of RTH involves ensuring that asymptomatic patients do not receive unnecessary treatment with thionamides, radioiodine ablation or thyroid surgery. Failure to differentiate RTH from primary thyrotoxicosis has resulted in the inappropriate treatment of nearly one-third of patients. Although occasionally desirable, no specific treatment is available for RTH. Screening and appropriate diagnosis of family members are also important to avoid therapeutic mistakes. RTH patients with symptoms of thyroid hormone deficiency may achieve clinical euthyroidism with very high doses of exogenous thyroid hormone (8). However, in RTH patients with tachycardia (due to the excess of $T_3$ action on the thyroid hormone receptor α), treatment with selective beta-blockers is indicated.

In summary, ISTSH is a rare condition but it usually makes a diagnostic challenge. The situation is relatively simple in cases of L-thyroxine replacement and amiodarone therapy when the high thyroxine is followed by a normal or low-normal triiodothyronine concentration. The parallel elevation of $fT_4$ and $fT_3$ provides a more complex diagnostic problem, the exclusion of analytical troubles, drug interactions or the differentiation of TSH-producing pituitary adenoma from resistance to thyroid hormone requires the collaboration of laboratory medicine specialist and endocrinologist. The evaluation of clinical presentation is obligatory to identify the direction of further investigations. The clarification of the proper diagnosis is the only way to avoid the unnecessary or harmful treatment.

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# 9. THYROID DISEASES AND OBESITY

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## 9.1 Introduction

Obesity has growing social and economic costs, and the efforts to treat or to prevent it are increasingly huge. In this period of pandemic alerts and fears, obesity may be, besides some infectious diseases that have a global burden, one of the few health problems that is really global.

Nowadays obesity is considered as a state of chronic low-grade inflammation, involving the adipose tissue and an array of co-morbidities. Although weight excess is generally associated with western lifestyle, evidence accumulates pointing to the growing obesity epidemic in the developing world. Another important aspect, thinking in terms of future trends, is that overweight in children and adolescents is also increasing. More direct consequences of obesity, measured as an increase in cardiovascular disease or diabetes mellitus (for example), are well established. Other aspects, as for example the social and psychological aspects of obesity, are being better studied and data shows the less educated as more vulnerable.

There are numerous studies showing that interventions with the objective of reducing weight , either pharmacologically or behaviourally, are not very successful for the big majority of those in need. On the other hand even modest decreases in weight are known to reduce some of the risk factors.

All this aspects have pushed obesity to the centre of many people's concern and research focus.

Thyroid hormones (TH) metabolic effects are well established. They cause an increase in heat production and oxygen consumption, augment the metabolism of carbohydrates, fats and proteins. Their production is highly regulated, and imbalances tend to have metabolic consequences, as highlighted by weigh gain in hypothyroidism and weight loss in hyperthyroidism. However the exact mechanisms that link TH and its metabolic consequences remain, at least partially, unexplained.

The main objectives of this presentation are, first, to try to look for epidemiological data relating obesity and thyroid function, second, to try to understand how obesity and thyroid diseases influence each other, and third to try to anticipated how the knowledge of TH action may be important to fight the obesity epidemic.

Finally, and with relevance for laboratory medicine, it is important to bear in mind that with the increase in longevity and in the number of obese people, the possibility of having more people with, simultaneously, thyroid diseases and obesity will be much higher in the future.

## 9.2 Body weight and thyroid function

Thyroid dysfunction, as already stated, is well known as a cause of changes in weight. Small differences in thyroid function, with thyrotropin (TSH) variations within the normal laboratory range, are associated with measurable differences in resting energy expenditure. In industrialized countries with an environment of food plenty and physical inactivity, this decreases in energy consumption may impact body weight. Recent studies have shown that there may be even a relation between small increases in serum TSH concentrations, within the reference range, and weight gain, both in men and women. This was also found in children and adolescents. Furthermore, besides the positive correlation between body mass index (BMI) and TSH, there's also a positive correlation between weight gain during 5 years and a progressive increase of TSH concentrations. In accordance with this, weight reduction leads to decrease of THs (not TSH) in children. On the other hand, reductions in fat mass after surgery do not cause changes in thyroid function. The real significance of this relation has not yet been proven, but these findings may signify that someone with higher TSH concentrations in the normal level is more likely to gain weight over time.

Still it is not clear if people gain weight because they have higher TSH, or if they have higher TSH because they are heavier.

Although obesity is generally associated with an increased risk of some of the more frequent adult cancers, studies on the relation between BMI and thyroid cancer give contradictory results. In men increased BMI was strongly associated with thyroid cancer, but may have a protective effect on women under 45 years old.

## 9.3 The relation of adipose tissue and thyroid hormones

The adipose tissue is no more considered as an "innocent bystander" in terms of metabolism. The adipocyte can be the source of a large number of proteins actively involved in energy homeostasis and in the regulation of neuroendocrine, autonomic and even immune functions. We may consider a group of paracrine and autocrine products, as cytokines/chemokines (TNF-α, IL-6, etc) and adipokines (adiponectin, leptin and resistin), and a second group with endocrine products like sex steroids and glucocorticoides. Also, obesity is associated with increased macrophage infiltration of adipose tissue. It is well established that there are various cytokines produced by adipocytes that may influence, among many others, the hypothalamus-pituitary-thyroid (HPT) axis. As an example, leptin has been demonstrated to alter the HPT axis. The close relation between leptin and TSH is outlined by the fact that concentrations change in a parallel manner in primary hypothyroidism (increasing) and in primary hyperthyroidism (decreasing). In starvation, circulating TH, TSH and leptin are all low.

Also, on the other hand, TH regulate many of the genes involved in adipocyte differentiation and also in lipogenesis, lipolysis and thermogenesis in the brown adipose tissue (BAT). TH levels also have direct effects on adipokine production.

## 9.4 Metabolic effects of thyroid hormones

Plenty of information, concerning metabolic effects of TH, came from the study of pathologic states of TH excess or deficiency. These hormones have been shown to modulate some metabolic pathways that may impact the basal metabolic rate. This basal rate, or resting

energy expenditure, is an important determinant of energy consumption (the other being physical activity). The possible mechanisms for this are the uncoupling of cellular metabolism from ATP synthesis or the existence of "futile cycles".

Adaptive thermogenesis, a way by which TH influence energy expenditure, is characterized by an uncoupling of oxidative phosphorylation in cold-exposed BAT, which is dependent on locally generated TH. This is achieved through the uncoupling of the mitochondrial proton gradient from ATP production promoting the generation of heat. BAT uses mitochondrial uncoupling to rapidly produce heat, which is used to warm up the body during cold exposure or to dissipate calories after a meal. This capacity of BAT to dissipate energy is considered as a possible therapeutic tool against obesity. For this to happen, type 2 deiodinase enzyme (D2) converts thyroxine (T4), a minimally active precursor, into T3, the potent TH form. This increase in T3 is such that the TH receptor saturation increases almost to 100%, upon cold exposure. Also, T3 concentrations in serum remain unchanged, because local D2 in BAT functions as an additional source of T3, increasing local TH signalling for adaptive thermogenesis.

The metabolic effects of TH are also highlighted by the fact that during fasting there is a reduction in its circulating levels, presumably as an adaptive answer to conserve energy in periods of food limitation o shortage. This adaptation takes place through changes in the HPT axis.

## 9.5 The relation between thyroid hormones and obesity

Thyroid function is usually normal in obese subjects. However, patients with thyroid diseases usually exhibit changes in body weight, thermogenesis and lipolysis in the adipose tissue. Increased levels of TSH in obesity, usually slightly above the normal range, may reflect hormone resistance, or just an adaptation to increase resting energy expenditure. This is in accordance with the fact that elevated TSH concentrations in obesity normalize after substantial weight loss. Moderately increased TSH concentrations are rather a consequence then a cause of obesity. Changes in thyroid hormone concentrations in obesity may be regarded as an adaptation process to increased bodyweight. Similarly, fasting and weight loss is associated with a decrease in TH concentrations and a reduction in resting energy expenditure, which may explain why it is difficult to maintain weight loss.

## 9.6 Bile acids- the link between obesity and thyroid hormones?

Bile acids (BA) increase T3 levels selectively in tissues where T3 can promote energy expenditure. This effect is mediated through type 2 deiodinase enzyme (D2) which converts T4, into T3. Although BAs activate various signalling pathways, this effect occurs by binding to the G protein-coupled receptor TGR5. The most important thermogenic tissue in humans, skeletal muscle, expresses both D2 and TGR5 increasing energy consumption upon exposure to BAs. In mice, the administration of BAs increases energy expenditure in BAT, preventing obesity.

The fact that BAs, at endogenous plasma levels, activate the D2 pathway in BAT reveals a way to mediate meal-induced energy expenditure

## 9.7 The use of thyroid hormones as a treatment for obesity

The rational for he use of TH in the treatment of obesity came from the knowledge that TH are the most potent thermogenic molecules produced in the human body. However, treatment of obesity with thyroid hormones has been a failure due to its pleotrophic effects in multiple tissues and organs (just mention, atrial fibrillation or accelerated bone loss as consequences). So, its use is unsafe and harmful, and the only feasible alternative is the manipulation of the TH pathway.

This lead to the emergence of thyroid hormone receptors beta-specific agonists, a breakthrough that may change the picture as they have proved capable to increase energy expenditure and to decrease serum cholesterol, without any important cardiac or bone effects.

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# 10. THYROID (DYS)FUNCTION AND PREGNANCY

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## 10.1 Hypothyroidism

Changes in maternal thyroid function during pregnancy require an additional challenge to the maternal thyroid gland. For this reason hypothyroid women treated with L-T4 need to increase their daily L-T4 doses. In both treated and untreated hypothyroid women, a rise in serum TSH levels indicates a worsening of the situation and therefore TSH should be monitored every 4–6 weeks. Indeed, hyperthyrotropinaemia is associated with deleterious effects in both mother and foetus, including gestational hypertension, preterm delivery, abortion, and severe congenital and neurodevelopmental malformations.

Many recent studies have underlined the frequent occurrence of adverse pregnancy outcomes in women with subclinical (SH) and overt hypothyroidism (OH) and in women with euthyroidism and autoimmune thyroid diseases. The general risk is clearly greater in OH patients, but it is also present in women with SH or even in those with euthyroid autoimmune thyroid diseases. L-T4 administration, at doses that maintain optimal TSH level, greatly decreased the frequency of obstetric complications, thus suggesting that any pregnant woman should be screened for the possible presence of hypothyroidism. In pregnancy, several lines of evidence show that the upper limit for TSH value should be 2.5 mU/l. However, no consensus has been reached for the lower TSH limit, variable from 0.4 to 1.0 mU/l. Recently, in a prospective study of 19 pregnant hypothyroid women followed with the aim of maintaining an adequate TSH level throughout pregnancy, it was suggested to increase L-T4 dose by approximately 30% as soon as pregnancy was confirmed. Moreover, Rotondi et al. proposed to increase L-T4 dose preconception in hypothyroid women of childbearing age, to optimize thyroid hormone supply during the first week of gestation. Finally, the recent Endocrine Society guidelines for the management of thyroid dysfunction during pregnancy recommend L-T4 treatment in SH.

We have performed a retrospective study on hypothyroid pregnant women referred to the out-patient department between January 2004 and December 2006. A total of 185 pregnant women were studied during gestation; 155 patients (76 SH, 52 OH, 27 PH) were already on L-T4 before conception and 30 (SH) started L-T4 therapy during gestation. Thyroid function and body weight were evaluated every 4–6 weeks.

In the group of patients already treated before conception,134 (86·5%) increased L-T4 doses during gestation one or more times, eight (6%) reached a definitive therapeutic dosage within the 12th week of pregnancy, 64 (47·8%) within the 20th week and 62 (46·2%) within the 31st week. This initial L-T4increase at the first evaluation during pregnancy was 22·9 ± 9·8 μg/day. The final L-T4 doses were significantly different depending on the aetiology, being 101·0 ± 24·6 μg/day in SH, 136·8 ± 30·4 μg/day in OH and 159·0 ± 24·6 μg/day in PH. The per cent increase of L-T4,expressedas Δ% of absolute dose, was +70% in SH, +45% in OH and +49% in PH as compared to baseline dose. In SH patients diagnosed during gestation, the

starting L-T4 dose was higher than L-T4 dose before pregnancy of SH patients already treated (75·4 ± 14·5 and 63·2 ± 20·1 μg/day, respectively), whereas the final doses were similar. L-T4dose was increased one or more times in 24 patients (80%), 8 the definitive dosage within the second trimester (33·3%) and 16 within the third trimester (66·7%). In conclusions, serum TSH and FT4 measurements are mandatory in pregnant patients and the optimal timing for increasing L-T4 is the first trimester of pregnancy, though many patients require adjustments also during the second and third trimester. The aetiology of hypothyroidism influences the adjustment of L-T4 therapy and SH patients needed as larger increase than OH and PH. Close monitoring during pregnancy appears to be mandatory in hypothyroid women.

## 10.2 Hyperthyroidism

Hyperthyroidism during pregnancy can be deleterious to the fetus as well as to the mother. Fetal complications include intrauterine growth retardation, prematurity, stillbirth, low birth weight, and neonatal hyperthyroidism. Maternal complications include obstetric complications, such as eclampsia, miscarriage, and placenta abruptio, as well as systemic complications, including congestive heart failure or thyroid storm.

The prevalence of hyperthyroidism during pregnancy is approximately 0.1% to 0.4%. Normal variation of thyroid function indices during pregnancy frequently includes significant suppression of TSH and elevation of free T4 and T3. During a normal first trimester of pregnancy, 18% of completely asymptomatic women have a TSH value less than the lower limit of normal. Of these, 50% have a TSH value that is completely suppressed and undetectable. Peak suppression of maternal TSH directly corresponds to the peak human chorionic gonadotropin (CG) concentration. This may be particularly confusing in multiparous pregnancies and in pregnancies complicated by hyperemesis gravidarum, because CG is increased in both situations. Thirty percent to 50% of women with hyperemesis gravidarum develop biochemical evidence of hyperthyroidism and may also develop clinical symptoms. A missense mutation in the TSH receptor has been implicated in formation of a supersensitive TSH receptor that amplifies the response to CG in hyperemesis gravidarum and may be responsible for the development of thyrotoxicosis. Familial gestational hyperthyroidism is a rare cause of hyperthyroidism in pregnancy; it occurs as the result of a mutation of the thyrotropin receptor that renders it more sensitive to CG. In this situation, gestational hyperthyroidism occurs with normal serum CG concentrations. True thyrotoxicosis should be suspected in pregnancy when TSH is less than 0.1. This is especially true if TSH suppression continues past 20 weeks of gestation; by that time, normal thyrotoxicosis of pregnancy generally resolves spontaneously. The prevalence of hyperthyroidism in pregnancy is 0.1–0.2%, and the commonest cause is gestational transient thyrotoxicosis (GTT), which is 10-fold more prevalent than Graves' disease. GTT is nonautoimmune hyperthyroidism in women with a normal pregnancy, is found more often in twins than in singletons, and is typically associated with hyperemesis gravidarum. The features of hyperthyroidism are usually not prominent and less florid than those of Graves' disease, although the latter can also present with hyperemesis gravidarum.

The most important understanding in the successful management of these pregnancies is that while adequate control is essential, maternal and /or fetal hypothyroidism due to excess treatment must be avoided. Most of the women with GTT improve spontaneously as pregnancy progresses, but a short course of beta-blockers or antithyroid medication may be required occasionally. For Graves' disease, women on antithyroid medications should be monitored by both clinical progress, for example weight gain and fetal growth, and the

measurement of maternal serum TSH and free thyroxine at 4-weekly intervals to maintain a high euthyroid or borderline hyperthyroid level with the lowest possible dose throughout pregnancy, adjusting the dose in each trimester if necessary. Treatment should be discontinued no later than 36–37 weeks if the maternal and fetal conditions are satisfactory. Women in clinical remission who have discontinued treatment at the time of pregnancy should be assessed at the first visit and then monitored with thyroid function test at least once per trimester. After delivery, maternal thyroid function should be reassessed at the time of the postnatal visit irrespective of treatment, and the mothers should be followed up until their appointment with an endocrinologist. The best and safe antithyroid medications in pregnancy is propylthiouracil. The only specific drug-related fetal anomaly is a rare condition called aplasia cutis congenita reported with the use of carbimazole or methimazole. The recommended doses of propylthiouracil range from 100 to 300 mg per day. Surgery may be indicated if higher doses are required, if features of fetal hypothyroidism occur despite the minimal effective dose for the mother or in cases of poor compliance. Surgery should be performed if necessary in the second trimester. Radioactive iodine is contraindicated in pregnancy. There were anecdotal cases where radioactive iodine had been given inadvertently before or during pregnancy, but pregnancy outcome was not affected adversely, and patients who received an ablative dose of radioactive iodine had increased incidence of preterm delivery but not of miscarriage. Nevertheless, pregnancy should be avoided within 1 year of radioactive iodine treatment to allow for radioactive iodine clearance and hormonalstabilization.

## 10.3 Thyroid neoplasia

Finally, differentiated thyroid cancer (DTC) represents the second more frequent tumor among those diagnosed during pregnancy. It is in fact well known that the high serum levels of chorionic gonadotrophin, which has close homology with TSH, may lead to a rapid increase of thyroid tumor size during pregnancy and may stimulate the growth of benign and malignant thyroid lesions. Others factors such as EGF, IGF1 and estrogens can contribute to the development or the evolution of thyroid nodules during pregnancy. Indeed the use of several estrogen-containing preparations (either oral contraceptives or postmenopausal estrogens) and a history of one or more pregnancies have been found to be associated with an increased risk of thyroid cancer.

In order to evaluate the outcome of DTC diagnosed during pregnancy, we studeied three groups of patients affected with DTC in relation to the timing of tumor diagnosis were studied: Group 1 including 47 women with diagnosis of DTC at least 1 year after the delivery; Group 2 including 15 women diagnosed during pregnancy and submitted to thyroidectomy during the second trimester or in the first year after delivery; Group 3: 61 women diagnosed and treated before pregnancy or nulliparous. The 3 groups did not differ as far as age and tumor staging concerns. In addition, immunohistochemical studies of estrogen receptor alpha (ERα) were performed in 39 PTC tissues from the 3 Groups. A significant better outcome was observed in patients of Group 1 and 3 compared to patients of Group 2 ($P<0.0001$). Accordingly, at the multivariate analysis including well known outcome predictors and the belonging to Group 2 as input variables, and persisting/relapsing disease as end-point, the diagnosis of DTC during pregnancy or in the first year post-partum, resulted to be the more significant indicator of persistent disease ($P=0.001$). Interestingly, ERα expression significantly differ among the 3 Groups, being detected in 5/16 (31%) of Group 1, in 7/8 (87.5%) of Group2, and in 0/14 of Group3 tumors ($P=0.01$). In conclusion, thyroid cancer diagnosed during pregnancy was found to be significantly associated with persistence or

relapse of the disease compared to patients, diagnosed before pregnancy or more than 1 year after the delivery, strongly suggesting that pregnancy has a negative impact on the outcome of thyroid cancer. The presence of ER in the majority of tumors during pregnancy firstly indicate that the poorer outcome of these cases could be related to the estrogen-mediated stimulus to grow.

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# 11. REPORTS ON THYROID DISEASES: TRUTHS, MYTHS AND PITFALLS

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Recently I was asked to talk about statistical knowledge of journal editors and professional reviewers during the peer review process of articles submitted to biomedical scientific journals (Editorial in Biochemia Medica, ref. 1). The problem is complex and therefore journals' scientific boards increasingly introduce statistical editors and professional statistical review to improve the quality of papers. As noted in the Editorial (1), statistical review combines the evaluation of both statistical and epidemiological methodology, with main goal to ensure that study was properly conducted, offered with appropriate presentation of results, but also to disclose possible errors in manuscript (2,3).

It might be even more interesting to raise a question on statistical knowledge of journal's readers – do they understand design of the study, methodology, subjects and variable selection, hypotheses, tests used in statistical analysis, as presented in the paper, i.e., do readers understand basic methodology of the study? Or they just believe authors, reviewers and editors considering with no doubt that only valid methodology was used and presented correctly, in a form that only the truth can be revealed from the paper (4,5).

The aim of this occasional study was just to open the question and discussion of truths, errors and pitfalls in statistical methodology of papers addressed to the topic of this postgraduate course – classification, diagnosis and management of thyroid diseases.

## 11.1 Material and methods

Basic idea of the study was to reveal statistical methodology in thirty recently published scientific papers available in electronic form, with the topic of thyroid disease research. Sample was considered as occasional but with assumption to be representative for papers published in respective international scientific publications accessible worldwide (6). Therefore, articles search was done using PubMed retrieval service for the most popular of bibliographical database, Medline of the US National Library of Medicine (7). Search was done at once on July 16, 2009 (Table 11.1.), at the computer using the Rijeka University School of Medicine network with granted free access to assorted biomedical journals.

***Table 11.1.*** *Search history*

- PubMed (*http://www.ncbi.nlm.nih.gov/sites/entrez*)
- July 16, 2009
- Department of Medical Informatics, Rijeka University School of Medicine, Croatia
- Key Word = " thyroid disease", N = 115.575 articles
- Limits 1: published in the last 5 years, only items with links to full text, Humans, English, Core clinical journals, N = 1,905 articles
- Limits 2: Clinical Trial, Meta-Analysis, Practice Guideline, Randomized Controlled Trial, Classical Article, Clinical Trial, Phase I, Clinical Trial, Phase II, Clinical Trial, Phase III, Clinical Trial, Phase IV, English, Core clinical journals, N = 654
- Limit 1 and 2, N = 182
- Random selection, N = 30

***Table 11.2.*** *List of papers (N = 30)*

| No. | Journal abbreviation* | Article** |
|---|---|---|
| 1-15 | J Clin Endocrinol Metab<br>(IF 5,493) | 2005 May;90(5):2666-74. Epub 2005 Feb 10<br>2007 Sep;92(9):3466-9. Epub 2007 Jun 19<br>2006 May;91(5):1934-42. Epub 2006 Jan 11<br>2007 Aug;92(8):3162-70. Epub 2007 May 15<br>2006 Dec;91(12):4873-80. Epub 2006 Sep 12<br>2007 Dec;92(12):4590-7. Epub 2007 Sep 18<br>2008 Apr;93(4):1351-8. Epub 2008 Jan 2<br>2006 May;91(5):1819-25. Epub 2006 Feb 14<br>2006 Sep;91(9):3389-93. Epub 2006 Jun 27<br>2007 Jul;92(7):2487-95. Epub 2007 Apr 10<br>2006 Dec;91(12):4786-91. Epub 2006 Sep 12<br>2007 Nov;92(11):4115-22. Epub 2007 Aug 21<br>2006 Feb;91(2):646-53. Epub 2005 Nov 8<br>2005 Jun;90(6):3350-9. Epub 2005 Mar 22<br>2006 Jul;91(7):2624-30. Epub 2006 May 2 |
| 16-18 | N Engl J Med<br>(IF 52,589) | 2008 Oct 23;359(17):1786-801<br>2008 Jul 3;359(1):31-42<br>2005 Sep 15;353(11):1105-13 |
| 19 | J Immunol (IF 6,068) | 2007 Mar 1;178(5):3281-7 |
| 20-21 | Arch Surg<br>(IF 3,485) | 2007 Dec;142(12):1182-7<br>2006 Jan;141(1):82-5 |
| 22 | J Gerontology (IF 2,932) | 2006 Nov;61(11):1194-200 |
| 23-24 | Surgery<br>(IF 3,004) | 2006 Dec;140(6):960-6; discussion 966-7<br>2008 Nov;144(5):775-9. Epub 2008 Aug 29 |
| 25 | Am J Ophthalmol (IF 2,628) | 2005 Aug;140(2):193-9 |
| 26 | Radiology (IF 5,561) | 2008 Jun;247(3):762-70. Epub 2008 Apr 10 |
| 27-28 | Am J Surg (IF 2,337) | 2006 Nov;192(5):675-8<br>2008 Jul;196(1):40-6. Epub 2008 Apr 16 |
| 29 | Transl Res (IF 1,325) | 2008 Apr;151(4):224-31. Epub 2008 Jan 29 |
| 30 | BMJ (IF 9,723) | 2007 Mar 10;334(7592):514. Epub 2007 Feb 19 |

**Journal abbreviations from the PubMed. IF – Impact factor for the journal for the year 2007.*
***Article notation as presented by PubMed search in http://www.ncbi.nlm.nih.gov/pubmed/: Year Month Date; Volume (Number): Pages from-to. Electronic publishing Year Month Date.*

Search through PubMed was done with only "thyroid disease" as keyword (115.575 articles found), but narrowed to original scientific papers with existing links to full text (electronic version) and published in English in core clinical journals in 2005 or later (details on setting of limits in Table 11.1.). In total, 182 articles were found and 30 among them were randomly selected as a sample for this study. In short, all 182 articles were sorted by PubMed according to the last name of the first author and designated with numbers from the first ($1^{st}$) to the last ($182^{nd}$). Random pattern of numbers 1–182 was constructed using generator from the Research Randomizer (Geoffrey C. Urbaniak and Scott Plous, Social Psychology Network, Site Statistics; available from *http://www.randomizer.org/*). Articles were selected into the final N = 30 sample from sorted list from PubMed consecutively, in sequence, using random numbers from the second set. Articles with no full access (N = 2), pay per view articles (N = 11) or otherwise unavailable articles (N = 1) were not included. In total 44 articles were checked in sequence and 30 were selected as accessible (Table 11.2.). Articles were downloaded in portable document format (PDF, Adobe Systems) for the purpose of this study only.

All thirty articles were read in details, from statistical reviewer point of view, following the rules for evaluating statistical and epidemiological methodology according to the checklist (Table 11.3.), and all errors, mistakes, blurs or questions raised were recorded. After evaluation, each article was graded as a range from 5 (excellent) to 1 (insufficient) for five methodological topics: study design, enrolment of subjects, presentation of statistical methodology, data presentation, and overall score for the whole paper. Average grade of article was calculated as arithmetic mean.

***Table 11.3.*** *Checklist for reviewing statistical methodology in articles from Table 11.2.**

| |
|---|
| A. General comments<br>• properly reported statistical methodology, rational concept of the study, appropriate study design<br>• valid assumptions about variables, general statistical acceptance of the paper |
| B. Study design<br>• clear aim of the study, hypotheses, power calculation and sample size calculation<br>• treatment and control groups, duration of the study, institutions |
| C. Methodology and data analysis<br>• source of subjects and data, inclusion and exclusion criteria, sample formation, sample size, randomization, allocation, stratification, matching, blocking, data on subjects recruitments, follow up, endpoints, censoring, blinding, and interventions<br>• primary and secondary outcomes, missing data, data measurements<br>• statistical methodology, unusual data loading, appropriate statistical analysis and appropriate use of statistical methods<br>• alpha and beta errors, level of significance |
| D. Data presentation<br>• baseline demographic and clinical characteristics, clear data presentation<br>• “data not presented” explanations, explanation of outliers<br>• appropriate precision, self-explanatory tables, figures, no repetition of data<br>• proper statistical language, terms, and measures |
| E. Data interpretation<br>• justified conclusions, data support findings, discussion based on results<br>• methodological limitations of the study, bias and limits of statistical inference |

**Accepted, shortened and adopted from the "Checklist for editing and reviewing statistical and epidemiological methodology in biomedical research papers, according to the suggestions published in biomedical literature" table from the Editorial in Biochemia Medica (1)*

## 11.2 Results and discussion

All articles were published between 2005 and 2008 years in eleven international journals, all of them with impact factor (IF) > 1 (Table 11.2., articles listed in sequence of random selection, in groups according to publishing journal).

Most articles (N = 26) were typical original studies conducted on samples with 10–39.002 subjects (median: 53 subjects per study) and two articles were meta-analysis reports of 14 and 41 original studies including 1.306 and 15.498 subjects, respectively. Two articles were scientific reports on gene expression and genome screening in thyroid diseases; because of its specific scientific structure they were excluded from further analysis.

Errors, mistakes, failures, blurs, and questions on statistical methodology were reported in 28 articles. After evaluation of whole sample of articles, all reviewing reports were systematized. Similar and comparable errors were grouped together, which formed seven more or less distinct error types, classes of errors, listed according to the subjective error strength from 1, minor, to 7, major (Table 11.4.).

***Table 11.4.*** *Number of articles with faulty statistical reports and scoring of presentation of statistical methodology**

| **Strength** | **Error, mistake, failure** | **N (articles)**** |
|---|---|---|
| 1 | Unnecessary data and double data presentation (figure, table) | 7 |
| 2 | Unclear data presentation, including not self reporting tables/figures | 15 |
| 3 | Unclear explanation of statistical methodology | 7 |
| 4 | Data not presented without note | 2 |
| 5 | Doubtful and unnecessary data precision, presentation of both parametric and nonparametric data, not reporting exact P values | 18 |
| 6 | No statistics presented | 2 |
| 7 | Doubtful statistical methodology, wrong tests, no adjustment for multiple comparisons, faulty interpretation | 6 |
| | **Score** | **Average** |
| | Study design | 4,68 |
| | Enrolment of subjects | 4,64 |
| | Presentation of statistical methodology | 3,71 |
| | Data presentation | 3,39 |
| | Overall score | 3,68 |
| | Average | 4,02 |

**Data presented for N = 28 papers. Two papers were excluded from observation (explanation in text).*
***N – number of articles with specified type of error; if the same error occurred more than once in the same article, it was not counted again.*

In total, 57 errors were found in 25 articles, with three articles found completely error free in three journals: The Journal Clinical Endocrinology & Metabolism, British Medical Journal, and New England Journal of Medicine. It might be perceived that all three journals have highest or almost highest IF in the selected group (IF 5,493–52,589, Table 11.2.), suggesting that general quality of journal implies quality of statistical methodology of published papers.

Unnecessary or double, and unclear data presentation was found in 7 and 15 articles (respectively, Table 11.4.). In reviewing the manuscript they are more often considered as statistical remarks or comments instead errors, but they are still measured as fault reporting of methodology that blurs understanding of the study. In this occasion unnecessary data reporting mostly included individual data information on all subjects from the study, typical for case reports, with no group information (case reports were not included it this study). Double presentation of results with both figures and tables simultaneously was found in two papers. Unclear data presentation was found in 15 articles, and included presentation of statistical measures with no explanation (average with no specification is it mean, median, or mode, for example), graphical presentations without explanation of all parameters in graphs, or generally presentation of more complex tables or figures that are not self reporting, as supposed (2).

Unclear explanation of statistical methodology was observed in seven articles. It was mostly considered as inexplicit or incomplete component of Statistics or Statistical Analysis, part of Materials and Methods section, like "data were analyzed with parametric tests", "all comparisons were done with analysis of variance", "chi-square and Fisher test were used", etc. In some articles explanation of statistics was blur and not clearly prepared for average reader who is not specialist in statistics.

In two occasions part of study results were discussed with no presentation of data (I used some time trying to find the data in tables and figures, but with no success). At times authors might find some results unimportant to present, mostly negative findings, so they write only statement on finding or conclusion in the paper, but then it is mandatory to note that "data (are) not presented."

Doubtful and unnecessary data precision, presentation of both parametric and nonparametric data, and not reporting of exact P values were found in 18 articles, about 64% of all analyzed. In most of articles with these type of errors patients age was, for example, measured in years as integers, but presented with decimals, some numerical variables were presented with both mean, median, standard deviation and full or interquartile range, some data were presented with mean and standard error of the mean and compared between two or more samples, data were presented with mean and compared with nonparametric tests, percentages for small and very small samples were calculated with decimals, etc. In some articles exact P values were not presented, or not presented on three decimals as supposed (1,2). Instead, some authors still use "P < 0,05" or "P < 0,01" expressions for considering something statistically significant, or "NS" notation when comparisons were "not significant."

In two articles some statistical tests and comparisons were announced in the Methods section but without later presentation of data. Therefore it is not clear were results of analysis considered worthless mentioning as negative or insignificant, or methods were mentioned by mistake.

Doubtful statistical methodology (unusual test with no explanation of data loading, for example), wrong usage of statistical tests (t-test for comparison of two very small samples, for example), no adjustment for multiple data comparisons, and faulty interpretation of results (low significant Pearson's correlation coefficient as a measure of high association of two parameters, for example) were found in six articles. Those errors were considered as the most serious – it is possible that data interpretation suggested by authors is not valid and therefore, article is not addressing the truth. But all of them occurred only once per article (i.e., six

individual errors in six different articles, with no repetition), so it is highly improbable that article as a whole might be considered as fault scientific report.

## 11.3 Concluding notes

Except errors of doubtful methodology, wrong testing, no adjustment and faulty interpretation that would fit into major category, all others errors were minor, and consequently, articles' grades reflect findings of errors (Table 11.4.). Study design and enrolment of subjects were graded over 4,5 in average, and presentation of statistical methodology, data presentation and article overall score were graded about 3,5 in average, presenting something that might be considered as very good quality reports from the methodological point of view. Seems that in the field of scientific reports on international level on thyroid diseases most articles fully address "the truth, the whole truth and nothing but the truth" (sworn testimony, ref. 8), with very rare pitfalls and almost no myths. It should be mentioned here that one earlier *ad hoc* study on methodological errors in the field of autoimmunity data revealed that remarkable number of papers suffered from statistical unclearness (9). Although samples from studies are not directly comparable, quite different findings are still intriguing and worth discussion and some further research.

At the end of this report short note should be given about two articles not included in analysis, dealing with gene expression and genome screening in thyroid diseases. Both papers were using methodology typical for gene research, well addressed in specific statistical books (10), but not common in general biomedical statistical literature. Therefore, articles were excluded from this study, but reviewed by the colleague with respectable knowledge on gene research and found as "highly adequate in methodology and data presentation."

## 11.4 Acknowledgement

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# 12. PREANALYTICAL AND ANALYTICAL ERRORS IN THYROID ASSAYS

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***Motto: Obtaining a false laboratory test result is worse than obtaining no result at all.***

## 12.1 General considerations

The process to provide a clinical laboratory test result involves at least three different steps or phases: 1/ preanalytical phase, 2/ the analysis itself and 3/ postanalytical phase (transferring data and interpretation of test results). Each step can be characterized by specific parameters and factors and unfortunately, each step might carry certain errors influencing the validity of the obtained data. There are many assessments regarding the frequency of errors in each phase however it might be stated that a vast majority of mistakes are done during the preanalytical phase (1).

## 12.2 Flow chart of getting laboratory test results

The route of obtaining exact laboratory parameters begins with examination of the patient and ends in transferring the clinical laboratory data to the requesting medical doctor who evaluates and interprets them. Figure 12.1. gives a schematic diagram of the major phases of the whole process indicating the estimated frequencies of errors during it (2).


***Figure 12.1.*** *Major phases and frequency of errors of the process obtaining laboratory test results.*

Please, note that the preanalytical phase is responsible for the occurrence of most of the possible errors in the flow of events. This fact is most probably due to human factors involving the patient and the medical staff as well. In the postanalytical phase the relatively high frequency of errors also might be related to human factors (e.g. knowledge and experience of the medical staff).

## 12.3 The preanalytical phase in general

The preanalytical phase is composed of many factors. The most important ones when dealing with blood samples are listed below (3).

### 12.3.1 Patient preparation

In most cases patients should be prepared in a proper way before sample collection. In general, prior to obtaining blood samples a 12-hour fast is necessary. If special tests are to be done the patient should discontinue medication influencing the parameters to be examined (e.g. glucose, plasma lipids, different hormones) and stop smoking and/or drinking alcohol.

### 12.3.2 Timing and methods of sample collection

It is best to obtain a venous blood specimen between 7:00 to 8:00 a.m. Since many parameters possess biological rhythms a standard timing of blood collection is essential. The technique of blood collection is of utmost importance. The use of a closed system is essential; this will ensure the cleanliness of the test tubes and also provides the appropriate anticoagulants or enzyme inhibitors if needed. In most cases, blood is drawn into plain tubes (a tube with separator gel is recommended) however, some labile parameters require special tubes and temperature (e.g. parathyroid hormone testing is done by collecting blood in EDTA-tubes, ACTH requires EDTA-tube immersed immediately in melting ice, etc.).

A prolonged strangulation of the vein should be avoided and also any manipulation that might cause lysis of the red blood cells in the sample. Previous physical exercise might influence many laboratory parameters.

### 12.3.3 Sample identification

Immediately before sample collection the tubes should be labeled appropriately. A bar code system is preferred where the bar code contains the patient's demographic data and the test requests having been input into the medical computer software system previously. It is desirable to have an online access from the hospital informatical system (HIS) to the laboratory informatical system (LIS) ensuring to send the lab requests online. In spite of the proper identification system there is still the possibility by human mistake to mismatch the samples and unfortunately the laboratory most often is not able to reveal this type of error.

### 12.3.4 Sample storage

If the samples can not be sent to the laboratory immediately a proper storage of them is essential. In most cases separated plasma or serum samples can be kept at $4^{o}$C for 1 – 2 days but certain parameters require immediate freezing (- $20^{o}$C, e.g. osteocalcin). There is a time window for many tests within which the analysis should be performed and the samples can not be stored (e.g. blood coagulation, blood picture, acid base balance tests). It is never allowed to keep coagulated whole blood in the fridge/freezer or uncoagulated whole blood in the freezer!

High temperature and exposure to sunlight might destroy many components in the blood.

### 12.3.5 Sample transport

Tubes should always be transported in stands and in closed containers. Frozen samples should be kept frozen during transport preferably by using dry ice or other cooling devices. It is very important that the labels on the tubes be not damaged during transport. A printed list of the patients' demographical data and the test requests should accompany the tubes even if there is an online connection between the sender and the lab.

### 12.3.6 Interfering factors in the sample

Exogenous (postprandial) and/or endogenous lipemia, hyperbilirubinemia, hemolysis are the most frequent interfering factors with spectrophotometric and immunochemical methods. Lipemia and hyperbilirubinemia usually cause physical interference with absorbance measurements, hemolysis besides physical factors results in release of intracellular components of the red blood cells (LDH, ASAT, potassium, etc.).

## 12.4 Major lab tests in thyroid and parathyroid diseases

### 12.4.1 Thyroid diseases

TSH (thyroid stimulating hormone)
Free T4 (free thyroxin)
Free T3 (free triiodothyronine)
TG (thyroglobulin)
TBG (thyroxin binding globulin)
Calcitonin
ATPO (anti-thyroid peroxidase antibody)
ATG (anti-thyroglobulin antibody)
TRAB (anti-TSH receptor antibody)

### 12.4.2 Parathyroid diseases

Intact PTH (parathyroid hormone)

## 12.5 Laboratory methods: assay principles

### 12.5.1 Immunoassays in general

An immunoassay is based on the quantitative estimation of an antigen by the use of labeled specific – preferably monoclonal – antibodies raised against the antigen or labeled antigen and specific antibody systems. By principle an immunoassay can be competitive or non-competitive. Technically, if the antigen or the antibody is bound to a solid phase the assay is called heterogeneous, if they are in solution the method is called homogenous. The assays are named after their labels (radioactive – RIA, fluorescent – FIA, chemiluminescent – LIA, enzyme – EIA, etc.). In all assays calibrators mimicking the sample matrix are used (4).

### 12.5.2 Competitive heterogeneous immunoassay

Figure 12.2. represents the assay principle when concentration of a small molecule is to be determined. Standard amount of the labeled analyte and unknown amount of the unlabeled analyte (in the patient's sample) is mixed and given to immobilized specific antibodies. Both analytes compete for the binding sites. The more analyte is in the patient's sample the less labeled analyte can bind to the antibodies and vice versa. After incubation and washing steps the signal is measured which is inversely proportional to the concentration of the molecules in the patient's serum.

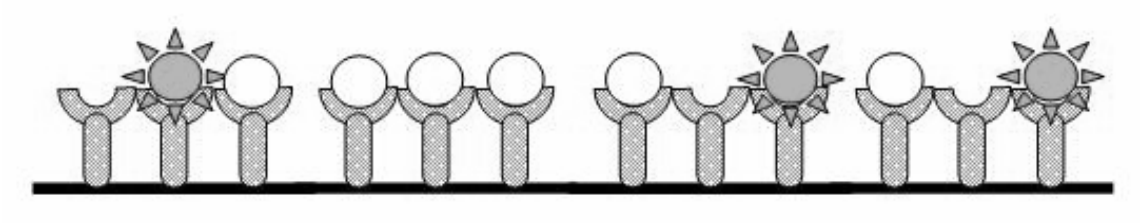

***Figure 12.2.*** *Principle of competitive heterogeneous luminescence immunoassay.*
✹ *labeled analyte;* ○ *analyte in patient's sample.*

This type of assay is used for determination of free thyroid hormones however, free hormones represent a minute fraction of total T4 and T3. The protein bound and free fractions are in equilibrium which ratio should not be changed under the assay conditions.

### 12.5.3 Non – competitive heterogeneous immunoassay

In non – competitive conditions usually a macromolecule (protein or peptide) is the antigen to be determined. Two monoclonal antibodies with different epitope specificity are used in two consecutive steps. During the first incubation the patient's sample is mixed with the immobilized primary antibody. After a washing step the secondary antibody carrying a label is added to the complex. After further incubation and washing the signal coming from the "sandwich" formed is measured and compared with those of the calibrators. The signal is directly proportional with the concentration of the analyte (Figure 12.3.). This type of assay is suitable for the measurement of peptides and proteins (e.g. TSH, TG, TBG, calcitonin, PTH).

Thyroid disease – related auto-antibodies are also protein macromolecules but their determination is based on competitive type of assays. Here auto-antibodies in the patient's serum compete with standard amount of labeled antibodies in the presence of recombinant target molecules.

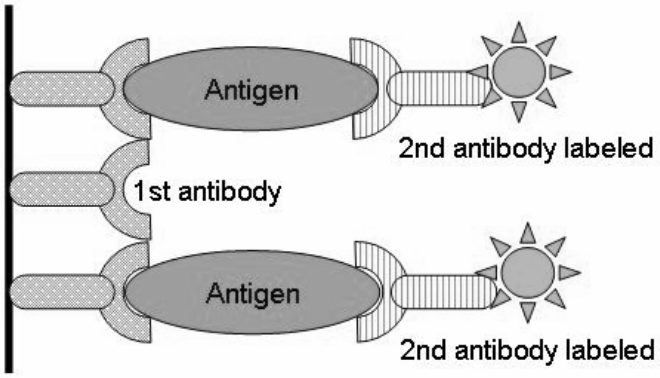


***Figure 12.3.*** *Principle of non – competitive luminescence immunoassay.*
*The secondary antibody is carrying a chemiluminescent label.*

## 12.6 Preanalytical factors influencing thyroid and parathyroid lab tests

In thyroid lab assays one should be aware of preanalytical factors which might result in erroneous test reports and/or misinterpretation of the data. Most of the preanalytical factors

are related to the patient and a minor part is in connection with medication or inappropriate sampling methods. In Table 12.1. the most important constellations are summarized (5).

***Table 12.1.*** *Preanalytical factors influencing thyroid lab test results*

| Anomalous binding of thyroid hormones (T4 & T3) to serum proteins | Altered reference intervals for thyroid hormones or TSH | Disrupted set point of the hypothalamic-pituitary-thyroid axis | Specimens |
|---|---|---|---|
| Genetic | Childhood | Non-thyroidal illness (NTI), including acute psychiatric illness | Free fatty acid (FFA) |
| Drug-induced | Pregnancy | Drugs | Heparin artifact |
| Disease-induced | Old age | Unusual thyroid conditions, including thyroid hormone resistance | |
| Pregnancy | | | |

### 12.6.1 Physiological variations in the TSH/T4 relationship

There are, in reality, many clinical situations where the TSH/FT4 relationship is disrupted, leading to discordant results. Examples include abnormalities in hypothalamic or pituitary function (such as TSH secreting pituitary tumors, central hypothyroidism etc.); postpartumthyroiditis; non-thyroidal illness (NTI); during the early phases of treating overt hyper- or hypothyroidism; or when changing the dose of thyroid hormone replacement drugs. In the latter 2 situations, it takes 6 – 12 weeks for pituitary TSH secretion to adjust to the new thyroid status, resulting in misleading TSH blood levels. During such transitional stages FT4 is the more reliable parameter of thyroid function.

### 12.6.2 Hormone binding protein abnormalities

There are pre-analytical artifacts that exist in many situations associated with binding protein abnormalities. Such examples include the following: genetic abnormalities in thyroid bindingproteins and medications that displace T4 from thyroid-binding proteins (both of which may result in spuriously high FT4 blood levels); during the critical phases of NTI; and in pregnancy. The point that needs to be made here is that it is far more common to encounter misleading totaland free thyroid hormone results than misleading TSH results. Given the improved sensitivity and specificity of TSH assays, the indirect approach (TSH testing) offers better sensitivity for detecting thyroid dysfunction than does thyroid hormone testing.

### 12.6.3 Effect of age on reference intervals

Awareness that age has an effect on TSH/FT4 levels is important in that failure to recognize this may lead to missed or undertreated thyroid disorders. This may happen when one reference range is quoted for all age groups in the laboratory reports. TSH levels tend to increase in older people. However, despite this well recognized fact, the current guideline recommendation is that a single adult reference range be quoted for all adult age groups. In children, the hypothalamic-pituitary-thyroid axis matures throughout infancy and childhood until puberty is reached. As a consequence of this maturation process, TSH and FT4 results in

children are higher than those of adults, particularly in the first week of life and throughout the first year. This fact is important to recognize because missed cases of congenital hypothyroidism may occur if the age-adjusted reference range is not taken into account.

### 12.6.4 Pregnancy

In pregnancy, laboratories should not be reporting test results using assays that measure total thyroid hormones (e.g. TT4, TT3). Total thyroid hormones are bound to proteins (unlike the free thyroxin FT4 which exists in the unbound form), and during pregnancy the concentration of these thyroid hormone-binding proteins increases due to the effect of increased estrogen production. The consequence is that of falsely elevated total thyroid hormone concentrations. Fortunately most, if not all, laboratories have circumvented this problem by utilizing free unbound thyroid hormone assays. Physiological changes occur during pregnancy that may affect blood TSH and FT4 levels in a certain proportion of normal pregnancies. In the first trimester, a decrease in blood TSH levels occurs (and hence 'subnormal' TSH results). This is attributed to thyroid gland-stimulating activity of human chorionic gonadotrophin (hCG) that is secreted by the growing placenta. hCG is genetically very similar to pituitary TSH and thus mimics TSH action. The fall in TSH is associated with a mild elevation in blood FT4 levels. In a very small proportion of cases, the blood FT4 may reach very high levels and when prolonged can lead to 'gestational transient thyrotoxicosis'.

### 12.6.5 Non-thyroidal illness (NTI)

A spectrum of thyroid function test abnormalities is often encountered in patients with both acute and chronic critical illnesses (physical and psychiatric) who usually do not have underlying thyroid dysfunction. The terms non-thyroidal illness and euthyroid sick syndrome are used interchangeably to describe such cases. Examples of illness include the following: sepsis, starvation, myocardial infarction, burns, trauma, surgery, malignancy, and psychiatric illness.

The underlying pathophysiology which results in patients with NTIs presenting with abnormal thyroid functions is unclear. It is thought to arise from a maladjusted hypothalamic-pituitary-thyroid axis. TSH, in the absence of dopamine or glucocorticoid administration (both of which decrease TSH secretion), is considered to be more reliable than FT4 and FT3 testing in NTI patients. The blood TSH levels are affected in variable degrees but may be mildly low during the acute phase of the non-thyroidal illness. TSH assessment in severe NTI depends on the sensitivity of the particular method that is used by the laboratory.

### 12.6.6 Effects of medication

Clinicians need to know if the patient is on medications that affect the interpretation of thyroid function tests. Medications do not interfere chemically with the tests (with the exception of heparin) – they interfere with the metabolism of thyroid hormone production and release, and affect TSH secretion. Table 12.2. illustrates common examples, although the list is by no means comprehensive (5).

***Table 12.2.*** *Effects of medication on thyroid functions and laboratory parameters.*

| Effects | Drugs | Results |
|---|---|---|
| Inhibit thyroid hormone synthesis or release from the thyroid gland | Lithium, sulfonylureas | ↓ FT4,↓ FT3, ↑ TSH |
| Decreases triiodothyronine hormone production by inhibiting peripheral conversion of FT4 to FT3 | Glucorticoids, propranolol, amiodarone, propylthiouracil | ↓ FT3 leads to ↑ FT4 |
| Stimulate TSH secretion | Iodine, lithium, dopamine antagonists, cimetidine | ↑ TSH |
| Inhibit TSH secretion | Glucocorticoids, dopamine agonists, somatostatin | ↓ TSH |
| Inhibit T4 and T3 binding to transport proteins | Phenytoin, sulfonylureas, diazepam, furosemide, salicylates | ↑ FT4, ↑ FT3 |
| Inhibit gastrointestinal absorption of ingested thyroid hormones for those on thyroid treatment | Cholestyramine, ferrous sulfate, aluminum hydroxide, and sucralfate | ↓ FT4, ↑ TSH |

### 12.6.7 Heparin artifact

Heparin causes an increase in blood free thyroid hormone (FT4) levels. This is an important phenomenon to recognize as it can lead to spurious FT4 results. Intravenous heparin administration induces lipoprotein lipase activity *in vitro,* resulting in the liberation of free fatty acids, which are known to displace thyroid hormones from the thyroid-binding proteins, leading to falsely elevated free thyroid hormone (FT4, FT3) blood levels. This effect is accentuated by incubation of blood at 37$^{o}$C and by increased blood triglyceride or low serum albumin concentrations.

### 12.6.8 Rare thyroid disorders

Thyroid function tests can result in misdiagnoses in the following situations, especially if the TSH first line testing is adopted: hypothalamic/pituitary disease leading to hypothyroidism (paradoxically normal or mildly elevated TSH, low FT4 blood levels); in cases of thyrotoxicosis caused by TSH-secreting pituitary tumors (rare, and represents < 1% of inappropriate TSH secretion and is characterized by non-suppressed TSH levels with no response to TRH stimulation, and MRI evidence of a pituitary mass); or in patients who are thyroid hormone-resistant, characterized by normal/slightly elevated TSH that responds to TRH stimulation with elevated FT4/FT3 levels. Such cases are often misdiagnosed as being hyperthyroid and subjected to inappropriate thyroid gland ablation. These cases can be diagnosed by non-suppressed TSH levels in response to TRH stimulation. This TRH response is appropriate despite elevated thyroid hormone levels, as the tissue response to thyroid hormone is reduced, requiring higher hormone levels to maintain the normal metabolic state.

### 12.6.9 Intraoperative PTH determination

In intact PTH assays the preferred specimen type is EDTA-anticoagulated blood. In serum, PTH is more unstable and a further advantage of EDTA-blood is that the sample can be analyzed immediately because there is no need to wait for coagulation. The possibility for immediate analysis is utilized in intraoperative PTH testing where a short turnaround time

(TAT) is essential. A major preanalytical interfering factor here is surgical manipulation of the PTH gland resulting in falsely elevated PTH levels in the circulation.

## 12.7 Analytical factors influencing thyroid and parathyroid lab tests

### 12.7.1 General considerations

Immunoassays are less sensitive to physical interfering factors e.g. lipemia, hyperbilirubinemia and hemolysis. However, depending on the actual assay there are certain concentration limits above which interference occurs. The sample type of immunoassays is usually serum with some exceptions where the stability of the tested parameter requires the use of a specified anticoagulant. When serum is recommended but anticoagulants are not excluded (e.g. Li-heparin, EDTA) one always should know the positive or negative effect of the anticoagulant on the test result. In automated systems incomplete coagulation of native blood before separation of serum may cause pipetting errors due to fibrin formation during the assay procedure. Cross reactivity (specificity of the antibodies used in the test) is of minor significance because in most assay systems monoclonal antibodies are applied. In non-isotopic immunoassays when alkaline phosphatase (AP) enzyme-labeled antibodies are used bacterial contamination might lead to falsely elevated signals due to activity of bacterial AP. An appropriate sample matrix is essential therefore dilution of samples of concentrations exceeding the linearity range of the test should be diluted only with special diluting fluid supplied by the manufacturer of the test system. In free thyroid hormone measurements dilution is not possible because it would alter the equilibrium between protein bound and free hormones giving erroneous results. Some more specific analytical interferences are discussed below (7).

### 12.7.2 Heterophile antibodies in the patient (HAMA)

Some patients have certain types of antibodies in their serum that have been induced by infections or exposure to therapeutic agents containing specific animal antigens or antibodies administered for diagnostic purposes (most frequently of mouse origin, here comes the name HAMA: heterophile anti-mouse antibody). They may also be polyreactive antibodies such as IgM rheumatoid factor. These heterophile antibodies may cross-react with any of the thyroid function test assay methods (immunoassay-based, utilizing animal antigens), leading to false results, which are more often inappropriately high. The inappropriate result may not necessarily be abnormal, but in fact inappropriately normal.

### 12.7.3 Interference caused by specific thyroid auto-antibodies in the patient

The presence of anti-TG antibodies in patients is quite frequent. A high titer of ATG interferes with TG immunoassays because of competing with anti-TG antibodies in the test system leading to erroneous results. This phenomenon is of high importance when absence of TG is intended to prove hypothyroidism (congenital or after thyroidectomy). Therefore, when a TG test is requested it is highly recommended to ask for ATG determination at the same time. The manufacturers exert efforts to avoid this interference in their TG reagents however, the problem has not been solved yet completely.

In autoimmune patients there might be auto-antibodies present to T4 and T3 also interfering with the assay system. When a patient is suspected to have auto-antibodies the thyroid tests should be repeated in another test system using different animal antibodies.

### 12.7.4 High dose hook effect

Apart from the linearity range of an immunoassay a problem arises when the analyte is present at an extremely high concentration in the patient's sample. In non-competitive assays the large amount of analyte molecules are crowding over the immobilized primary antibody and mask part of the bound antigen. In this case the sandwich can not be formed properly leading to a falsely low result. This type of error can not be noticed by the lab experts. The clinician should consult with the laboratory staff (data not plausible) and the problem is solved by dilution of the sample until the result does not change any more. This type of error is usually seen when tumor markers are determined (e.g. calcitonin).

## 12.8 Examples (data measured at our Institute of Laboratory Medicine)

TSH receptor auto-antibody determinations are used for the differential diagnosis of hyperthyroidism. The auto-antibodies can stimulate the TSH receptor molecules and induce an increased thyroid hormone production without elevation of TSH in the blood. The test is most useful in exclusion of autonomous thyroid gland function and it has a prognostic value in Graves's disease. In pregnant women with Graves's disease the TRAB levels serve as a good basis for estimation the risk of development of hyperthyroidism in the fetus. In Figure 12.4. an example is shown for interpretation of TRAB levels. Our data suggested a diagnostic cutoff of TRAB in our laboratory of > 4 mU/l. In this group all patients' free thyroid hormone concentrations were outside the reference range.

Intraoperative PTH testing has been possible since the introduction of automated non-isotopic immunoassay systems. In our laboratory the TAT time of intraoperative PTH testing is less than 30 minutes. Figure 12.5. shows some representative data where the preanalytical error caused by surgical manipulation resulted in falsely elevated PTH concentrations in the unsuccessful surgery patient group.

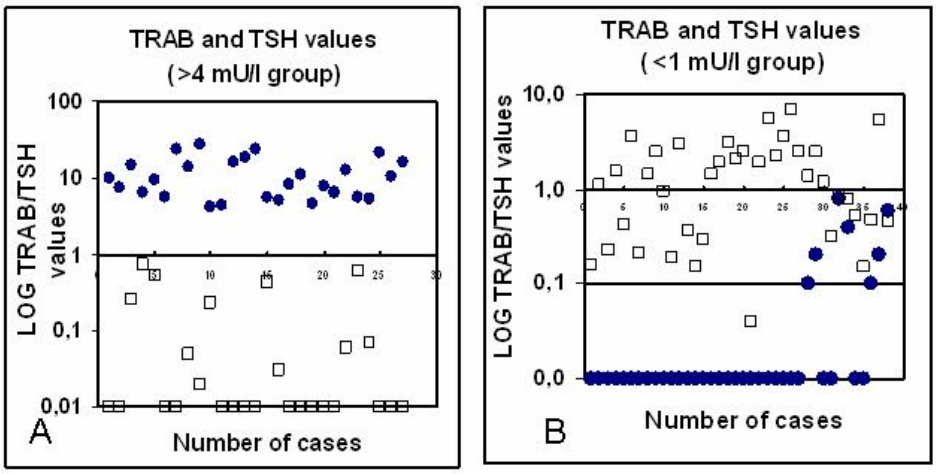


***Figure 12.4.*** *Serum TRAB levels ☐ serum TSH levels. Please note that concentrations are expressed in logarithmic form. In diagram A patients above the diagnostic cutoff of TRAB values are shown while in diagram B TRAB levels were within the reference range.*

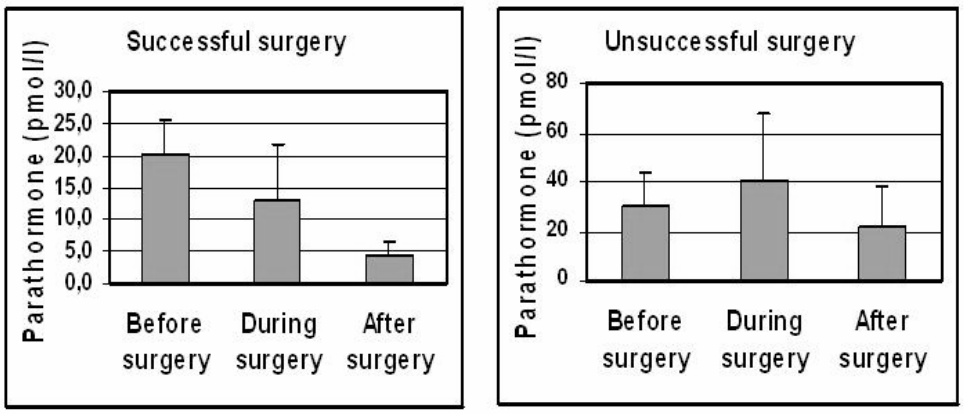


***Figure 12.5.*** *Intraoperative PTH testing. In the successful surgery group PTH levels decrease as expected (4 minutes half life of PTH). In the unsuccessful surgery group there is a temporary increase during operation and the after surgery levels still remain high. Patient number = 20.*

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# 13. BIOLOGICAL VARIATION OF THYROID AUTOANTIBODIES AND THYROGLOBULIN

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Biological variation of components is used for three purposes: 1) goals for the analytical quality, 2) Index of individuality and 3) reference change value (1,2). For most of the naturally occurring quantities measured in serum, plasma and blood, biological within- ($CV_{within\text{-}subject}$) and between-subject ($CV_{between\text{-}subject}$) coefficients of variations have been estimated and the results are available in data banks, published in scientific journals, and on the internet (3,4).

Very few papers describe the biological variation for the thyroid hormones (5-7) and thyrothropin and no papers described the biological variation for the thyroid antibodies before this study.

Definitions according to 'biological variation'

- Variation between subjects, (CVbetween-subject)
- Variation within-subject (CVwithin-subject) – analytical variation not included
- Variation of replicates
- Variation for the Pooled Coefficient of Variation within-subject (CVwithin-subject)

Presence of thyroid autoantibodies increases the likelihood of having or developing subclinical as well as overt thyroid disease. Therefore elevated serum thyroid peroxidase antibodies (TPOAb) and/or thyroglobulin antibodies (TgAb) often results in treatment with thyroxine, sometimes regardless of clinical symptoms and whether serum thyrotropin (TSH) is elevated. In the lecture I will present results from our studies of the within-subject of biological variation of the thyroid antibodies.

Because thyroid disease is more common in women than men, our first project was including only fertile women. The aim was to investigate whether there was a systematic variation of the thyroid autoantibodies related to the menstrual cycle.

To deal with biological variation in fertile women do have some challenges

## 13.1 The participants

Twenty-four healthy, fertile women with a regular menstrual cycle participated. None were pregnant, or breastfeeding and none had chronic diseases. The median age was 35.5 years (range 23 to 46 years). Six women were taking oral estrogens

## 13.2 The blood sampling

Blood samples were obtained twice a week in all 24 women. Twenty women had 9 blood samples, the remaining 4 individuals had 7, 8 10 and 11 samples drawn due to the length of their cycles. The sampling period varied between 25 and 36 days. An overlap between the first and the last blood sample in the individual cycles was allowed.

All samples were collected by one laboratory technologist to reduce the pre-analytical variation.

Blood samples were obtained from each individual between 7.30 and 11.00 a.m. from a cubital vein. Prior to collection of blood each individual sat for 5 minutes. First day of the present menstrual cycle was registered and so were several life stile habits and facts, such as height and weight.

Eleven ml of serum, in 1 ml tubes, was frozen at -800C within three hours of sampling.

## 13.3 Harmonisation of the menstrual cycle

The length of the menstrual cycle, was 28 days for women using estrogens as contraceptives while it varied from 27 to 32 days for the remaining women. Consequently, the specimens were ordered from first day in the cycle for each woman, and for each sample the 'harmonised day' was calculated as 'day in the menstrual cycle' times 28 divided by the 'actual length of the cycle'. Then the 'harmonised days' were ranked in groups of four days resulting in seven sub-groups.

## 13.4 Normalisation of measured concentrations

For each woman and for each component the mean concentration was calculated and each measured concentration was divided by this mean resulting in values distributed around 1.0 for each woman.

## 13.5 Estimation of coefficients of variation

A one-way analysis of variance was applied to the seven subgroups with the normalised values as replicates, and coefficients of variation were calculated for within- and between-variation of the seven subgroups. A considerable between-variation compared to the within-variation would indicate a systematic course through the menstrual cycle.

## 13.6 Estimation of parameters of coefficients of variation

### 13.6.1 Between-subject variation and pooled (common) within-subject variation.

For each component (value > zero) the natural logarithm (ln or $\log_e$) was calculated, and an analysis of variance for repeated sub-sampling at three levels was applied to the ln-concentrations, resulting in the coefficient of variation parameters $CV_{between\text{-}subject}$ and $CV_{within\text{-}subject}$ together with the analytical, $CV_{analytical}$ . The CV-values can be expressed both as fractions and as percentages: CV % = CV*100.

### 13.6.2 Within-person variation for each individual.

The individual variation for each person, $CV_{within\text{-}person}$, is estimated for each woman based on the ln-concentration values.

By using ln-values, the estimated standard deviations, $s_{lnx}$, are the best estimates of coefficients of variation: $CV \sim s_{lnx}$. The more correct formula is $CV \approx (\exp(s_{lnx}^2) - 1)^{½}$, but up to a directly calculated value of CV = 0.400 the underestimation is negligible, as the more correct is CV = 0.417, which is less than a 5 % underestimation).

There were no systematic variations during the menstrual cycle for the total group of 24 women for any of the investigated components. The between-group coefficients of variation are below 3 % compared to the within-group variation of 10 to 18 %. For the six women taking oral contraceptives, however, there was a decrease of approximately 20 % in the Tg-concentration from the first to the next period of four days in the menstrual cycle, followed by a slow rise to the original value during the remaining cycle. For these women, the between-group variation was 9.6 %, which is of the same order as the within-group variation of 12.5 %. There was no systematic course for the thyroid autoantibodies in the six women using estrogen.

## 13.7 Variance homogeneity

If a number of calculated variances are samples from one and the same general variance, then it is considered as 'variance homogeneity', and consequently, the ranked and cumulated distribution of these variances are distributed as $\chi^2$/DF (Chi-square distribution for DF = degrees of freedom according to the sample sizes).

This hypothesis is illustrated by cumulating the ranked individual coefficients of variation, $CV_{within\text{-}person}$-values, and plotting them as fractions in a rankit plot together with the theoretical pooled $CV_{within\text{-}subject} * (\chi^2/DF)^{½}$. If the curve for cumulated CV-values follows the theoretical, then the variances can be considered homogeneous. Test of variance homogeneity can also be performed with Bartlett's test or Cochran's test.

The distribution of TPOAb-coefficients of variation do not follow the $\chi^2$-distribution and is therefore not homogeneous. Separating CV-values below and above the upper reference limit also results in inhomogeneous distributions of both. The TgAb-distribution, however, is close to the $\chi^2$, as if they all came from the same common Gaussian distribution.

Only one woman had all TRAb-measurements above the detection limit, so there is no distribution of individual CV-values. For Tg, three women had several measurements below the detection limit, implying that the distribution, by definition, may be considered inhomogeneous.

## 13.8 Description of variability within- and between-subject variations

The individual concentrations and $CV_{within\text{-}person}$-values can be visualized with the individual means and 95 % confidence intervals on a logarithmic scale for all 24 women and for TPOAb, TgAb and Tg, Reference intervals and decision limits are indicated.
The prevalence of antibodies was 38 % (90 % CI : 22 to 54 %),

Nine women had antibody levels above the upper reference limit of the laboratory (6 had TPOAb above 10 kIU/L, 6 had TgAb above 20 kIU/L, and one woman had TRAb above 0.75 IU/L). Seven women had Tg below the lower reference limit of 2 μg/L, five of whom had elevated TgAb.

## 13.9 Relationship between thyroglobulin and its antibody

TgAb and Tg are interrelated and the combined values for each blood sample in all 24 women illustrate that Tg-concentrations above 5 μg/L correspond to TgAb-concentrations within the reference interval. Below Tg-values of 5 μg/L there is a crude inverse relation with TgAb-concentrations. The combinations of the two components are very reproducible within each individual. A high serum concentration of TgAb reduces Tg considerably, and this may result in Tg-concentrations below the detection limit. This is expected from the direct relation between antigen and its antibody as described by Feldt-Rasmussen et al. (8). They demonstrated that subtotal thyroidectomy released Tg in high concentrations, which could reduce the TgAb considerably, and diminish it to below the detection limit, if the previous TgAb-concentration was moderate. When the release of Tg stopped, TgAb could rise to original levels within two days. It follows that the level of TgAb is sensitive to changes in Tg.

With the homogeneous variances of TgAb, the conditions for using RCV to disclose clinical important changes in TgAb are present.

**Reference change value, (RCV)** is calculated for each of the parameters as $(z \times \sqrt{2} \times CV_{total})$ where $z = 1.96$ and $CV_{total} = (CV_{total\ analytical}^2 + CV_{within\text{-}subject}^2)^{½}$ (9)

90 % confidence interval for a fraction, p: $p \pm 1.64*(p*(1-p)/n)^{½}$ (8).

The calculated 95 % RCV's are between 35 and 45 % for TPOAb, TgAb and Tg.

For individuals with an assumed concentration of 28 μg/L (Tg), 28 kIU/L (TPOAB), 28 kIU/L (TgAb) and 2.8 U/L (TRAb) the next measurement should exceed 41, 40, 38 and 3.3, respectively, in order to exceed the 95% reference change value.

## 13.10 Variance homogeneity and Reference Change value

The variances of TgAb are $\chi^2$-distributed and thus homogeneous, which means that one and the same coefficient of variation is applicable to all women when monitoring with serum TgAb. In contrast, TPOAb variances are not homogeneous, and the use of one and the same within-subject value for all women may lead to erroneous interpretation of measured differences, when compared to the reference change values. This is so since an individual with a narrow variation needs larger changes – as calculated in individual CV-values - to surpass the RCV, which is based on the common pooled CV, whereas a woman with a high CV may show an increased fraction of false positive differences. Thus, the RCV can be applied to TgAb without limitations, whereas for TPOAb caution is suggested. For TPOAb, estimations of biological within-subject variation in each single individual previous to clinical use would be very valuable.

**The analytical quality** specifications for imprecision (desirable analytical performance) is calculated from the formula: $CV_{analytical} < ½\ CV_{biological}$. (10)

## 13.11 Estimated parameters for biological variation

9 women had antibodies above the upper reference limit of the laboratory (6 had TPOAb above 10 kIU/L, 6 had TgAb above 20 kIU/L and one woman had TRAb above 0.75 IU/L). Seven women had Tg below the lower reference limit, five of whom had elevated TgAb.

Variations in the thyroid antibodies were random and not related to the menstrual cycle.

For TPOAb (2.5 to 258 kIU/L), the CV-biological was 11.3 % while the CV analytical was 10.6 %.

For TgAb (5.6 to 148 kIU/L) CV biological was 8.5 % and CV analytical was 9.0%. The woman with TRAb had a CV biological of 4.8 %, while the analytical variation in duplicates was 3.9 % at a level of 2.8 IU/L.

Values of $CV_{within\text{-}subject}$ are around 10 % for TPOAb and TgAb and are mainly the same below and above the upper reference limits. The analytical imprecisions are of the same size and about twice that of the analytical quality specifications for imprecision.

$CV_{within\text{-}subject}$ for TRAb is approximately 5 %, but based on only one subject.

For Tg, the $CV_{within\text{-}subject}$-variation is about 16 %, whereas, the analytical imprecision is low and within the quality specification.

## 13.12 Other studies

The investigation was performed in order to establish the biological variation for the thyroid antibodies in healthy fertile women. Surprisingly, 9 of 24 (38 %) of the healthy volunteers had thyroid antibody concentration above the upper reference limit. The prevalence of elevated TPOAb was 25 % (6 of 24), for TgAb it was 25 % (6 of 24), and for TRAb 4 % (1 of 24). This prevalence of elevated TPOAb of 25 % is higher than the 14 % found in another Danish investigation using the same methods (7) and in other studies using various criteria for having antibodies. For instance with a prevalence of 14 % using a cut-off of 0.5 kIU/L, to a prevalence of 3.5 %, using a cut-off of 200 kIU/L However, the latter studies applied more rigorous rule-out criteria based on other parameters, e.g. ultrasound scanning and use of medication or having a family history of thyroid disease. The total prevalence of 38% of any thyroid antibody, in our study, is higher than that found by others. On the other hand, the fact that six women had elevated TPOAb and/or TgAb makes the estimated within-subject coefficients of variation more reliable.

Our within-subject variation for serum Tg was approximately 16 %, which is of the same order of magnitude as found by Feldt-Rasmussen et al. (5). Our high between-subject variation seems to be owing to the considerable scatter (also without the women with Tg-concentrations below the detection limit), whereas an older study comprised only five women, who by chance were only slightly scattered with a value of 25 %.

## 13.13 Index-of-individuality

There are no calculations of index-of-individuality, as the between-subject variations of TPOAb and TgAb are extremely high, due to the fact that 25 % of the women have concentrations above the upper reference limits.

## 13.14 TPOAb and IgG

The measured thyroid antibodies are of the IgG type. Consequently, the estimated within-subject variation of about 10 % should be compared to the within-subject variation of IgG, which is estimated to be 3.0 % in the same samples. This factor of about three may be explained by the fact that the individual specific antibodies each has its own turn-over, whereas total IgG includes all different antibodies with varying synthesis and catabolism, and therefore the variation of the total IgG is equalized. According to Ricos et al. (3,4) the median value from published data is 4.5 %. This is somewhat higher than our estimate, but confirms the order of difference between the specific antibodies and the total IgG.

## 13.15 Conclusion in the first study

There was no relationship between concentration of TPOAb or TgAb and time in the menstrual cycle.

The biological within-subject variation and imprecision for TPOAb, at a serum concentration of 2.4 to 258 IU/L, was 11.3% and 10.6 %, respectively. For TgAb, at a serum concentration of 5.6 to 148 IU/L, it was 8.5 % and 9.0 %, respectively.

In the second study the biological variation over a period of 10 years was investigated

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# 14. NATIONAL ACADEMY OF CLINICAL BIOCHEMISTRY LABORATORY SUPPORT FOR THE DIAGNOSIS AND MONITORING OF THYROID DISEASE: A PERSONAL SUMMARY

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## 14.1 Foreword and introduction

Over the past fifty years, improvements in the sensitivity and specificity of thyroid tests have dramatically impacted clinical strategies for detecting and treating thyroid disorders. In the 1950s only the protein bound iodide (PBI) technique -an indirect estimate of the total T4 concentration- was available. Improvements in the sensitivity of assays to measure the pituitary thyroid stimulating hormone, thyrotropin (TSH) now allow TSH to be used for detecting both hyper- and hypothyroidism The development of immunoassays methods have progressively improved the specificity and sensitivity of thyroid hormone testing. Currently, serum-based tests are available for measuring the concentration of both the total (TT4 and TT3) and free (FT4 and FT3) thyroid hormones in the circulation. Measurements of the thyroid hormone binding proteins (TBP), thyroxine binding globulin (TBG), transthyretin (TTR) formerly called thyroxine binding pre-albumin (TBPA) and albumin (SA) are also available. Assay of the prothyroid hormone protein, thyroglobulin (Tg) as well as the measurement of calcitonin (CT) in serum, have become important tumor markers in differentiated and medullary thyroid carcinomas. Autoimmunity, a major cause of thyroid dysfunction, is now investigated using sensitive and specific tests for autoantibodies to thyroglobulin (TgAb), the TSH receptor (TRAb) and, more recently, thyroid peroxidase (TPOAb). Current thyroid tests are usually performed on serum by automated methods that employ specific antibodies and highly efficient chemical markers. Furthermore, urine iodide concentrations are measured to estimate dietary iodide intake.

Improvement in quality laboratory testing allows accurate diagnosis and cost-effective management of thyroid disorders by physicians, endocrine specialists as well as general practitioners. The use of laboratory parameters differs depending on the clinical status of the patient. In clinically overt hyperthyroidism in a woman or the presence of a thyroid mass, TSH testing is sufficient to confirm the clinical suspicion of hyper- or hypothyroidism. In most patients however, thyroid dysfunction symptoms may be tenuous so that only laboratory testing can establish the diagnosis. Such a situation is observed mainly in women with “sub-clinical hypothyroidism. In such instance, the collaboration between the physicians and clinical laboratory scientists is essential for optimal, cost-effective management of the patient with thyroid disease.

## 14.2 Pre-analytic factors

Most pre-analytical factors have little effect on serum TSH assay, the first -and only- thyroid test to be used to assess thyroid status in ambulatory patients. Pre-analytic variables and interfering substances present in specimens may influence the binding of thyroid hormones to plasma proteins and thus decrease the diagnostic accuracy of thyroid hormone measurements, more frequently than serum TSH.

TSH and FT4 values may be diagnostically misleading in patients with severe diseases not related to thyroid dysfunction (nonthyroidal illness or NTI). Hospitalized patients with normal thyroid function, frequently, have abnormal serum TSH and free thyroid hormone concentrations as a result of NTI or medications that might interfere with hormone secretion or synthesis. Various situations, such as genetic abnormalities or variation during pregnancy in thyroid binding proteins, may impact the reliability of thyroid testing. Iatrogenic factors such as nonthyroidal medications e.g. glucocorticoids or beta-blockers and specimen variables, including autoantibodies to thyroid hormones and Tg as well as heterophilic antibodies (HAMA, see below) can affect the diagnostic accuracy resulting in test result misinterpretation.

### 14.2.1 Physiological variables

Levels of thyroid hormones as well as their precursor protein, thyroglobulin (Tg) is quite stable within individuals over 1 to 4 years. All thyroid analytes display a greater inter-individual variability compared to intra-individual variability. The stability of intra-individual serum T4 concentrations reflects the long half-life (7 days) of T4. The stability of intra-individual T3 concentrations reflects autoregulation of the rate of T4 to T3 conversion. Inter-individual variability is especially high for serum Tg concentrations. Serum TSH levels also display high variability, both within and between individuals. This, primarily, reflects the short half-life of TSH (~60 minutes) together with its circadian and diurnal variations, the levels peak during the night and reach a nadir sometime between 10:00 to 16:00.

Variables such as gender, race, season, phase of menstrual cycle, cigarette smoking, exercise, fasting and several others parameters have less effects than method-to-method differences on the reference intervals for thyroid tests. Regarding adults, age-adjusted reference ranges for thyroid hormones and TSH are unnecessary despite the wider serum TSH variability seen in older individuals. In contrast, the problem of age related differences persist in neonates and infants (higher TSH and FT4 concentrations), as manufacturers have not established age-specific reference intervals. In pregnancy, significant variations of thyroid testing parameters are observed. They ought to be taken into account as thyroid dysfunction during pregnancy may have a detrimental effect on fetal outcome (fetal wastage, lower infant IQ,).

### 14.2.2 Pathological factors

They may lead to misinterpretation of laboratory results and inappropriate diagnoses, unnecessary further testing and escalating health care costs.

Medications can cause both in vivo and in vitro effects on thyroid tests. In general, the serum TSH level is affected less by medications than thyroid hormone concentrations. Glucocorticoids, dopamine, propranolol, iodide containing solutions or prescriptions, particularly the anti-arrhythmic drug Amiodarone, lithium, furosémide, and héparine.

As stated above, in patients who are seriously ill (acute or chronic NTI), often have abnormalities in their thyroid tests but usually do not have thyroid dysfunction.

### 14.2.3 Specimen variables

Studies suggest that thyroid hormones are relatively stable whether stored at room temperature, refrigerated or frozen. Manufacturers recommend serum as the preferred specimen. Serum can be stored at 4-8°C for up to one week. Storage at -20°C is recommended if the assay is to be delayed for more than one week. Collection of serum in barrier gel tubes does not affect the results of most TSH and thyroid hormone tests. TSH and TT4 in dried whole blood spots used to screen for neonatal hypothyroidism are also stable for months when stored with a desiccant.

Hemolysis, lipemia, and hyperbilirubinemia do not produce significant interference in immunoassays, in general. However, free fatty acids can displace T4 from serum binding proteins

Heterophilic antibodies may be encountered in patient sera. They are either human anti-mouse antibodies (HAMA) or specific human anti-animal immunoglobulins (HAAA). Manufacturers are currently employing various approaches to deal with the HAMA/HAAA issue with varying degrees of success

### 14.2.4 Thyroid test performance standards

As for other biological parameters, analytical performance of thyroid testing is typically assessed in the laboratory by parameters such as:

1. Within and between-run precision assessed at different analyte concentrations
2. Minimum detection limit (analytical sensitivity)
3. Functional Sensitivity (defined by % CV, relative to the analyte-specific biologic and methodologic variation)
4. Linearity of measurements made across the reportable range of values
5. Recovery of analyte added to the standard matrix
6. Normal reference interval (mean +/- 2 standard deviation of values) for a cohort of subjects without disease
7. Correlation with a reference method

It should be stressed that for thyroid testing, as for any other biological testing, analytical performance goals should be established on biological principles (within- and between-individual variation). In the case of thyroid, the specificity of the thyroid function –and dysfunction- should be considered, particularly the large intra- and inter-individual variations.

For diagnostic testing, thyroid test results are reported together with a "normal" reference interval that reflects inter-individual variability. Reference ranges, however, cannot be used to determine whether differences exist between two consecutive test results, made during the monitoring of a patient's treatment, constitute a clinically significant change, or merely reflect the technical (between-run imprecision) and biologic (intra-individual variance) variability of the measurement. The "normal" reference interval is usually irrelevant during the post-operative clinical management when using tumor markers such as Tg. Clearly, method bias and precision goals need not be as stringent when a measurement is used for diagnostic

testing compared with using serial measurements for patient monitoring. While the "normal" reference interval stated on the typical laboratory report helps the physician to make a primary diagnosis, it does not give relevant information to help the physician assess the significance of changes resulting from treatment.

## 14.3 Thyroid tests for the clinical biochemist and physician

### 14.3.1 Thyroid hormones

Thyroxine (T4) is the principal hormone secreted by the thyroid gland. All the T4 in the circulation is derived from thyroidal secretion. In contrast, only about 20% of circulating triiodothyronine (T3) is of thyroidal origin. Most of the T3 in blood is produced enzymatically in nonthyroidal tissues by 5'-monodeiodination of T4. In fact, T4 appears to function as a pro-hormone for the production of the biologically active form of thyroid hormone, T3.

In the circulation, most (~99.98%) T4 is bound to specific plasma proteins, thyroxine-binding globulin (TBG) (60-75%), TTR/TBPA (prealbumin / transthyretin) (15-30%) and albumin (~10%). Approximately 99.7% of T3 in the circulation is bound to plasma proteins, specifically TBG. This represents a 10-fold weaker protein-binding than seen for T4 Protein-bound thyroid hormones do not enter cells and are thus considered to be biologically inert and function as storage reservoirs for circulating thyroid hormone. In contrast, the minute free hormone fractions readily enter cells by specific membrane transport mechanisms to exert their biological effects. In the pituitary, the negative feedback of thyroid hormone on TSH secretion is mediated primarily by T3 that is produced at the site from the free T4 entering the thyrotroph cells.

Total hormone concentrations (TT4 and TT3) are measured at nanomolar levels whereas free hormone concentrations (FT4 and FT3) are measured in the picomole range and to be valid, must be free from interference by the much higher total hormone concentration.

#### *14.3.1.1 Total thyroxine (TT4) and total triiodothyronine (TT3) methods*

Total T4 and total T3 assay methods (TT4 and TT3) have evolved through a variety of technologies over the past four decades. The PBI tests of the 1950s that estimated the TT4 concentration as "protein-bound iodide" were replaced in the 1960s first by competitive protein binding methods using TBG and, later in the 1970s, by radioimmunoassay (RIA) methods. Currently, serum TT4 and TT3 concentrations are measured by competitive immunoassay methods that are now non-isotopic and use enzymes, fluorescence or chemiluminescence molecules as signal. Total hormone assays necessitate the inclusion of an inhibitor (displacing or blocking agent) such as 8-anilino-1-napthalene-sulphonic acid (ANS), or salicylate to release the hormone from binding proteins. Nowadays, TT3 assay is no more in use and TT4 barely use in Europe, as the diagnostic accuracy of total thyroid hormone measurements is less than that of free hormone assay.

#### *14.3.1.2 Free thyroxine (FT4) and free triiodothyronine (FT3) estimate tests*

Thyroxine in blood is more tightly bound to serum proteins than is T3, consequently the free T4 (FT4) bioavailable fraction is less than free T3 (0.02% versus 0.2%, FT4 versus FT3, respectively). Unfortunately, the physical techniques used for separating the minute free

hormone fractions from the predominant protein-bound moieties are technically demanding, inconvenient to use and relatively expensive for routine clinical laboratory use. Methods that employ physical separation of free from bound hormone (i.e. equilibrium dialysis, ultrafiltration and gel filtration) are typically only available in reference laboratories. Routine clinical laboratories typically use a variety of free hormone tests that estimate the free hormone concentration in the presence of protein-bound hormone. Accordingly, a nomenclature of free T4 and free T3 estimate methods has been set up:

- The free hormone methods used by most clinical laboratories (indexes and immunoassays) do not employ physical separation of bound from free hormone and do not measure free hormone concentrations directly! These tests are typically binding protein dependent to some extent and should more appropriately be called "Free Hormone Estimate" tests, abbreviated FT4E and FT3E.
- In general, Free Hormone Estimate tests overestimate the FT4 level at high protein concentrations and underestimate FT4 at low protein concentrations.

Each method has plus and minus when compared to the others. Accordingly, when it is suspected that a FT4 result is discrepant with the clinical status, FT4 should be checked using a different manufacturer's method (usually measured in a different laboratory). Serum FT3 measurement has little specificity or sensitivity for diagnosing hypothyroidism, since enhanced T4 to T3 conversion maintains normal T3 concentrations until hypothyroidism becomes severe. Patients with NTI or caloric deprivation typically have low total and free T3 values. Serum FT3 measurements, interpreted together with FT4, are useful to diagnose complex or unusual presentations of hyperthyroidism and certain rare conditions. In other world, FT3 should be used in few situations.

#### *14.3.1.3 FT4 method validation*

Most free hormone estimate methods receive inadequate evaluation prior to their introduction for clinical use. Manufacturers rarely extend the validation of their methods beyond the study of ambulatory hypo- and hyperthyroid patients, pregnant patients and a catchall category of "NTI/hospitalized patients". There is currently no consensus as to the best criteria to use for evaluating these free T4 estimate methods.

#### *14.3.1.4 Interferences with thyroid tests*

Thyroid hormone test should display zero interference with any compound, drug or endogenous substance (i.e. bilirubin) in any specimen, at any concentration. Studies available from manufacturers vary widely in the number of compounds studied and in the concentrations tested.

#### *14.3.1.5 Serum FT4 and FT3 normal reference intervals*

Physical separation methods are used to assign values to the calibrators employed for most FT4 estimate tests. There is closer agreement between the reference intervals of the various ligand assays used by clinical laboratories than there is between the various methods that employ physical separation. Reference intervals for FT4 immunoassay methods approximate 9-23 pmol/L (0.7 –1.8 ng/dL). In contrast, the upper FT4 limit for methods such as equilibrium dialysis that employ physical separation extends above 30 pmol/L (2.5 ng/dL). Reference intervals for FT3 immunoassay methods approximate 3.5-7.7 pmol/L (0.2 – 0.5

ng/dL). FT3 methods that employ physical separation are currently only available as research assays. Problems are expected and effectively occur from time to time with "new methods" put on the market.

### 14.3.2 Thyrotropin/thyroid stimulating hormone (TSH)

TSH methods are capable of detecting not only increases in TSH, characteristic of primary hypothyroidism, but also the low TSH values typical of hyperthyroidism. Methods are often based on non-isotopic immunometric assay principles and are available on a variety of automated immunoassay analyzer platforms. Most of the current methods are capable of achieving a functional sensitivity of 0.02mIU/L or less, which is a necessary detection limit for the full range of TSH values observed between hypo- and hyperthyroidism. With this level of sensitivity, it is possible to distinguish the profound TSH suppression typical of severe Graves' thyrotoxicosis (TSH < 0.01 mIU/L) from the TSH suppression (0.01 – 0.1 mIU/L) observed with mild (subclinical) hyperthyroidism and in some patients with a non-thyroidal illness (NTI). It is recognized that the TSH measurement is a more sensitive test than FT4 for detecting both hypo- and hyperthyroidism.

As a result, some countries now promote a TSH-first strategy for diagnosing thyroid dysfunction in ambulatory patients. Other countries still favor the TSH + FT4 panel approach, because the TSH-first strategy can miss patients with central hypothyroidism or TSH-secreting pituitary tumors.

#### *14.3.2.1 Assay problems*

TSH is a heterogeneous molecule and different TSH isoforms circulate in the blood and are present in the pituitary extracts used for assay standardization (Medical Research Council (MRC) 80/558) as well as in recombinant human TSH (rhTSH) preparations that might be used as primary standards.

As for other assays,TSH assay functional sensitivity is defined by the 20 % between-run coefficient of variation (CV) determined by the recommended protocol TSH immunoassays.

#### *14.3.2.2 TSH reference intervals*

Despite some gender, age and ethnicity-related differences in TSH, it is not considered necessary to adjust the reference interval for these factors in clinical practice. Serum TSH levels exhibit a diurnal variation with the peak occurring during the night and the nadir, which approximates to 50% of the peak value, occurring between 1000 and 1600 hours. This biologic variation does not influence the diagnostic interpretation of the test result since most clinical TSH measurements are performed on ambulatory patients between 0800 and 1800 hours and TSH reference intervals are more commonly established from specimens collected during this time period. Serum TSH concentrations is normally distributed when log-transformed. For reference range calculations, it is customary to log-transform the TSH results to calculate the 95% reference interval (typical population mean value ~1.5 mIU/L, range 0.4 to 4.0 mIU/L in iodide-sufficient populations). However, given the high prevalence of mild (subclinical) hypothyroidism in the general population, it is likely that the current upper limit of the population reference range is skewed by the inclusion of persons with occult thyroid dysfunction.

#### *14.3.2.3 Clinical use of serum TSH measurements*

TSH should be assayed in most if not all situations in which thyroid dysfunction is suspected, provided that the TSH assay used has a functional sensitivity at or below 0.02 mIU/L:

- Screening for Thyroid Dysfunction in Ambulatory Patients
- Elderly Patients
- L-T4 Replacement Therapy
- L-T4 Suppression Therapy
- Serum TSH Measurement in Hospitalized Patients with NTI
- Central Hypothyroidism
- Inappropriate TSH Secretion Syndromes
- TSH-Secreting Pituitary Tumors
- Thyroid Hormone Resistance

### 14.3.3 Thyroid autoantibodies (TPOAb, TgAb and TRAb)

Autoimmune thyroid disease (AITD) causes cellular damage and alters thyroid gland function by humoral and cell-mediated mechanisms. Cellular damage occurs when sensitized T-lymphocytes and/or autoantibodies bind to thyroid cell membranes causing cell lysis and inflammatory reactions. Alterations in thyroid gland function result from the action of stimulating or blocking autoantibodies on cell membrane receptors. Three principal thyroid autoantigens are involved in AITD. These are thyroperoxidase (TPO), thyroglobulin (Tg) and the TSH receptor. Other autoantigens, such as the Sodium Iodide Symporter (NIS) have also been described, but as yet no diagnostic role in thyroid autoimmunity has been established.

TSH receptor autoantibodies (TRAb) are heterogeneous and may either mimic the action of TSH and cause hyperthyroidism as observed in Graves' disease or alternatively, antagonize the action of TSH and cause hypothyroidism. The latter occurs most notably in the neonate as a result of a mother with antibodies due to AITD.

TPO antibodies (TPOAb) have been involved in the tissue destructive processes associated with the hypothyroidism observed in Hashimoto's and atrophic thyroiditis. The appearance of TPOAb usually precedes the development of thyroid dysfunction. Some studies suggest that TPOAb may be cytotoxic to the thyroid.

The pathologic role of TgAb remains unclear. In iodide sufficient areas, TgAb is primarily determined as an adjunct test to serum Tg measurement, because the presence of TgAb can interfere with the methods that quantitate Tg. In iodide deficient_areas, serum TgAb measurements may be useful for detecting autoimmune thyroid disease in patients with a nodular goiter and for monitoring iodide therapy for endemic goiter.

Laboratory tests that determine the cell-mediated aspects of the autoimmune process are not currently available. However, tests of the humoral response, i.e. thyroid autoantibodies, can be assessed in most clinical laboratories. Although autoantibody tests have inherent clinical utility in a number of clinical situations, these tests should be selectively employed.

#### *14.3.3.1 Clinical significance of thyroid autoantibodies*

TPOAb and/or TgAb are frequently present in the sera of patients with AITD *(251)*. However, occasionally patients with AITD have negative thyroid autoantibody test results. TRAb are present in most patients with a history of or who currently have Graves' disease. During pregnancy, the presence of TRAb is a risk factor for fetal or neonatal dysfunction as a result of the transplacental passage of maternal TRAb *(252,253)*. The prevalence of thyroid autoantibodies is increased when patients have non-thyroid autoimmune diseases such as type 1 diabetes and pernicious anemia *(254)*. Aging is also associated with the appearance of thyroid autoantibodies *(255)*. The clinical significance of low levels of thyroid autoantibodies in euthyroid subjects is still unknown *(256)*. However, longitudinal studies suggest that TPOAb may be a risk factor for future thyroid dysfunction, including post-partum thyroiditis (PPT) as well as the development of autoimmune complications from treatment by a number of therapeutic agents *(50,257,258)*. These include amiodarone therapy for heart disease, interferon-alpha therapy for chronic hepatitis C and lithium therapy for psychiatric disorders *(75,259-262)*. The use of thyroid autoantibody measurements for monitoring the treatment for AITD is generally not recommended *(263)*. This is not surprising since treatment of AITD addresses the consequence (thyroid dysfunction) and not the cause (autoimmunity) of the disease. However, changes in autoantibody concentrations often reflect a change in disease activity.

#### *14.3.3.2 Nomenclature of thyroid antibody tests*

The nomenclature used for thyroid autoantibodies has proliferated, particularly in the case of TSH receptor antibodies (LATS, TSI, TBII, TSH-R and TRAb). The terms used in this monograph, TgAb, TPOAb and TRAb are those recommended internationally. These terms correspond to the molecular entities (immunoglobulins) which react with the specified autoantigens recognized by the laboratory test. Method differences may bias the measurement of these molecular entities, e.g.: methods may detect only IgG or IgG plus IgM; TPOAb or Ab directed to TPO and other membrane autoantigens; TSH inhibiting and/or TSH stimulating TRAb.

#### *14.3.3.3 Recommended uses for TPOAb measurement*

- Diagnosis of autoimmune thyroid disease
- Risk factor for autoimmune thyroid disease
- Risk factor for hypothyroidism during Interferon alpha, Interleukin-2 or Lithium therapy
- Risk factor for thyroid dysfunction during amiodarone therapy (see Guideline 5)
- Risk factor for hypothyroidism in Down's syndrome patients
- Risk factor for thyroid dysfunction during pregnancy and for post-partum thyroiditis
- Risk factor for miscarriage and in-vitro fertilization failure

#### *14.3.3.4 Recommended uses for TgAb measurement in non-neoplastic conditions*

- In iodide sufficient areas, it is not usually necessary or cost-effective to order both TPOAb and TgAb, because TPOAb-negative patients with detectable TgAb rarely display thyroid dysfunction.
- In iodide deficient areas, serum TgAb measurements may be useful for detecting autoimmune thyroid disease when patients have a nodular goiter.

- Monitoring iodide therapy for endemic goiter.

#### *14.3.3.5 Recommended uses for TgAb measurement in differentiated thyroid carcinomas (DTC)*

- TgAb concentration should be measured in ALL patient sera prior to Tg analysis because low levels of TgAb can interfere with serum Tg measurements causing either falsely low or undetectable or high values depending on the Tg method used.
- TgAb should be measured in every serum specimen sent to the laboratory for Tg testing.
- Serial TgAb measurements should be made on all TgAb-positive DTC patients using the same manufacturer's method because serial TgAb values have prognostic significance for monitoring response to DTC treatment.
- TgAb methods should be immunoassay not agglutination, because low levels of TgAb can interfere with serum Tg measurements made by most methods, and serial measurements must be quantitative not qualitative.
- Serum Tg recovery tests do not reliably detect the presence of TgAb and should be discouraged as a method for detecting TgAb (Guideline 46).
- Before changing the TgAb method, the laboratory should inform physician users and evaluate the relationship between the old and proposed new method values. Patients should be re-baselined if the difference between the methods is >10% CV

#### *14.3.3.6 Recommended uses for TRAb measurement*

- To investigate the etiology of hyperthyroidism when the diagnosis is not clinically obvious.
- A declining TRAb concentration during long-term antithyroid drug therapy is suggestive of remission. However TRAb measurements can be misleading in 25% of such patients.
- TRAb measurements are useful to diagnose Graves' disease patients and for relating TRAb values to a treatment algorithm.
- To evaluate patients suspected of "euthyroid Graves' opthalmopathy". Undetectable TRAb however, does not exclude the condition.
- Although TSAb assays have theoretical advantages, some believe that TBII tests which detect both stimulating (TSAb) and the rare cases of blocking (TBAb/TSBAb) antibodies are equally useful.
- For pregnant women with a past or present history of Graves' disease. Note: Pregnant women who are euthyroid after receiving prior antithyroid drug treatment for Graves' disease have a negligible risk for fetal or neonatal hyperthyroidism.
- Euthyroid pregnant women (± L-T4 treatment) who have had prior radioiodide treatment for Graves' disease should have TRAb measured both early in pregnancy when a high value is a risk factor for fetal hyperthyroidism (2-10%), and during the third trimester to evaluate the risk of neonatal hyperthyroidism.
- Pregnant women who take antithyroid drugs (ATD) for Graves' disease to maintain a euthyroid state during pregnancy should have TRAb measured in the third trimester. A high TBII value should prompt a clinical and biochemical evaluation of the neonate for hyperthyroidism, both at birth (cord blood) and at 4 to 7 days after the effects of transplacental passage of ATD have been lost.
- The assessment of the risk of fetal and neonatal thyroid dysfunction necessitates the detection of either blocking or stimulating TRAb when mothers have no intact thyroid

following past therapy for Graves' hyperthyroidism.
- To identify neonates with transient hypothyroidism due to the presence of TSH receptor blocking antibodies.

### 14.3.4 Thyroglobulin (Tg)

Thyroglobulin (Tg), the precursor protein for thyroid hormone synthesis is detectable in the serum of most normal individuals when a sensitive method is used. The serum Tg level integrates three major factors:

- the mass of differentiated thyroid tissue present;
- any inflammation or injury to the thyroid gland which causes the release of Tg;
- the amount of stimulation of the TSH receptor (by TSH, hCG or TRAb).

An elevated serum Tg concentration is a non-specific indicator of thyroid dysfunction. Most patients with elevated serum Tg have benign thyroid conditions. The primary use of serum Tg measurements is as a tumor marker for patients carrying a diagnosis of differentiated thyroid cancer (DTC). Approximately two thirds of these patients have an elevated pre-operative serum Tg level that confirms the tumor's ability to secrete Tg, and validates the use of serum Tg measurements as a post-operative tumor marker *(307)*. In contrast, when the pre-operative serum Tg concentration is not elevated above normal, there is no evidence that the tumor is capable of Tg secretion, and the value of an undetectable post-operative serum Tg value is less reassuring. In such patients a detectable post-operative serum Tg could represent a large amount of tumor. In general, changes in serum Tg post-operatively represent changes in tumor mass, provided that a constant TSH level is maintained with L-T4 therapy.

#### *14.3.4.1 Standardization*

Serum Tg concentrations measured by either RIA or IMA methods, vary widely. A study sponsored by the Community Bureau of Reference of the Commission of the European Communities has developed a new international Tg reference preparation, CRM-457. The bias between different Tg methods may result from differences between the Tg-free matrix used to dilute standards and patient serum, or differences in the epitope recognition by the different Tg antibodies used by individual manufacturers. Ideally, the diluent used for standards should be Tg-free/TgAb-free human serum or alternatively, a non-serum matrix that has been selected to produce a signal (radioactive counts, relative light units etc) that is identical to Tg-free/TgAb-free human serum. It is critical that physicians be informed before the laboratory changes its Tg method to allow for a re-baselining of DTC patients.

The widespread adoption of the CRM-457 standard was projected to reduce, but not eliminate the significant method-to-method variability that exists with this procedure. It was hoped that worldwide standardization would facilitate better agreement in the literature from different studies as well as improve the clinical use of serial Tg monitoring of DTC patients who sometimes have serum Tg measurements determined by different laboratories. Unfortunately, the use of the new CRM-457 standard has not eliminated the problems of between-method variability as much as initially thought. Currently, serum Tg levels determined by methods that use CRM-457 standards can differ by as much as four-fold. These method-to-method differences are greater than the goal for maximum imprecision required for monitoring individual patients and precludes the interchangeable use of different Tg methods for long-term follow-up of thyroid cancer patients.

#### *14.3.4.2 For laboratories considering changing their Tg method*

Select a Tg method on the basis of its performance characteristics not cost or expediency. Before changing the Tg method the laboratory should consult with physician users and compare results between the old and proposed new method using specimens from both TgAb-negative and TgAb-positive patients.

- TgAb-negative patients: If the bias between the old and new method results is > 10%, physicians should be informed and given sufficient time to re-baseline critical patients.
- TgAb-positive patients: The laboratory should warn physicians about the likely direction of interference in the presence of TgAb.
- If serum Tg values are to be reported for TgAb-positive specimens, an appropriate cautionary comment should be displayed on each laboratory report:

*For IMA methods:*
IMA methods may give inappropriately low or underestimate serum Tg levels when TgAb is present. Undetectable serum Tg results cannot be used to indicate the absence of tumor in a TgAb-positive patient. A detectable Tg level indicates that Tg is present, but concentrations may be underestimated.

*For RIA methods:*
RIA methods may give inappropriately higher- or underestimated serum Tg values when TgAb is present (depending on the method). Detectable serum Tg results should not be used as the sole factor for determining the presence of residual thyroid tissue or tumor.

#### *14.3.4.3 Tg messenger RNA (mRNA) testing*

The clinical value of Tg mRNA measurements in peripheral blood has yet to be established. Before Tg mRNA testing can be used to facilitate the therapeutic decision-making for DTC, questions regarding the sensitivity and tissue specificity of Tg mRNA in peripheral blood need to be resolved

#### *14.3.4.4 Clinical uses of serum Tg measurement*

14.3.4.4.1 Non-neoplastic conditions

Serum Tg is elevated when patients have a goiter or in most hyperthyroid conditions. A low serum Tg concentration can be a useful parameter for confirming the diagnosis of thyrotoxicosis factitia and/or investigating the etiology of congenital hypothyroidism

14.3.4.4.2 Differentiated thyroid carcinomas (DTC)

In the setting of DTC, the serum Tg concentration reflects thyroid mass (tumor or normal remnant), thyroid injury (surgery or FNA) and TSH receptor stimulation (endogenous or rhTSH) *(122)*. Since the TSH level is a major regulator of serum Tg concentrations, it is difficult to interpret serum Tg values without knowing the TSH status of the patient. Although there is no “normal Tg reference range” for treated DTC patients, the normal relationship between thyroid mass and serum Tg provides an important reference point. Specifically, one gram of normal thyroid tissue releases ~1 μg/L (ng/mL) Tg into the circulation when the serum TSH is normal and ~0.5 μg/L (ng/mL) when the serum TSH is suppressed below 0.1 mU/L.

### 14.3.5 Calcitonin (CT) and RET Proto-oncogene

Medullary carcinoma of the thyroid (MTC) arises from a malignant transformation of the parafollicular C cells of the thyroid and accounts for ~5-8% of all cases of thyroid cancer. Approximately 75% are sporadic in presentation and 25% are hereditary. In a study of thyroid nodules, the prevalence of MTC is reported as 0.57%. The behavior and management of MTC differs from that of well-differentiated follicular-derived thyroid carcinomas . The inherited forms of MTC come under the heading of multiple endocrine neoplasia (MEN) types 2A and 2B. These are autosomal dominant inherited multiglandular syndromes with age-related penetrance and variable expression. Familial MTC (FMTC) is characterized by the occurrence of MTC without any associated endocrinopathy. In 1993, genetic mutations in the RET proto-oncogene were discovered. The gene responsible for these diseases is known to be located on the chromosome sub-band 10q11.2.

#### *14.3.5.1 Calcitonin assays*

- Mature (32 amino acid) CT is the principal tumor marker for MTC.
- CT measurements used for the diagnosis of MTC and for monitoring purposes should be performed using two-site immunometric assays that are specific for the mature 32 amino acid monomer of CT.
- Currently, the lower normal threshold for CT is generally accepted as being less than 10 pg/ml (ng/L).
- As new, more sensitive CT kits become available, the lower CT threshold should be redefined.

#### *14.3.5.2 Provocative calcitonin-stimulation tests used for diagnosing MT*

Provocative stimuli, such as calcium and pentagastrin (Pg) and when Pg is unavailable, omeprazole, have been used to expose C-cell abnormalities, since they induce an increase in the CT level at all stages of MTC.One advantage of these tests is that they are able to detect hyperplasia of the C cells before MTC appears in earnest. In countries like the United States where genetic testing is readily available, surgery for gene carriers is based on genetic testing alone and provocative tests are rarely used. In some countries Pg has become difficult to obtain and the majority of surgeries are now performed based on genetic testing alone. Provocative tests are usually employed:

- To confirm the diagnosis of MTC preoperatively when basal CT levels are only mildly elevated (less than 100 pg/ml).
- To detect C-cell disease in *RET*-positive gene carriers
- For pre-surgical monitoring of *RET*-positive children
- For post-operative monitoring for tumor recurrence
- When genetic testing is not readily available

#### *14.3.5.3 Postoperative follow-up of MTC*

- Serum CT and CEA should be measured just prior to, and 6 months after, surgery for MTC. Serum CT levels fall slowly in some patients. The first post-operative CT measurement should not be made until 2 weeks after surgery.
- The presence of residual tissue or a recurrence of MTC can only be ruled out if both basal and post pentagastrin or calcium-stimulated CT levels are undetectable.

#### *14.3.5.4 Elevated calcitonin levels in conditions other than MTC*

Elevated calcitonin levels have been observed in other pathologies besides MTC and neuroendocrine tumors. Increased serum calcitonin release occurs with autoimmune thyroid diseases (Hashimoto's thyroiditis or Graves' disease). Non-thyroid conditions where elevated CT has been noted include severe renal insufficiency, hypercalcemia and hypergastrinemia, acute pulmonary inflammatory conditions and other local or general forms of sepsis (Biermer's disease, iatrogenic disorders, etc.).

#### *14.3.5.5 Detection of MTC by measuring the RET Proto-oncogene*

Until 1987, the only method available for detecting subjects at risk for MTC was to perform repeated stimulated CT measurements on family members of MTC patients. The subsequent identification of the locus 10q11.2 responsible for MEN 2 on chromosome 10 then made it possible to detect at-risk subjects by genetic screening. It has now been established that several types of mutations on chromosome 10 can activate the proto-oncogene *RET*, that is responsible for MEN 2 .This now allows physicians to screen for the condition before the first biological signs appear. Currently in many developed countries, genetic studies are the first line approach for this diagnosis. For accurate disease prediction however, it is necessary that positive genetic screening results be followed with an exhaustive survey of both the healthy and affected members of the family

### 14.3.6 Urinary iodine measurement

An adequate dietary intake of iodine is required for normal thyroid gland hormone production and to maintain a euthyroid state. It follows therefore that the measurement of iodine intake from foodstuffs or medications has clinical relevance. In the clinical laboratory, iodine measurements are used primarily for epidemiological studies or for research .To date, the major application of iodine analysis is to assess the dietary iodine intake of a given population.This is an issue of considerable importance, since it has been estimated that iodine deficiency disorders (IDD) potentially affect 2.2 billion people throughout the world. Even in developed countries such as the USA and Australia, a decline in dietary iodine intake has been demonstrated, while borderline dietary intake has long been characteristic in much of Europe .

As the majority of ingested iodine is excreted in the urine, the measurement of urinary iodine excretion (UI) provides an accurate approximation of dietary iodine intake . In most circumstances the determination of UI provides little useful information on the long-term iodine status of an individual, since the results obtained merely reflect recent dietary iodine intake. However, measuring UI in a representative cohort of individuals from a specific population provides a useful index of the iodine level endemic to that region . Besides estimating the UI concentration in people, other applications of iodine measurements include determining iodine in milk, food products and drinking water . Iodine measurement in thyroid or breast tissue has been performed as part of research studies. Since low inorganic iodine concentrations in serum (~ 1pg/dL) are associated with relatively abundant hormonal iodine, the measurement of plasma inorganic iodine (PII) has been restricted to research studies in pregnancy .

## 14.4 The importance of the laboratory – physician interface

It is essential that clinical laboratory scientists develop an active collaboration with the physicians using their laboratory services in order to select thyroid tests with the most appropriate characteristics to serve the patient population in question.

An active laboratory-physician interface ensures that high quality, cost-effective assays are used in a logical sequence, to assess abnormal thyroid disease presentations and to investigate discordant thyroid test results.

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