Thyroid Diseases in Childhood

Recent Advances from Basic Science to Clinical Practice

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Mario De Felice and Roberto Di Lauro

1.1 Where Does the Adult Thyroid Come from?

The thyroid gland has a characteristic shape consisting of two elongated lobes connected by a narrow isthmus. In humans, it is located in front of the trachea at the base of the neck, between the thyroid cartilage and the V-VI tracheal ring. The gland has a distinctive histological organization characterized by the presence of spheroidal structures, called follicles. Follicles consist of a closed cavity (follicular lumen) surrounded by a layer of epithelial cells known as thyroid follicular cells (TFC) devoted to the production and export of thyroid hormones. In addition to TFCs, the most abundant other endocrine cells, C cells responsible for calcitonin production, are scattered in the interfollicular space.

The processes leading to the formation of the adult thyroid involve both the assembly of distinct embryonic structures originated in different locations (the *thyroid anlage* and the ultimobranchial bodies) and the differentiation of multipotent cells toward a highly specialized phenotype. Such processes require the harmonized action of genetic factors acting outside (extrinsic) and inside (intrinsic) the precursors of thyroid cells [1].

Thyroid development in humans (summarized in O'Rahilly [2] and more recently in Polak [3]) has been accurately described, at microscopic level, since the first

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decades of the 1900s. In the last 20 years, the molecular pathways underlying thyroid organogenesis have been systematically analyzed in animal models, mostly in mice. *Zebrafish* (Danio rerio), a tropical freshwater teleost fish, has also been used as a valuable tool in developmental biology including thyroid morphogenesis [4]. Data from animal models and insights from patients presenting thyroid dysgenesis (i.e., impaired thyroid development) have allowed to dissect the genetic mechanisms controlling the development of the thyroid gland that are to a large extent conserved among vertebrates [5].

1.2 A Morphological Description of Thyroid Development in Humans

The adult thyroid gland is assembled from different embryonic components: a median component (the thyroid bud, derived from the *thyroid anlage*) and two lateral structures (the ultimobranchial bodies).

According to Weller [6], in human embryos the "median thyroid primordium" (*thyroid anlage*) is visible at Carnegie stages (C)9 (length crown-rump ca. 1.5–2.5 mm; 1–3 pairs of somites; ca 20 days) as a fold in the ventral wall of the primitive pharynx. At this stage, the thyroid primordium consists of an accumulation of endodermal cells; the cells are closely packed but do not appear different from the other cells of the surrounding endoderm. At C10, embryonic day (E) 22, the thyroid primordium is clearly evident as a midline endodermal thickening in the floor of the primitive pharynx caudal to the region of the first branchial arch which forms the *tuberculum impar*. This thickening, 2 days later at CS 11, forms a small endodermal pit-like depression and then an outpouching of the endoderm so that the thyroid appears as a bud (spherical nodule) [2]. The thyroid bud is a nonhomogeneous structure, formed by high and pseudostratified epithelial cells different from those lining the pharynx [7]. As a consequence of the outgrowth and budding of the thyroid anlage, the developing thyroid by E26 appears as a flask-like structure with a narrow neck. This structure becomes an endodermally lined diverticulum that starts from the midline of the dorsum of the tongue and extends caudally. The distal part of the diverticulum represents the anlage of the gland itself connected with the floor of the pharynx by the thyroglossal duct. By E30, the thyroid anlage becomes larger while the thyroglossal duct becomes rather thinner and starts to fragment by CS 15 (ca 7–9 mm; E33). At CS16, the developing thyroid is a bilobate structure still not connected with the pharynx [6]. Finally, the thyroid reaches its destination in front of the trachea between E45 and E50 and shows its definitive external form - two lobes connected by the narrow isthmus – by CS20 (ca 18–22 mm; ca E51).

The ultimobranchial bodies (UBBs) – the so-called lateral thyroid component - are first evident at E24, as an outpouching of the ventral region of the fourth pharyngeal pouches. By E35, UBB primordia appear as long-necked flasks which start to migrate a few days later, losing any connection with the pharynx. After a ventral to caudal migration, UBBs are visible in close contact with the developing thyroid

at CS18 (E44). Then, the two structures merge; around E55, cells from UBBs and median thyroid are completely fused.

The subsequent formation of the peculiar follicular organization of the thyroid requires several weeks [8]. This process begins after 7 weeks of gestation when thyroid tissue is composed of strands of unpolarized thyroid cells. By the 10th week, small canaliculi appear in the thyroid tissue. Then, these small canaliculi enlarge, and around the 11th week, small follicles are clearly visible Progressive follicular growth occurs by the 12th week onward [9]. T4 is first detected at the 11th week, but a robust hormone production begins around 15-16 weeks [10].

1.3 Thyroid Development in Animal Models

1.3.1 Ontogenesis

The process of gastrulation divides the embryonic epiblast into three germ layers, ectoderm, mesoderm, and endoderm, the most internal layer of the vertebrate embryo (Fig. 1.1a). Embryos of different vertebrate species show divergent morphogenetic modes; however, after gastrulation and just before the organogenesis processes begin, all embryos share similar body plans when the endoderm generates a primitive gut tube. The tube, which runs along the anterior-posterior (craniocaudal) axis of the embryo, is subdivided into the foregut - the most anterior (cranial) region of the tube, midgut, and hindgut (Fig. 1.1b). The endoderm contributes to a remarkable number of cell lineages which form the epithelial components of many organs, such as intestine, liver, pancreas, stomach, lungs, thymus, and thyroid, the anterior-most organ that originates from the foregut.

Shortly after formation, the gut tube is formed of a single layer of largely unspecified epithelial-like cells. Subsequently, due to complex phenomena, only partially clarified, the endodermal cells acquire a positional identity along the anterior-posterior axis, and distinct groups of cells can be identified by the expression of specific signaling molecules and transcription factors. Endodermal cells are recruited into specific cell lineages (this initial, still reversible, step of the differentiation program is called *specification*); as a consequence, the tube, previously monotonous, is patterned in defined domains (*endoderm regionalization*). In many cases, the first visible event is the appearance of a multilayered structure from which, eventually, organ primordia emerge as outpockets (buds) from the tube (Fig. 1.1b).

As in humans, also in mouse embryos (19.5 day-long gestation), the earliest morphological proof of thyroid specification is the appearance, at E 9–9.5, of an endodermal thickening in the ventral wall of the primitive pharynx (Fig. 1.2). However, a day before this thickening is evident, a group of cells of the pharynx endoderm (*thyroid anlage*) is distinguishable from any other endodermal cells for the simultaneous expression of the transcription factors Nkx2-1, Pax8, Foxe1, and Hhex [11]. These cells can be defined as precursors of thyroid follicular cells (pTFC), i.e., developing thyroid cells that do not yet synthesize thyroid hormones. It is worth noting that, even if the ultimobranchial bodies participate in the organogenesis of the gland, thyroid

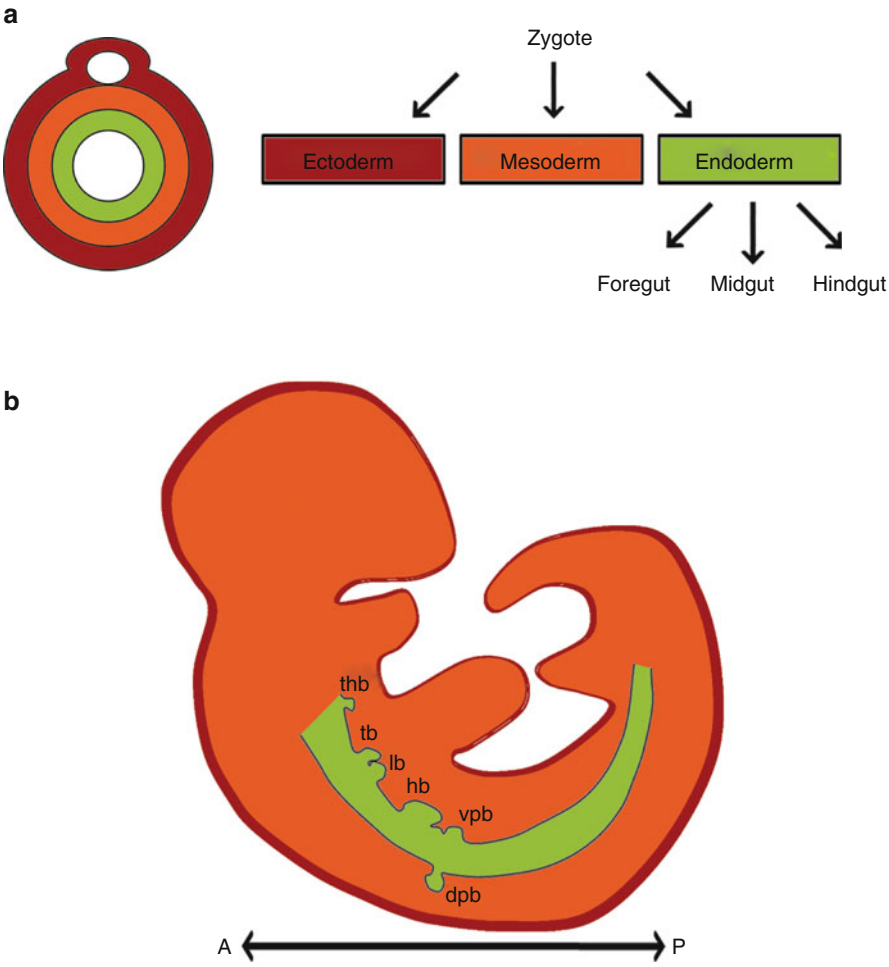

Fig. 1.1 Early stage of mammalian development. Panel **a**: Schematic cross-section of a mammalian embryo after gastrulation. The position of three germ layers, ectoderm (*brown*), mesoderm (*orange*), and endoderm (*green*) is indicated. The endoderm is the internal-most layer of the embryo. Panel **b**: The endoderm lineage. The endoderm generates a primitive gut tube which runs along the anterior-posterior axis of the embryo. The tube is subdivided into the foregut (the most anterior region of the tube), midgut and hindgut. Panel **c**: Schematic representation of an E10 mouse embryo. The embryonic structures derived from ectoderm, mesoderm and endoderm are indicated in brown, orange and green respectively. At this stage the primordia of organs derived from the foregut are evident as buds (outpockets) from the primitive endodermal tube. *thb* thyroid bud, *tb* trachea bud, *lb* lung bud, *hb* liver bud, *vpb* ventral pancreatic bud, *dvb* dorsal pancreatic bud. A ← → P: anterior-posterior axis of the embryo (Courtesy of Dott. MT De Angelis)

hormone-producing cells derive only from the thyroid anlage. This assumption has been proven formally using lineage studies in zebrafish [12] but not yet in mammals where thyroid primordium and UBBs merge in the definitive gland.

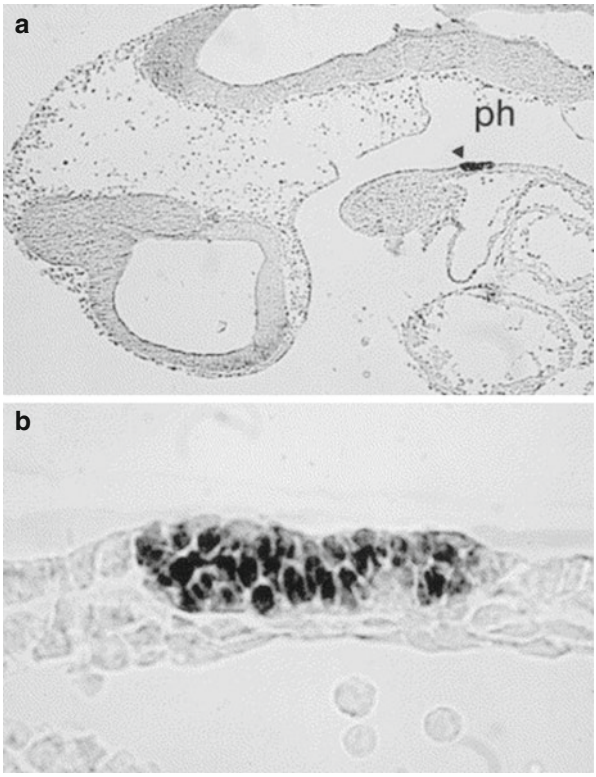

Fig. 1.2 Thyroid anlage in mouse embryo. Panel **a**: Sagittal section of mouse embryo at E9.5. Precursors of Thyroid follicular cells are specifically stained with anti Pax8 antibody. Thyroid anlage (*arrow head*) appears as a restricted area of the endodermal epithelium of the pharynx floor (*ph*). Panel **b**: Enlargement of the same section (From: Missero et al. [44])

The *prime mover* responsible for the specification of the thyroid anlage in mice is still rather obscure. However, data obtained in other organisms such as zebrafish and chicks have been offering valuable information to extend our knowledge of early steps of thyroid development in mammals.

The development of some endoderm-derived organs (liver, pancreas, lungs) requires inductive signals from the surrounding mesoderm. In the case of the thyroid, we know that in the mouse by E8.5, the *thyroid anlage* is very close to the aortic sac, from which embryonic heart outflow and pharyngeal arteries originate [13]. The developing thyroid could be targeted by signals coming from the cardiac mesoderm or endothelial lining of the aortic sac. This hypothesis has received strong support by studies in zebrafish [14]. It has been demonstrated that early thyroid development absolutely requires both the expression of the transcription factor Hand2 in the cardiac mesoderm surrounding the thyroid anlage and fibroblast growth factor (FGF)-signals. In humans, a cross-talk between the developing thyroid and heart is supported by epidemiological studies. In fact, a number of patients affected by thyroid dysgenesis also present cardiac malformations at birth [15]; furthermore, DiGeorge syndrome includes both congenital heart defects and an increased risk of congenital hypothyroidism [16].

1.3.2 Translocation and Lobe Formation

In mouse embryos, by E9.5 the thyroid anlage expands and then forms a bud that moves caudally in the surrounding mesenchyme. In the early phase of morphogenesis, the proliferation rate of pTFC is lower than that of the other endodermal cells [13]. Hence, enlargement of the developing thyroid probably depends on the recruitment of other endodermal cells of the primitive pharynx into the thyroid anlage.

The migration of pTFC is a notable feature of thyroid organogenesis. Morphogenesis of the neck encompasses a number of events that can contribute to the translocation of pTFC in the direction of the trachea. However, translocation mostly involves active migration of the precursor cells, which probably move through a process of "collective cell migration" by which cells move in concert without completely disrupting their cell-cell contacts [17]. The genetic basis of the thyroid bud migration has been, at least in part, elucidated; the presence of transcription factor Foxe1 in pTFC is required for thyroid bud migration [11, 18].

At E11.5, after the thyroid primordium is no longer connected to the pharynx, the process of lobulation begins, attested by a lateral enlargement of the developing thyroid; one day later, thyroid appears as an elongated structure close to third pharyngeal arch arteries that will participate in the formation of the definitive carotid vessels. These reports suggest that inductive signals originating from adjacent vessels could be relevant in the lobulation of the gland. This hypothesis was validated using genetically modified mice deprived of sonic hedgehog [19] (a morphogen relevant in vertebrate organogenesis) or Tbx1 [16] (a transcription factor regulated by sonic hedgehog itself). In these models, the development of vessels is impaired, and the thyroid bud does not stay in contact with them. At the same time, the lobulation process is disturbed, and the gland does not separate into two distinct lobes but appears as a single mass. However, in addition to extrinsic signals, pTFC specific mechanisms are required for correct lobulation. In fact, in mice partially deficient for both the transcription factors Nkx2-1 and Pax8, thyroid hemiagenesis is frequently observed [20].

1.3.3 Functional Differentiation and Enlargement

By E14, precursors of TFCs proliferate and begin to undergo a differentiation program completed at E16.5, when the thyroid is composed by *bona fide* adult TCFs, able to synthesize thyroid hormones. TCFs express, according to a precise temporal pattern, a number of specific proteins such as thyroglobulin (Tg), TSH receptor (Tshr), thyroid peroxidase (TPO), sodium/iodide symporter (NIS), thyroid NADPH oxidases (Duox's), and pendrin (PDS). At the same time, the gland accomplishes the proper follicular organization required to guarantee a correct hormone supply to the organism.

In the postnatal thyroid, mechanisms triggered by TSH upon its interaction with Tshr are extremely relevant in the regulation of both proliferation and function of

TFCs. The finding that in the mouse Tshr is detectable in thyroid cells by E14 and TSH by E15 could suggest a role for TSH/Tshr signaling in the control of functional differentiation and proliferation of TFC in the developing thyroid. However, in mouse embryos lacking a functional Tshr, the size of the fetal thyroid is quite similar to that in wild-type mice [21]. In addition, the number of proliferating cells in the thyroid is comparable in mutants and wild-type embryos. On the contrary, adult mice lacking TSH signaling have a clearly hypoplastic thyroid. These data indicate that in the mouse, TSH/Tshr signaling does not have a relevant effect on the proliferation of thyroid cells during embryonic life [22]. This scenario could be different in humans. In fact, in the mouse, thyroid organogenesis is complete just at the end of gestation, and the functions of the hypothalamic-pituitary-thyroid axis are accomplished only after birth. In humans,, the thyroid is realized by 12–13 weeks; during the rest of gestation, the thyroid continues to grow and the hypothalamic-pituitary-thyroid axis is functioning. While the role of Tshr/TSH signals in controlling the growth of fetal thyroid remain rather obscure, molecular analysis reveals that both TPO and NIS are almost absent in mouse embryos lacking a functional Tshr. Thus, TSH induced signals are required to express key molecules for hormone biosynthesis and to complete the differentiation program of the thyroid follicular cells [21].

Finally, TSH signaling is not required for folliculogenesis because normal thyroid follicles are present in mouse embryos lacking a functional Tshr. Signals from endothelial cells are relevant in forming follicles. In fact, in animal models characterized by a thyroid-specific severe reduction of vascular density, the gland appears as a multilayer mass of nonpolarized cells [23].

1.3.4 Merging of Ultimobranchial Bodies

In placental mammals, the ultimobranchial bodies are embryonic transient structures derived from the fourth pharyngeal pouch, which will merge with the developing thyroid.

In mice by E10, the fourth pharyngeal pouches are detectable as lateral extroflexions of the primitive foregut expressing the transcription factor Islet1 [24]. One day later, the caudal portion of the pouches grows forming UBB primordia identified for the expression of the factors Hes-1 [25], Isl1, and Nkx2-1 [26]. UBBs begin to migrate and reach the primordium of the thyroid at E13. By E14.5, UBB cells begin to disperse within the thyroid parenchyma, and only remnants of UBBs can be distinguished in the thyroid gland.

The expression of Nkx2-1 is required for the survival of UBBs [26], while Hes-1 plays a role in the merging of these structures into the thyroid [25]. UBB defects have been also described in animal models with an impaired expression of Hox3 genes, encoding factors involved in the morphogenesis of several structures. In these models, UBBs do not merge into the thyroid but remain as bilateral vesicles composed exclusively of calcitonin-producing cells (persistent ultimobranchial bodies) [27].

1.4 Thyroid Toolkit Genes

The generation of an increasing number of mouse models carrying mutations *on demand* of specific loci has been offering biomedical research an invaluable tool for the identification of the functions of proteins *in vivo* and the dissection of the pathways underlying developmental processes. Also in the case of the thyroid, a large part of our knowledge of molecular mechanisms of its morphogenesis is due to studies of phenotype mice, in which genes encoding proteins expressed during thyroid development have been inactivated by gene targeting (knockout mice).

Molecular genetics of thyroid development was inaugurated by the discovery of a number of transcription factors expressed in the thyroid anlage, Nkx2-1 (formerly called TTF-1), Foxe1 (formerly called TTF-2), Pax8, and Hhex. These genes, which can be considered thyroid "toolkit genes," remain expressed in both precursor and differentiated thyroid follicular cells. Although these factors are also expressed in other tissues, the coexpression of all four is a peculiar hallmark of thyroid cells [11].

In the next paragraphs, the focus will be on the role of these genes as deduced by their distribution and by the phenotype of knockout animals; the consequences of mutations in these genes in the pathogenesis of thyroid diseases will be exhaustively treated in later chapters.

1.4.1 Nkx2-1

During embryonic life, Nkx2-1 is expressed in the developing brain, lungs, and thyroid. In the brain, the factor is present in some areas of the developing diencephalon, such as the hypothalamic areas and the infundibulum, and in the neurohypophysis [28]. As for the lungs, Nkx2-1 is detectable in the epithelial cells of the developing trachea and lungs. In the thyroid, it is expressed in both precursor and differentiated TFC, in the C cells [26], and in the epithelial cells of the fourth pharyngeal pouches forming the ultimobranchial body. In human embryos, the expression pattern of Nkx2-1 is not different from that of mice [29]. The phenotype of *Nkx2-1* knockout mice reflects the expression domain of this gene: severe defects in lung and forebrain morphogenesis, lack of pituitary and thyroid [30]. Notably, in absence of Nkx2-1, the thyroid anlage forms and buds, but already by E10 the thyroid primordium appears smaller than in wild-type embryos and soon undergoes degeneration. The ultimobranchial bodies undergo the same process and disappear by E12 [26]. Thus, Nkx2-1 is required not for the initial specification of both follicular and parafollicular thyroid cells but for the survival of these cells. In addition, Nkx2-1 is also involved in the folliculogenesis. In fact, in mice in which this gene is made inactive by the middle of organogenesis, the postnatal thyroid shows a reduced number of dilated follicles [31].

1.4.2 Pax8

During embryonic life, Pax8 is expressed since E8.5 in the kidneys, brain, and thyroid primordium [32]. In the developing kidney, at an early stage, Pax8 is expressed in the nephrogenic cord and in mesonephric tubules; then, it is present in the cortex of the metanephros. In the brain, Pax8 is transiently expressed in the myelecephalon, through the entire length of the neural tube, otic vesicle, and at the midbrain-hindbrain boundary. In the thyroid, Pax8 is expressed in the thyroid follicular cells and in their precursors. In *Pax8* knockout embryos, the thyroid primordium forms, buds from the gut, and begins its migration [33]. However, the thyroid primordium is almost undetectable by E12. In newborn mice, the thyroid lacks thyroid follicular cells and is composed almost completely of calcitonin-producing C cells. The animals are affected by a severe hypothyroidism and die within 2–3 weeks after birth. In mice in which the *Pax8* gene is inactivated in the late fetal life, the thyroid shows a severe hypoplasia and reduced expression of genes required for hormone biosynthesis. Thus, Pax8 is required for the survival and the differentiation of thyroid follicular cells.

1.4.3 Foxe1

At variance with Nkx2-1 and Pax8, whose presence in the gut is restricted to the thyroid anlage, during development Foxe1 is expressed in tissues which are developed from the pharynx and pharyngeal arches: thyroid, tongue, epiglottis, palate, choanae, and esophagus [34]. In addition, Foxe1 is also detected in the whiskers and hair follicles, which derive from the ectoderm [35].

Foxe1 knockout mice die within 48 h of birth [18]. These mice display a severe cleft palate, an absent thyroid or ectopic thyroid, and lack of thyroid hormones. In embryos in absence of Foxe1 at E8.5, the thyroid anlage is specified, but at E9.5 thyroid precursor cells are still on the floor of the pharynx, showing a clear defect of migration. At later stages of development, in the absence of Foxe1, thyroid follicular cells either disappear or form a sublingual small thyroid remnant. In conclusion, Foxe1 has a specific role in controlling the migration of thyroid follicular cell precursors but is not relevant for the specification and differentiation of the thyroid anlage.

1.4.4 Hhex

Hhex is expressed during embryonic life in several organs derived from the foregut endoderm including the thyroid [36]. In the adult, in addition to the thyroid, Hhex expression is maintained in the liver and lungs. In absence of this factor, many developmental processes are impaired and *Hhex* knockout embryos die at

midgestation (E13.5-E15.5); they show severe defects in the liver, forebrain, heart, and thyroid [37]. At E8.5, the thyroid anlage is visible, but already at E9.5, the thyroid primordium is absent or severely hypoplastic. These data suggest that Hhex is required for the survival of precursors of TFC.

1.4.5 Gene Interactions

As summarized above, knockout mice demonstrate that Nkx2-1, Hhex, Pax8, and Foxe1 are required for correct thyroid development. In addition, these transcription factors are linked in a regulative network during the process of thyroid development [11].

In the thyroid anlage, at E9, the expression of Titf1, Hhex, and Pax8 is not dependent on the expression of each other, since the absence of any of these genes does not affect the expression of the others. At a later stage, Nkx2-1, Hhex, and Pax8 contribute to a common network of reciprocal regulatory interaction since each of them controls the maintenance of the expression of the others. The concurrent presence of Nkx2-1, Hhex, and Pax8 is required for the expression of Foxe1. According to this scenario, in the developing human thyroid, the expression of both NKX2-1 and PAX8 precedes the onset of FOXE1 expression [29].

1.5 How Many Genes to Build the Thyroid?

It is obvious that other genes, in addition to *Nkx2-1*, *Foxe1*, *Pax8,* and *Hhex*, are required for a correct thyroid morphogenesis. The global gene expression of the thyroid bud at E10.5 has been defined and compared to genes expressed in the whole embryo. Using this approach, it has been possible to identify a list of about 450 genes strongly enriched in the thyroid bud. It is plausible that this list includes many novel genes required for thyroid morphogenesis [38].

On the other hand, the study of the phenotype of knockout mice has revealed an important role in the development of the gland for some genes which are not thyroid specific but are expressed in many tissues. Some of these genes are described below.

1.5.1 Nkx2-5

Nkx2-5, like *Nkx2-1*, is a gene belonging to the Nkx2 family. It is transiently expressed in the thyroid anlage [39]. In *Nkx2-5* knockout embryos, the number of thyroid precursor cells is strongly reduced, and the developing thyroid appears hypoplastic [40]. The mutant embryos die at early stages of development, and hence, it is not possible to address the role of this factor in the differentiated thyroid. However, in humans, mutations in *NKX2-5* have been associated with thyroid dysgenesis [40].

1.5.2 Fgfr2

Proteins belonging to the Fgf family recognize and activate specific receptors (Fgfr) present in many types of epithelial cells. pTFCs express Fgr2-IIIb (fibroblast growth factor receptor 2, isoform IIIb), a tyrosine kinase receptor able to bind specific Fgfs that are expressed in the mesenchyme. A relevant role of FGF/FGFR interactions in thyroid morphogenesis is demonstrated by the absence of the thyroid gland in knockout mice for Fgr2-IIIb [41].

1.5.3 Hox3 Genes

Hoxa3 is expressed in many tissues, including the developing thyroid, and in the precursors of C cells. As described above (see 1.3.4), in *Hoxa3* knockout mice, ultimobranchial bodies do not merge into the thyroid [27]. In addition, a reduction in the number of thyroid follicular cells and hypoplasia of the thyroid have been described. These defects appear more severe in mice carrying simultaneous mutations in *Hoxa3* and in its paralogs *Hoxb3* and *Hoxd3* genes [42].

1.6 From Embryology to Clinical Practice

The animal models described above demonstrate that mutations in a number of genes involved in thyroid morphogenesis cause defects in thyroid development, i.e., cause congenital hypothyroidism with thyroid dysgenesis (CHTD). According to the "medicine translational approach," it seemed conceivable to investigate whether patients affected by CHTD carry mutations either in the toolkit thyroid genes or in other genes known to be relevant for thyroid morphogenesis. This working hypothesis was revealed to be correct, and it has been currently reported that about 3–5 % of cases of CHTD are associated with mutations in either the toolkit thyroid genes or other genes (mainly *TSHR*) [3, 5]. This data indicate that, albeit in only a small fraction of human patients, CHTD is caused by inheritable genetic defects. However, some points are worth highlighting:

(a) The mode of transmission of inheritance of the phenotype could be different between humans and mice. Humans carrying heterozygous mutations in either *NKX2-1* or *PAX*8 are affected by CH, while mice heterozygous for either *Nkx2-1* or *Pax8* do not present an overt hypothyroidism.
(b) In mice, Foxe1 is required for thyroid bud migration. In humans, impaired thyroid migration (ectopic thyroid) has never been found to be associated with mutations in the *FOXE1* gene.
(c) There is no model for thyroid agenesis, i.e., absence of the gland due to an impaired specification of thyroid bud. Genes required for the initiation of thyroid morphogenesis are still unknown in mammals.

(d) Some cases of CHTD could be due to mutations in not yet identified genes. The identification of new genes and mechanisms involved in thyroid development will be valuable for the elucidation of the molecular pathology of thyroid dysgenesis.

1.7 Making Thyroid *In Vitro*

A recent spectacular achievement by the group of Costagliola [43] (see also Chapter XX – Antonica) has demonstrated that it is possible to form functional thyroid tissue starting from multipotent mouse embryonic stem cells. This work demonstrates that the forced expression of Nkx2-1 and Pax8, combined to exposure to TSH, leads stem cells to differentiate into functional TFC that reorganize in follicles, thus reinforcing the important role of these factors in thyroid differentiation. In addition, this seminal accomplishment opens the way to different approaches for the therapy of thyroid diseases and provides an excellent model to investigate in better detail the processes leading to thyroid formation and function.

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Thyroid Hormones in Fetal Development

2

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Normal fetal growth and development depend on several endocrine, metabolic, and nutritional factors [1]. Among them, an important role is played by thyroid hormones (TH) (T4 thyroxine and T3 triiodothyronine) both of maternal and fetal origin. The supply of maternal TH to the human fetus depends mainly on the mother's thyroid function and on several placental transport mechanisms. Moreover, during pregnancy, double the normal iodine intake of the mother is required to preserve normal TH concentrations [2]. In fetus hypothalamus-pituitary-thyroid–target tissue axis is activated shortly after the thyroid gland has reached its anatomical site in the first weeks of gestation. A full maturation of the complex system including TH transport, receptor availability, and normal function of postreceptoral mechanisms are however needed to ensure the specific biological action at target tissues.

2.1 Implantation Window

Several experimental studies (extensively reviewed by Colicchia et al. [3]) pointed out a possible action of TH and TSH during the "implantation window," i.e., the very short period of time (lasting about 4 days) between the prereceptive and the refractory endometrial phases. This action fits very well with clinical experience showing that thyroid dysfunction of the mother may often be associated with

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complications affecting both mother and fetus [4]. In the last few years, new data underlying the activities of TH in the first phases of implantation and of embryo development has been derived by reproduction technologies and animal models [3].

On the basis of this new knowledge, it has been proposed that TH act throughout different mechanisms.

Two of them, however, seem to be the most important:

(1) Angiogenesis is crucial for successful implantation and is regulated by several factors but mainly by vascular endothelial growth factor (VEGF) and angiopoietins [5, 6]. TH exert a proangiogenic activity inducing VGEF transcription [7] and gene expression.
(2) Natural killer (NK) cells are the most represented leukocyte subpopulation in utero during the implantation period. Throughout their immunomodulation activity, they regulate the subsequent phases of normal implantation, i.e., decidualization [8], placental trophoblast growth [9], and invasion [10]. TH seem to improve this action, and more generally a reciprocal modulation of immune system and TH has been recently suggested [11].

2.2 Placenta

During pregnancy, the placenta transports iodide, which is crucial for a normal synthesis of TH, utilizing a sodium-iodine symporter which is codified by a specific gene expressed in the placenta since the first gestational weeks [12]. In humans as in other mammals, the maternal TH are able to cross the placental barrier, and this is particularly relevant in the period in which the fetal thyroid is not yet able to synthesize TH. Free T4 is preferentially transported across the placenta [13], and its level in fetal blood,after reaching in the second trimester a concentration which is about half of that in maternal blood, increases during the following trimester maintaining high levels until birth [14]. Six different types of transporters for TH have been described in the last few years: the monocarboxylate transporter MCT8 seems to be specific for TH while the remaining five (belonging to the groups of L-type amino acid transporters (LAT) and of organic anion transporting polypeptides (OATP)) are not specific for TH.

Loubiere et al. [15] studying mRNAs encoding TH transporter expression have shown that it starts around 6 weeks of gestation and that the patterns of the different transporters is different during pregnancy.

They demonstrated a lower expression of MCT8, MCT10, OATP1A2, and LAT1 before 14 weeks compared to term as well as a nadir expression in the early second trimester. These AA speculate that these variations could fit well with the necessity to limit maternal-fetal TH transfer at a time when fetal thyroid begins its activity (second trimester) and with the need of increasing maternal TH supply during the last trimester as suggested by other AA [16].

The different role of the TH transporters is however not fully elucidated and merits further investigation. Recently, for example, it has been demonstrated that

transthyretin (TTR), whose role in the transport of TH and retinol has been recognized, is fully expressed in the first weeks of pregnancy in the trophoblast cells under conditions of low oxygen levels and could thereafter contribute to bind maternal T4 and transfer it to fetal circulation [17].

Finally, D3-deiodinase enzyme is highly expressed in the placenta where it may prevent an overexposure to maternal TH catalyzing diodination of T4 to rT3 and of T3 to T2, i.e., from biologically very active to inactive hormones.

2.3 Fetal Hypothalamic-Pituitary-Thyroid Axis (FHPT)

Activity of the fetal axis, of metabolism of TH in utero, and of TH transporters and receptors in fetal tissues have been recently widely reviewed by Forhead and Fowden [18].

In Table 2.1, the developmental stage of several anatomical and functional parameters of FHPT are summarized in relation with the gestational weeks. It is clear that FHPT undergoes a progressive maturation during pregnancy reaching its complete functionality and sensitivity after birth.

During pregnancy, the pattern of fetal blood levels of T4,T3, and TSH is quite different because T4 and TSH levels progressively rise while T3 remains low until the 30th week of gestation when it begins to increase [19].

At birth, TSH peaks, decreasing thereafter in the first few days of postnatal life while T3 and T4 increase in response to various stimuli, such as increase of cortisol (the most important hormonal trigger in the complex phenomenon of adaptation of life). The role of TH at birth seems, however, to be more supportive than pivotal

Table 2.1 Timing of developmental stage in human fetus

Developmental stage	Gestational weeks
Thyroid gland organogenesis	
Pre-colloid	7–13
Colloid	13–14
Follicular	>14
TRH in hypothalamus	10–12
TSH in anterior pituitary gland and circulation	10–12
TSH receptor in thyroid gland	10–12
Iodide uptake in thyroid gland	10–12
Thyroglobulin synthesis	10–12
Iodinated amino acids	14
Synthesis and secretion of thyroid hormones	16–18
Rise in plasma T3	30
Gene and protein expression of thyroid hormone transporters	7–9
Thyroid hormone receptor binding	10–16

as demonstrated by the fact that the adaptation of postnatal life of the hypothyroid fetus is normal [20].

2.4 TH Metabolism and Biological Activity

TH levels, however, are not only due to the FHPT axis with negative feedback at pituitary and hypothalamus levels but depend also on the metabolism of TH after their synthesis and secretion. At peripheral level, two enzymatic systems regulate the metabolism of TH in more biologically active (T3) or relatively inactive (rT3) form. The metabolism is regulated firstly by the deiodinase enzyme activity (D1,D2, and D3 deiodinases). It is interesting to note that these three deiodinases which catalyze the deiodination of T4 to T3 could not only act with different kinetics but are also differently expressed in several tissues. For example, the deiodination in liver regulated by D1 seems to be the most important source of T3 in blood [21] while D2 products of catalyzation are mainly responsible for tissue concentrations. The role of D3, as previously discussed, is prominent in the placenta.

T4 is metabolized to less active or inactive forms not only by deiodinase activity but also by sulfation. It has been demonstrated that more or less 80 % of T4 secreted by the thyroid gland could be processed to sulfate inactive metabolites [22]. Sulfation is a reversible chemical reaction by the activity of specific sulfatase enzymes present in several fetal tissues. This reversibility could be very significant in hypothyroidism because sulfate forms of T3 could be transformed into more active T3 reducing potential damage of the brain.

In experimental animals, it has been demonstrated that in hypothyroidism peripheral fetal tissues could modulate the metabolism of TH. Polk et al. [23] showed, for example, that in hypothyroid sheep fetus deiodination ot T4 in liver is reduced due to a less active D1 while in brain D2 activity, which acts on transformation of T4 to T3, is enhanced.

In other words, we should consider concentrations of TH both in blood and in fetal tissues as a result of a complex balance between the synthesis of these hormones, under the control of the FHPT axis, and their metabolism in different tissues. This complex homeostatic system is flexible because, as discussed before, metabolism of TH could be modulated in different fetal tissues on the basis of the specific metabolic condition (like hypothyroidism).

Moreover, the system might change during development. A clear example of this could be the increase of T3 levels during the second trimester and the peak of this hormone seen near the term of pregnancy, due to a change of tissue deiodinase activity.

In order to reach full biological activity determined by the entrance of T3 in the cell nucleus and its binding to nuclear coactivetors, many other metabolic steps are involved [24]. In particular, a normal action of thyroid hormone transporters could be necessary, but our knowledge of the development of these transporters in the fetus is very limited [18]. Also, the expression of the specific

TH receptors (THr) and of their different isoforms at various developmental stages need further research. Several evidences show, however, that in a species-specific manner TRr binding capacity varies in different tissues during development [18].

2.5 TH Metabolism and Biological Activity in Brain

It is well known that absence or insufficiency of TH during fetal life causes cerebral damage which could clinically manifest itself postnatally by different grades of cognitive impairment ranging from mild forms to overt cretinism. Therefore, several studies have been addressed to better understand TH action in the brain. Herewith, we summarize some aspects.

TH enter the brain mainly crossing the blood-brain barrier of the choroid plexus transported by specific transporters or indirectly via the blood–CSF barrier. MCT8 seems to be the most important transporter as demonstrated by the fact that the mutation of its gene coding could cause serious developmental impairment in the affected fetus [25].

The transport system seems to involve mainly T4 and contributes to maintain a TH concentration in the brain at about 20 % in comparison to blood levels. TH metabolism is regulated by deiodinase: deiodinase 2 is active in astrocytes where T4 is converted to T3 while deiodinase 3 is expressed in neurons where TH are converted into less biologically active rT3 and T2. The balance between the activities of the deiodinase is a key factor to protect the brain from the detrimental effects of hyper-or hypothyroidism [26].

Next step of TH metabolism is the binding of TH to specific receptors (TR). It has been demonstrated that the two major isoforms of TR, i.e., TRα1 and TRβ1, are expressed at different times during fetal life. TRα1 is detected by 8 weeks while TRβ1 is expressed later; moreover, TRα1 shows a stronger response to T4 than TRβ1. On the basis of several experimental data, it has been recently hypothesized that T4 could act in the brain not only as prohormone, as always suggested, but also as a direct-acting hormone [26].

2.6 TH and Fetal Growth

As underlined at the beginning of this chapter, intrauterine growth is determined by multiple hormonal and nutritional drivers [1]. Hormones stimulating growth might exert an anabolic effect directly on the tissues and modulate the fetal growth according to the nutrients [27].

Among the hormones involved in fetal growth, insulin and insulin-like growth factors (IGFs: IGF-1 and IGF-2) seem to play a pivotal role. In fact, both hypoinsulinemia, due to mutation of insulin promoting factor 1 gene [28], and mutation of IGF-1 and IGF-2 gene are responsible for intrauterine growth retardation [29, 30].

Changes in the IGF system were seen up to the 35th week of gestation in cord serum suggesting that by that time the system was mature in the fetus [31].

Moreover, in our experience [32], IL-6 gene expression and protein content in the placenta were significantly increased in IUGR newborns and were positively related with IGFBP-1 and IGFBP-2 gene expressions, suggesting cytokine and IGF system relationships in the placenta. However, relationships are difficult to demonstrate in observational studies, in particular, when linear statistical methods are being applied to virtually nonlinear relationships; thus, "the system of intra-uterine growth" was further analyzed using biology approaches that identified these as important hubs of the system [33].

A role of TH in regulating fetal growth has been suggested by clinical observations showing that low birth weight could be associated with several thyroid dysfunctions during pregnancy [34, 35]. Moreover, a positive association between FT4 in cord blood at birth and birth weight, birth length, head circumference, and sum of skinfold thicknesses has been recently reported [36].

As demonstrated by several experimental studies, TH act directly on growth regulating the oxidative metabolism of tissues; their blood concentration is positively correlated with the total oxygen fetal consumption, and its action is tissue specific being more evident on fat and skeletal muscle [18]. On the other hand, TH levels could influence fetal growth indirectly by modulation of several hormones and growth factors including expression of IGFs and GH [18].

In experimental animals, it has been recently demonstrated that bone formation but not reabsorption is altered in induced hypothyroidism underlying the important role of TH on skeletal growth [37].

Further information on TH action during fetal life could be derived by studies of intrauterine growth retardation (IUGR). IUGR is primarily due to an abnormal placental development and could be associated both to an increased fetal mortality and to a postnatal impairment of cognitive functions, intelligence quotient, and neurodevelopmental delay [38–40].

Several lines of experimental evidence seem to indicate that abnormalities in thyroid homeostasis could contribute to the etiology of these derangements. A reduction of TH blood levels has been reported in IUGR fetus through cordocentesis [15], and more recently Chan et al. [41] published, for the first time, data on human tissues demonstrating that the transporter MCT8 is less expressed in the central neuro system of IUGR supposing that this abnormality could contribute to the neurodevelopment impairment.

2.7 TH and Fetal Maturation

It is well known that near term, several biological mechanisms are activated for the transition from intra- to extrauterine life. A rise in fetal cortisol concentrations allows the activation of a series of physiological processes involving pulmonary gas

exchange, cardiac function, hepatic gluconeogenesis, thermogenesis and is considered the most significant phenomenon.

Cortisol acts on the deiodinase system stimulating T4 deiodinazation to T3 which otherwise play an important role in mediating cortisol maturational effects through different mechanisms which are not yet completely understood and which merit further investigation. The most important actions have been recently reviewed by Forhead and Fowden [18] and can be summarized as follows:

2.7.1 Lung

Cortisol and T3 synergistically induce liquid absorbance in the lung [42] and improve surfactant synthesis and release by type 2 pneumocytes [43].

T3 contributes to upregulation of pulmonary angiotensin-converting enzyme stimulating the fetal renin angiotensin system and therefore acting on the maturation of cardiovascular and renal function.

2.7.2 Heart

On the basis of experimental data in animals both under physiological conditions and after manipulation of thyroid function (thyroidectomy or TH infusion), it has been shown than TH might not only contribute to cardiomycyte growth and differentiation and to the expression of contractile proteins but may also interfere with changes of several receptors for angiotensin II and catecholamines which are of primary importance in cardiovascular adaptation in postpartum life.

2.7.3 Liver

A synergistic activity of cortisol and T3 seems to be necessary to activate hepatic glucogenesis at birth.

2.7.4 Adipose Tissue

At birth, thermogenesis is activated by brown adipose tissue through an uncoupling oxidative metabolism from ATP synthesis in the mitochondria, with the release of heat [44].

This uncoupling is mediated by uncoupling protein 1 (UCP1) level increase in the brown adipose tissue during late gestation in response to a local conversion of T4 to T3 and to induction of UCP1 synthesis in response to the increasing cortisol levels in the fetal plasma as term approaches [20].

2.8 Maternal Thyroid Function and Its Influence on the Fetus

It is well known that overt maternal hyper-and hypothyroidism are associated with increased morbidity both in the mother [45] and in the fetus [46].

It has been estimated that overt hyperthyroidism, mainly due to Grave's disease, occurs in 0.1–0.4 % of pregnancies [47]. A treatment with antithyroid drugs is recommended [48], and propylthiouracil (PTU) should be the first choice because methimazole (MMI) seems to cause a higher than normal incidence of fetal congenital malformations [49]. On the other hand, liver damage may be associated with PTU treatment [48].

The incidence of overt hypothyroidism is quite similar to that of the hyperthyroidism [50], and the treatment with levothyroxine is recommended [48].

It is interesting, however, to note that overt hypothyroidism is usually defined by elevated TSH and low FT4 serum levels, but according to Canaris et al. [51] it could be difficult to be clinically diagnosed because only about one third of pregnant women diagnosed as overtly hypothyroid on the basis of biochemical analysis were symptomatic.

While overt hyper-and hypothyroidism are relatively rare, a subclinical thyroid derangement seems to be more frequent and may be detected only by some forms of screening (universal or case-finding). How to screen is a matter of discussion mainly because prospective interventional studies are lacking; therefore, the guidelines published by professional associations or scientific societies are different [52, 53].

We point out some examples to better explain why it is not clear which policy should be followed during pregnancy to eliminate or at least minimize the possible damage of the mother and/or fetus.

(1) Subclinical hyperthyroidism, defined as low TSH with normal TH levels, has been considered not harmful by some AA [54], while recently, some other AA [55] have found an association between relatively high T4 levels in the early pregnancy and increased incidence of low weight at birth. This discrepancy could be due to several factors, but one of the most meaningful is the difficulty to find a general agreement on the definition of what is low or normal for TH levels.

    In fact, it has been longly recognized the need of specific laboratory standards for the period of pregnancy (a decline of TSH in the first trimester is physiological) and a "gold standard" to measure free T4 i.e., liquid chromatography-tandem mass spectrometry or comparing the method of one's laboratory with the gold one is necessary [56].

(2) Observational studies on maternal and fetal outcomes of subclinical hypothyroidism, characterized by elevated TSH and normal FT4 serum levels, have given contrasting results ranging from no adverse reactions to negative outcomes both in the mother (miscarriages, eclampsia) and in the fetus (preterm,

low birth weight). Therefore, L-thyroxin treatment is not universally recommended although some guidelines suggest the therapy in mothers with subclinical hypothyroidism, particularly when positive for autoantibodies (TPO) [53].

This last advice is mainly based on the result of the prospective study by Negro et al. [57] showing a positive effect of this approach on the maternal-fetus unit.

(3) Finally, further studies are necessary for isolated hypothyroxinemia and for auto-antibody positivity since available data are contradictory and not robust enough to allow any definitive statement [53].

In conclusion, while waiting for results of ongoing prospective studies, it seems reasonable that gynecologists be aware of the possible maternal-fetal damages induced by an alteration of thyroid function in pregnancy and that particular attention be directed in selecting mothers at risk. It remains to be determined whether universal screening is valid also in terms of cost/benefit.

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Etiology of Congenital Hypothyroidism

3

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Congenital hypothyroidism (CH) is a condition of thyroid hormone deficiency present at birth and is the most common inborn endocrine disorder [1]. Many different classifications have been proposed. According to the duration of thyroid hormone deficiency, it may be classified into transient and permanent CH. *Transient CH* refers to a temporary deficiency of thyroid hormone, detected at birth but then recovering to euthyroidism in the first few months or years of life. *Permanent CH* refers to a persistent deficiency of thyroid hormone requiring lifelong replacement therapy.

CH can be further classified according to the anatomic location of the pathogenic defect into primary, secondary (or central), and peripheral CH (Table 3.1). In *primary CH*, the defect involves the thyroid gland development or migration (thyroid dysgenesis) or one of the steps of thyroid hormone synthesis (dyshormonogenesis) or alterations in TSH binding or signal transduction. *Secondary CH* includes defects of thyrotropin-releasing hormone (TRH) formation or binding and TSH production at hypothalamus/pituitary level. *Peripheral CH* results from defects in thyroid hormone transport, metabolism, or resistance of peripheral tissues to thyroid hormone action. In addition, some forms of CH are associated with defects in other organ systems, and they are classified as *syndromic CH*.

Transient CH may be caused by maternal or neonatal factors. Maternal factors include exposure to iodine deficiency or excess (amiodarone or iodine antiseptic compounds), transplacental passage of thyrotropin receptor blocking antibodies, and use of antithyroid medications by mothers with hyperthyroidism. Neonatal factors include neonatal iodine deficiency or excess [2], congenital liver hemangiomas (with increased deiodinase type 3 activity), and mutations in the genes DUOX2 and DUOXA2.

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Table 3.1 Classification of congenital hypothyroidism

CH etiology	Associated mutations	TSH	T3 and T4
A. Primary CH			
Thyroid dysgenesis	PAX8	high	low
	TTF2		
	NKX2.1		
	NKX2.5		
Thyroid dyshormonogenesis	NIS/SLC5A5	high	low
	TPO		
	DUOX2, DUOXA2		
	Tg		
	DEHAL1/SECISBP2		
	SLC26A4		
Resistance to TSH	TSH-R	high	low
	Gs alpha		
B. Central (secondary) hypothyroidism			
Congenital hypopituitarism	HESX1	low	low
	LHX3		
	LHX4		
	PIT1		
	PROP1		
Isolated TSH deficiency	TSH b	low	low
TRH deficiency		low	low
TRH resistance	TRH-R	low	low
C. Peripheral hypothyroidism			
Thyroid hormone resistance	THRb	normal/high	mildly high
Abnormalities of thyroid hormone transport/metabolism	MCT8	normal	high T3, low T4

Permanent primary CH in iodine-sufficient areas is caused in 85 % of cases by thyroid *dysgenesis*, resulting from an aberration of the embryological development of the thyroid gland.

Thyroid dysgenesis encompasses three major forms, thyroid ectopy, athyreosis, and hypoplasia, accounting for 30–45 %, 35–45 %, and 5 % of cases, respectively [3–5]. *Thyroid ectopy* refers to an ectopic location of the thyroid gland, inferior or superior to the hyoid bone or above the thyroid cartilage. A thyroid remnant is usually found along the normal pathway of the thyroglossal duct [6, 7]. *Athyreosis* refers to the complete absence of thyroid tissue and *thyroid hypoplasia* to an aberrant development resulting in reduced thyroid volume. Thyroid dysgenesis is generally sporadic in occurrence. However, a genetic component is evident in about 2 % of cases [8]. Genes implicated as a cause of thyroid dysgenesis include thyroid transcription factor 2 (*TTF-2*) [9], *NKX2.1* (also known as *TTF-1*) [10, 11], NXK2.5 [12], and paired box gene eight (PAX8) [13, 14]. These genes encode for transcription factors expressed both during thyroid embryogenesis and in the normal

functioning gland. They are also present in other tissues and are associated with syndromic CH. Thus, patients with homozygous missense mutations in *TTF-2* present with spiky hair, cleft palate, cohanal atresia, and thyroid dysgenesis (Bamforth-Lazarus syndrome) [9]. Mutations in *NKX2.1* cause CH associated with neonatal respiratory distress, ataxia, and chorea [10, 11]. In patients with NKX2.5 mutations, CH is associated with cardiac malformations [12]. In the presence of PAX8 mutations, genitourinary malformations may be detected along with CH [15, 16].

About 15 % of cases of permanent primary CH are caused by *dyshormonogenesis*, encompassing various defects in thyroid hormone production. The defects are usually inherited in an autosomal recessive manner and include mutations in genes encoding the sodium-iodide symporter (NIS) (*SLC5A5* gene), thyroperoxidase (TPO), hydrogen peroxide generation factors, i.e., thyroid oxidase, and dual oxidase maturation factors (*DUOX2* and *DUOXA2* genes), thyroglobulin (Tg), iodothyronine deiodinases (DEHAL1 or SECISBP2 genes), and the transmembrane protein pendrin, which acts as a chloride-iodide transporter both in the thyroid and in the inner ear (Pendred's syndrome, due to SLC26A4 gene mutations, is characterized by hypothyroidism, goiter and deafness) [17].

Permanent primary CH may also be due to *resistance to TSH* binding or signaling, caused by mutations in the TSH receptor gene (TSH-R) or by mutations in the alpha subunit of the stimulatory guanine nucleotide binding protein (Gs alpha), leading to pseudohypoparathyroidism type 1a [18–20].

Central CH is generally caused by defects of TSH production, usually in a picture of congenital hypopituitarism. *Congenital hypopituitarism* may be associated with midline defects such as septo-optic dysplasia or cleft lip and/or palate and may be due to mutations in genes regulating pituitary gland development, such as PIT1, PROP1, HESX1, LHX3, LHX4, and GLI2. In these cases, TSH deficiency is rarely isolated and more frequently associated with the deficiency of other pituitary hormones, including growth hormone, adrenocorticotrophic hormone, and antidiuretic hormone, which may preexist or follow TSH deficiency. Less frequent causes of central CH are *isolated TSH deficiency*, caused by mutations in the TSH b subunit gene, and thyrotropin-releasing hormone (*TRH*) *deficiency or TRH resistance*, resulting from mutations in the TRH receptor gene (TRH-R) [21].

Peripheral CH may be due to resistance of target tissues to thyroid hormone action. In most cases, this is determined by mutations in genes encoding for thyroid hormone receptor b (THRb). The clinical characteristics vary according to the level of thyroid hormone hyporesponsiveness: affected individuals are generally euthyroid; however, hypothyroid subjects have been reported. Circulating T3 and T4 are mildly elevated without suppression of TSH. *Thyroid hormone resistance* is also known as Refetoff's syndrome [22]. Peripheral CH may also depend on defects in thyroid hormone transport and metabolism. Mutations in the gene encoding monocarboxylase transporter 8 (MCT8) cause hypothyroidism associated with mental retardation and neurological abnormalities. This condition, characterized by high serum T3, low T4, and normal TSH levels, is also known as Allan-Herndon-Dudley syndrome [23].

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Neonatal Screening for Congenital Hypothyroidism: What It Has Taught us About Thyroid and Brain Development

4

Johnny Deladoëy, Guy Van Vliet, and Yves Giguère

4.1 Premises

Congenital hypothyroidism (CH) is a relatively common disorder with a prevalence of 1 in 2,500 live births [1]. CH is characterized by a significant deficiency of thyroid hormones starting in the perinatal period, which can lead to severe intellectual disabilities if left untreated but which was only recognized clinically at a median age of 9 months [2]. Therefore, biochemical screening for CH is now routinely performed at 2 days of life, enabling the initiation of thyroid hormone therapy during the second week of life, if required. The implementation, since the 1970s [3], of a universal newborn screening (NBS) for CH has prevented severe intellectual disability in numerous patients [4, 5]. This tremendous success might suggest that the "problem of CH has been solved" and that it now suffices to refine the screening procedure to "diagnose" all possible cases, even the most benign. This belief leads to the following misperceptions:

1. CH is mainly an alteration of thyroid function, and if confirmed, additional diagnostic procedures (e.g., thyroid scintigraphy or genetic analysis) are seen as superfluous.

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2. Consequently, the management of CH is based on restoring thyroid function to normal, but to know the cause of CH is not necessary [6].
3. To diagnose all cases of CH, the solution is to increase the sensitivity of the NBS.

Taken together, these misperceptions combine themselves into a vicious circle:

1. CH is defined on the basis of a blood test cutoff value on NBS, confirmed by a second blood test value at diagnosis.
2. Specificity of the NBS is often evaluated afterward (2–3 years later), with another blood test, the result of which will determine whether to stop or to continue T4 therapy; in the latter case, CH is defined as permanent.
3. Permanent CH is seen as serious (worthy of treatment), regardless of its severity or cause, and therefore, the goal of NBS for CH is to identify the maximal number of cases of "permanent CH."

Given that the initial screening and intermediary and final diagnoses are mainly based on a blood test, this closed system leads to self-validation and self-correlation. Moreover, at each step, the cutoff may vary widely, and ultimately sensitivity and specificity of the NBS also vary depending on the definition used to label a CH case as permanent; this self-fulfilling prophecy is also refractory to any test and refutation. This has led to a steady increase in the incidence of CH (defined again with a blood test alone) in the USA [7]. Even though this increased incidence was essentially due to an increased incidence of benign cases, failure to identify the underlying cause of CH through imaging complicates our understanding of the significance of this phenomenon.

This situation illustrates the need for

1. A clear diagnosis with the use of thyroid scintigraphy [8], thyroid echography, or both [9]
2. An assessment of maternal thyroid function and thyroid autoantibodies
3. A molecular diagnosis in either syndromic cases (e.g., Brain-Lung-Thyroid Syndrome [10, 11]) or in cases of familial hyperthyrotropinemia (e.g., TSH-receptor mutations [12, 13])

Thus, even if the benefit of NBS of CH is unquestionable, a thorough diagnostic procedure will help by avoiding CH overdiagnosis due to liberal NBS cutoffs by providing valuable feedback on the performance of NBS. With these premises in mind and considering the recently published guidelines [14], we will discuss in this chapter the epidemiology of CH based on screening, the continuous health burden of CH [15], the initial diagnostic procedure and follow-up, and the emergence of molecular diagnosis and personalized medicine for patients with CH.

4.2 Newborn Screening and Epidemiology of CH

Early detection and treatment of CH through NBS prevents the severe intellectual disability which was frequently observed in the prescreening era, when 8–27 % of CH patients had IQ of less than 70 and the mean IQ was ~85, 15–20 points below the

Table 4.1 Breakdown of CH incidence stratified for diagnostic subgroups in different jurisdictions

CH incidence	Québec, Canada	Milan, Italy	Albert, NZ[a]	Castanet, France		
Period	1990–2009		1993–2010	1980–1998		
TSH threshold (mU/L)	15- 15	15–5	20	12–10	15	Variable over time
Global	1: 2,900	1: 2,450	1: 2,654	1: 1,446	1: 3,850	1: 3,560
Dysgenesis	1: 4,200	1: 4,510	1: 4,740	1: 4,520	1: 6,250*[1]	1: 5,000
Dyshormonogeneis	1: 29,300	1: 37,800	NA	NA	1: 17,000	NA
Normal gland *in situ*	1: 22,250	1: 9,850	NA	NA	NA	NA

[a]Incidence among Caucasians only

population mean [4]. Because primary hypothyroidism is at least 10-fold more common than central hypothyroidism and because only 19 % of cases of central hypothyroidism have T4 below the cutoff [16], primary TSH screening is the most sensitive test [14]. In practice, many jurisdictions use a combination of TSH and T4 strategies with various TSH and T4 cutoffs [1, 17], which explains the differences observed in the reported global incidence. Furthermore, CH is a heterogeneous condition with multiple causes (see chapter BONA_CH etiology) (see reviews [18, 19]). NOSTRO

Therefore, to better compare different NBS screening strategies, firstly the etiological category (established through thyroid scintigraphy) is extremely helpful, and secondly the ethnic composition of the screened population has to be considered, given that thyroid dysgenesis is common in Caucasians but rarely observed in Blacks [20] and that thyroid dyshormonogenesis might be enriched in certain ethnic groups [21] or in ethnic groups with a high rate of consanguinity [22]. Of note, when ethnic background is taken into account, the incidences of thyroid dysgenesis and dyshormonogenesis (i.e., the severe forms of CH) are almost similar across countries (Table 4.1), whereas the incidence of functional CH as defined above varies greatly even within the same jurisdiction using different screening procedures over time [1, 23]. This explains why estimates of global CH incidence vary greatly (1:1,600 to 1:3,500) across the world, whereas the incidence of thyroid dysgenesis (~1:5,000) remains stable within an ethnic group over time [24]. Contrasting with the stable incidence of thyroid dysgenesis [24], that of functional CH may be affected by toxic contaminants such as dioxin. Indeed, slightly increased TSH values have been observed in neonates born to mothers contaminated in 1976 after the Seveso accident [25]. Altogether, to better assess and compare NBS programs, a diagnostic evaluation as complete as possible should follow a positive screening result. Appropriate treatment of confirmed cases is crucial, but follow-up data on health and developmental outcomes should also be collected prospectively.

4.3 Initial Diagnostic Procedure and Follow-Up

NBS is only the start of a process that should lead to diagnosis, management, and outcome evaluation of CH [14]. First, the detection of a high TSH concentration on screening should be communicated quickly to the parents, and the newborn should

be referred to experienced physicians to confirm the diagnosis and to start the treatment as early as possible [26]. Initial work-up should include (1) assessment of venous TSH and free T4, (2) an X-ray of the knee to assess the severity of intrauterine hypothyroidism by the presence or absence of femoral and tibial epiphyses, and (3) either thyroid scintigraphy or echography (or both) to establish the anatomical diagnosis (i.e., ectopy, athyreosis, normal gland, or goiter). Nowadays, it is possible to perform all these diagnostic procedures on the same day to avoid any delay in the initiation of the treatment.

A thorough diagnostic procedure will serve both the patient and the population. First, a clear diagnosis with explanation based on imaging will increase adherence to treatment [27], a point which is not negligible given the high proportion of poorly compliant CH patients, a proportion which may reach as much as 38 % after 36 months of treatment [28]. Second, an accurate diagnosis allows a better assessment of NBS performance and a better assessment of the possible causes of incidence variations [1, 21, 23, 24]. This said, even if advantages of thyroid imaging for the diagnosis of CH are obvious, the diagnosis of all cases of thyroid dysgenesis (even the subclinical cases of thyroid ectopy or hemiagenesis) should not become the new goal of NBS for CH. Indeed, one should keep in mind that some patients with ectopic thyroid maintain normal serum thyroxine throughout life [29] and may not need LT4 treatment. Therefore, it is acceptable that some cases with thyroid ectopy are missed by CH [1]. Immediately after the diagnostic procedures, LT4 should be started. An optimal treatment of CH is essential for neurodevelopment, so reliable L-T4 preparations are crucial [30]. The treatment should be started no later than 2 weeks after birth and an initial dose of L-T4 of 10-15 μg/kg per day is recommended [14]. This practice of early and high dose treatment is based on observational studies [26, 31] and only one randomized controlled trial [32]. However, calls for additional RCTs on this question [33] raise ethical issues. Considering the controversies regarding the reliability of generic L-T4 [34, 35], brand rather than generic L-T4 tablets should be used [14] and switching brands (with inherent variable bioequivalence) should be avoided, as it requires additional blood draws to measure TSH levels [30]. The first follow-up visit should take place 2–3 weeks after initiation of L-T4 treatment, at which time the TSH level should be normalized. The patients should then, be followed every 1 to 3 months until 1 yr of age, and every 2 to 4 months until 3 yrs of age; during these visits, assessment of global development is important, and we suggest that infants with severe CH should be referred to the audiologist given the high proportion of hearing impairment in CH patients (see paragraph below) [36]. Thereafter, evaluations should be carried out every 6 to 12 months until growth is completed.

4.4 Continuous Health Burden of CH

Since the 1970s, NBS for CH followed by appropriate treatment allows most affected children to attain their full intellectual potential, with one tablet of thyroid hormone daily. However, intellectual disability may still be observed, especially in

severely affected children, and this is reflected, in some cohorts, in a mean loss of 10 IQ points compared to controls drawn from the general population [37]. Although milder than in the prescreening era, such mean loss of intellectual potential has significant social and economic impact when aggregated across hundreds of individuals [15, 38, 39]: given that (1) thyroid ectopy and athyreosis account for 60 % of CH cases and that (2) each IQ point raises worker productivity by 1.7 to 2.3 % (for a lifetime earning of 700,000$), we estimate (for a country of 35 million inhabitants such as Canada) a global economic benefit of 1–1.3 million $ per year for each IQ point gained in patients suffering from severe CH, i.e., thyroid ectopy and athyreosis [38]. Even early high-dose thyroxine treatment cannot fully prevent neurocognitive deficit in the most severe cases [40]. Moreover, a fourfold increase of hearing impairment is observed in the French national cohort of CH patients [36, 39]. Consistent with the neurocognitive deficit reported in the Swiss cohort [37, 40], Leger et al. reported that male patients with athyreosis and with low socioeconomic background have an increased likelihood of not graduating from high school [39]. Of note, even if CH prognosis has improved considerably over time, some patients diagnosed in the early years of screening displayed comorbidity and mortality due to various neurodevelopmental disorders and associated malformations [41]. Finally, lower fertility has recently been reported in women (but not in men) suffering from the most severe form of CH [36].

All these data should remind us that, 40 years after the first implementation of routine screening, the health burden of CH has not disappeared, perhaps because the cause of the severe forms of CH (i.e., mainly thyroid ectopy and athyreosis) remains mostly unknown. This represents a critical barrier to further improving the outcome of CH. It is therefore important to consider the role of possible genetic markers in making an even earlier diagnosis of these most severe forms of CH.

4.5 New Persepctives: Emergence of Molecular Diagnosis and Personalized Medicine for Patients with CH?

The genetic causes of dyshormonogenetic goiters are well described, and these conditions follow classical Mendelian models of inheritance (reviewed in [19]). Those of functional CH are being unraveled and have significant clinical implications: the demonstration of a heterozygous mutation in TSHR may justify stopping treatment with thyroxine, on the basis that the persistent hyperthyrotropinemia overcomes the TSH resistance [13]. The finding of mono- or even biallelic inactivation of *DUOX2* also justifies testing treatment withdrawal, since CH may be transient [42].

On the other hand, the majority of cases of thyroid dysgenesis (thyroid ectopy and athyreosis being the most frequent and severe) have no identified genetic cause. Underscoring the lack of clear genetic determinants is the fact that thyroid dysgenesis is predominantly not inherited (98 % of cases are sporadic [43]); thyroid dysgenesis also has a high discordance rate (92 %) between monozygotic (MZ) twins and a female and ethnic (Caucasian) predominance [20, 44]. Germline mutations in the thyroid-related transcription factors NKX2.1, NKX2.5, FOXE1, and PAX-8

have been identified by candidate gene screening in at most 3 % of patients with sporadic thyroid dysgenesis [19, 45]. Linkage analysis has excluded these genes in rare multiplex families with thyroid dysgenesis [46]. Moreover, evidence of non-penetrance of mutations in genes such as *NKX2.5* in close relatives of patients [47] suggests that modifiers, possibly additional germline mutations such as copy number variants (CNVs) and/or somatic mutations, are associated with thyroid dysgenesis. Indeed, a higher rate of CNVs is expected in congenital disorders [48], and Thorwarth et al. found a high rate of CNVs in thyroid dysgenesis but exclusively in athyreosis and thyroid hypoplasia [49]. Even though this study did not find recurrent CNVs and did not show any functional analyses, it provides the "proof of concept" that differences in gene copy number could account for the small heritable component of thyroid dysgenesis and shows that it is reasonable to use a higher-resolution platform to screen for CNVs and single-nucleotide variants (SNVs) in thyroid dysgenesis. Given the unprecedented high sensitivity of these "omics" methods, functional studies are now necessary to assess the pathogenicity of the genetic variants identified, and in this respect, the zebrafish model has proven its utility [50, 51].

Conclusion

Intellectual disability, health impairment, and increased mortality may still be observed in a subset of patients with CH despite the universal NBS and early treatment of this condition [36, 37, 39, 41]. To better treat children with thyroid dysgenesis, we need to (1) diagnose them even earlier, (2) better predict the severity and extent of potential neurocognitive deficits as well as the presence of additional developmental abnormalities unrelated to CH *per se*, and (3) propose even better treatment avoiding under- and overtreatment [52]. It is therefore crucial to better understand the cause of thyroid dysgenesis that future studies focus on the biological mechanisms responsible for thyroid dysgenesis (i.e., the severe forms of CH for which data clearly show that early treatment provides neurodevelopmental benefits) and for which NBS was originally implemented. Hopefully, this will generate novel therapeutic modalities for children affected with severe CH.

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Laboratory Aspects in Neonatal Screening of Congenital Hypothyroidism

5

Carlo Corbetta and Luisella Alberti

5.1 Introduction

The aim of a newborn screening program is to identify affected babies in the newborn population. This implies that screening tests and laboratory methodologies are characterized by performance parameters (accuracy, analytical range, analytical specificity, blank reading, detection limit, interferences, precision, reagent stability, etc.) able to meet the medical usefulness of a newborn screening program and specific criteria such as sensitivity (positivity in disease), specificity (absence of disease), predictive value of positive test (percent of patients with positive test results who are diseased), predictive value of negative test (percent of patients with negative test results who are nondiseased) [1] .

For what strictly concerns newborn screening for congenital hypothyroidism (CH), many aspects should be taken into consideration. A crucial aspect is to define the spectrum of pathologies to be screened: primary CH, secondary (central) CH, severe forms of CH, mild forms of CH. Moreover, the complex regulation of thyroid hormone biosynthesis, as well as the rapid modification of reference intervals of thyroid and pituitary hormones in the first weeks of life, may affect many laboratory aspects. In addition, there are features concerning the biological matrix universally used for newborn screening, the *dried blood spot* (DBS). DBS is collected on a special filter paper with a high degree of uniformity and designed to absorb a specific volume of blood. The quantitative nature of DBS allows the quantitative assessment of biomarkers of many congenital diseases and the definition of cutoff values to differentiate asymptomatic newborns that may have a disease from those who

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may not [2]. These features meet the needs of the analytical process and guarantee efficiency and effectiveness of the newborn screening program.

It is universally accepted that only high-technology laboratories which use standardized operating procedures and have appropriately trained staff with large experience in automated immunoassay procedures, quality assurance policy, and information technology [3] can successfully process the high workload requested by a newborn screening program for CH. In fact, it is recommended that the number of babies screened yearly by a screening center is at least 35,000-50,000 [4].

The criteria on the basis of which a laboratory test may be applicable to a newborn screening program have been clearly defined [5, 6]. A screening test must be applicable to DBS samples and feasible for high-throughput platforms. Furthermore, it should have high sensitivity and specificity in order to identify the highest number of affected babies in the newborn population. It should also be inexpensive. Finally, in the USA, the Centers for Disease Control and Prevention (CDC) classified the biochemical genetic testing and newborn screening as essential laboratory services for the screening, detection, diagnosis, and monitoring of inherited metabolic diseases, endocrine and hemoglobin disorders, and other rare diseases [7]. Biochemical genetic tests and newborn screening tests are considered high-complexity tests. Laboratories that perform these tests must meet more severe regulations for the total testing process in comparison with general laboratories. Moreover, the qualification of laboratory personnel, including training and experience, is a critical factor for ensuring the quality of laboratory test results.

5.2 Biomarkers and Screening Strategies Used in Newborn Screening for Congenital Hypothyroidism

In general terms, the laboratory tests [8] most commonly used to evaluate thyroid hormone dysfunction are classified into

- Hormone concentration tests:
  - Total thyroxine (T_4)
  - Total triiodothyronine (T_3)
  - Free thyroxine (fT_4)
  - Free triiodothyronine (fT3)
  - Thyrotropin (Thyroid-stimulating hormone) (TSH)
  - Reverse Triiodothyronine (rT_3)
- Serum-binding protein tests:
  - Thyroxine-binding globulin (TBG)
  - Thyroxine-binding prealbumin (Tranthyretin) (TBPA)
- Autoimmune Thyroid Disease tests:
  - Antithyroglobulin antibodies (TgAb)
  - Antithyroid peroxidase antibodies (TPO Ab)
  - TSH receptor antibodies (TRAb)

- Other Hormones and Thyroid-Related Protein tests:
  - Thyrotropin-releasing hormone (TRH)
  - Thyroglobulin (Tg)
  - Calcitonin (CT)

Some of these analytes (TSH, T_4, fT_4, fT_3, TBG) can be detected in DBS samples (http://www.cdc.gov/labstandards/pdf/nsqap_analyte_list.pdf).

According to the clinical target of a newborn screening program for CH, i.e., detection of babies with primary CH and/or central CH, the screening protocol should use appropriate biomarkers in order to obtain a highly effective screening program [11, 12]. Generally, an analyte expressing the effect of a genetic and/or functional defect is an optimal biomarker. For example, phenylalanine is the best analyte in newborn screening for phenylketonuria (PKU). This rare inherited disorder is caused by a genetic defect causing the lack of Phe-hydroxylase, an enzyme needed to process phenylalanine. Without the enzyme, phenylalanine increases in blood. This vision explains why T4 was identified as an optimal biomarker when newborn screening for primary CH was introduced. However, it was realized that T_4 is not an ideal indicator of thyroid status, in part because of the effects of variations in serum-binding protein levels and also because the relationship between T_4 and T_3 (the primary active thyroid hormone) is not always predictable [8]. Differently, TSH serum (or blood) concentration reflects an integrative action of all thyroid hormones in one of its target tissues, the pituitary cells that secrete TSH. Moreover, TSH serum or blood levels are inversely proportional to T4 concentration, and a twofold change in FT_4 causes an approximate 100-fold change in serum (or blood) TSH concentration [13]. This log-linear ralationship explains why little changes in T_4 level are reflected in great variations in TSH levels. This feature, which allows the identification of subclinical thyroid diseases, together with the increasing accuracy of TSH measurement which has been achieved over the years, are the main factors on the basis of which TSH is considered the best biomarker to identify primary CH in the first days of life. Even though the measurement of TSH alone is not appropriate to identify babies with central CH, which requires the combined use of other biomarkers (mostly T4 or FT4), the TSH-centered strategy for screening evaluation of thyroid function in newborns is both cost effective and medically efficient.

Although TSH as primary screening test is the most used screening strategy for CH worldwide, other strategies are possible:

1. T4-backup TSH
2. Tandem T4 and TSH (in all samples)
3. T4, TSH, TBG (combined method)
4. Tandem fT4 and TSH

In the T4-backup TSH strategy, T4 is the primary screening test and TSH is measured only in samples whose T4 concentrations are below the 10th centile. In the 1990s, this strategy was the most used worldwide. Over the years, the

improvement of the analytical and functional sensitivity of TSH assays, as well as the increasing use of the retest at 2–4 weeks of life (*serial testing*) in preterm or sick newborns [14], has increased the use of TSH as primary screening test for CH. At present, T4 is used as primary screening test only in some states of the USA and in Israel, whereas the strategy with simultaneous (*tandem*) T_4 and TSH testing is applied only in a limited number of screening programs [15]. The widespread use of TSH as primary screening test for CH is also confirmed by annual reports of the most common international program of quality assurance for newborn screening, the *Newborn Screening Quality Assurance Program* (NSQAP) of the Centers for Disease Control, Atlanta [16]. In 2013, 578 newborn screening laboratories in 73 different countries took part in the Program. Among the 492 laboratories participating in the Proficiency Testing Program, 310 participated for TSH and only 84 for T4. Similarly, among the 445 laboratories participating in the Quality Control Program, 294 participated for TSH and only 72 for T4.

It is important to understand which strategy provides the best performance metrics in relation to the aims of a newborn screening program. In a recent study, performance metrics of four screening strategies used from 1994 to 2010 were compared: T4-backup TSH, tandem T4 and TSH, TSH (no serial testing), TSH plus serial testing [17]. In terms of effectiveness, the TSH plus serial testing strategy resulted to have the best performances, although the tandem T4 and TSH strategy allows the identification of cases with central CH. It has been reported that the measurement of fT4 can be used instead of T4 in tandem strategy. The use of fT4 avoids the effects of variations in serum-binding protein levels on the T4 measure. In Japan, some NBS laboratories are using the fT_4 measure as primary marker with the simultaneous measure of TSH in all DBS samples: the fT4 measurement enables the detection of CH of central origin, with an estimated incidence in Japan's regions of 1:30.833 live births [18, 19]. In the Netherlands, the Dutch NBS program is using a different strategy: primary T4 test with sequential TSH measured in the lowest 20 % and TBG measured in samples with the lowest 5 % of T4 values. Also, this strategy allows the detection of both primary and secondary CH, the latter with an incidence of 1:15.000 live borns [20].

5.3 Analytical Methods and Technology Used for Screening of Primary CH

In the last 60 years, the measurement of TSH and thyroid hormones has been performed by means of immunochemical methods based on the antigen-antibody reaction, i.e., the antigen is detected by an antibody which is used as a reagent. The availability of labeled antigens and antibodies has allowed the development of highly sensitive and specific immunochemical tests for the assessment of hormones in biological samples.

Immunochemical methods can be classified in "competitive" and "noncompetitive" methods. In a competitive immunoassay, all reactants are mixed together, simultaneously or sequentially with different sensitivity. In a noncompetitive

immunoassay, a capture antibody is adsorbed or covalently bound to a surface of a solid phase. The antigen to measure reacts with the solid-phase capture antibody. After a washing action to remove other proteins, a labeled antibody is added and reacts with the bound antigen through a second distinct epitope: after a new washing action, the bound label is determined, and its concentration or activity is directly proportional to the concentration of the antigen. The invention of noncompetitive immunoassay technology has generally been credited to Miles and Hales, who in 1968 labeled anti-insulin antibodies with ^{135}I and used them in a noncompetitive two-step immunoradiometric assay (IRMA) of insulin [21]. The analytical detection limits of competitive and noncompetitive immunoassays are determined principally by the affinity of the antibody and the detection limit of the label used. Since the sensitivity of competitive assays is defined by the association constant of antibodies, while the sensitivity of noncompetitive assays is defined by the total error, nonspecific binding, and the affinity of antibodies, it has been possible to create noncompetitive assays that are several orders of magnitude more sensitive than competitive assays. The principal examples of labeled immunoassay are

1. Radioimmunoassay (RIA): developed in the 1960s, RIA methods use radioactive isotopes of iodine (^{125}I, ^{131}I) and tritium (^{3}H) as labels.
2. Enzyme Immunoassay (EIA): EIA methods use the catalytic properties of enzymes to detect and quantify immunochemical complex. EIA assays are classified in
   (a) Enzyme-Linked Immunosorbent Assay (ELISA)
   (b) Enzyme Multiplied Immunoassay Technique (EMIT)
   (c) Cloned Enzyme Donor Immunoassay (CEDIA)
3. Chemiluminescence Immunoassay (CLIA): chemiluminescence is the name given to light emission produced during a chemical reaction. Isoluminol and acridium esters are two examples of chemiluminescent labels used in CLIA.
4. Fluoroimmunoassay (FIA): FIA methods use a fluorophore as label. In this assay, the problem of the background fluorescence has been overcome by the use of rare earth (lanthanide) chelates and background rejection (time-resolved) procedures.

Recent data of the CDC NSQAP show that the time-resolved fluoroimmunoassay (TR-FIA) is widely used in newborn screening programs for congenital endocrinopathies (CH and congenital adrenal hyperplasia). In this technology, the design of the immunoassay involves the use of a lanthanide chelate fluorophore (generally, europium) and its detection by means of time-resolved fluorometry. Typically, the fluorescence from a europium chelate lasts many times longer than from a conventional fluorophore. This means that the measurement of the signal can take place well after nonspecific interfering fluorescence has faded away. Again, the wavelength of the fluorescence light is significantly different from that of the light used to excite the europium chelate. This difference in wavelength is known as "the Stokes shift": a large Stokes shift allows a more sensitive measurement of the fluorescence. The time-resolved fluorometry relies on two important properties of the fluorophore which contribute to the sensitivity of the assay: the long decay time and the wide "Stokes shift."

Preanalytical and analytical phases of the newborn screening process need technologies with a high sensitivity and suitable for sampling a small volume of blood . Generally, DBS is a blood punch of 3.2 mm of diameter, in which the mean serum volume is about 1–1.5 μl according to the neonatal hematocrit. Only technologies characterized by high sensitivity and reproducibility can guarantee an accurate, precise, and sensitive measure of a biomarker. From this point of view, FIA methods are highly reliable.

In the last two decades, there has been a technological evolution of newborn screening laboratories toward methods and immunoassay analyzer platforms able to guarantee high sensitivity, high analytical speed, and a high workload. This technological evolution has determined a roughly 100-fold improvement in the lower detection limits of analytical methods to measure TSH, the availability of highly automated immunoassay platforms, and the development of multiplex assays by using tandem mass spectrometry. Specifically, the improvement in the lower detection limits of analytical methods has led to a widespread use of blood TSH as primary screening marker for CH. It is possible to classify TSH methods according to the concept of "functional sensitivity" which is the functional detection limit of serum (and blood) TSH assays determined on the basis of low-end interassay precision characteristics. The Nomenclature Committee of the American Thyroid Association recommended that precision [as coefficient of variation (CV)] at the lower reporting limit should be in serum 10–15 % and no worse than 20 % [22]. At present, the functional sensitivity is defined as the lowest concentration of TSH at which an interassay CV of 20 % can be achieved. The first-, second-, and third-generation methods have functional sensitivity of 1.0, 0.1, and 0.01 mIU/L, respectively. Some more recent methods (fourth-generation) declare a detection limit of 0.001 mIU/L.

Concerning the availability of automated immunoassay platforms, automation of preanalytical phase has been greatly improved by the introduction of multiplex platforms punching in 96-well microplates and able to analyze a high number of samples with a complete traceability. Currently such preanalytical systems run parallel with highly automated immunoassay platforms able to simultaneously test for multiple diseases from a single sample (*multiplex platforms*). The widespread use of multiplex platforms started in the 1990s with the introduction of tandem mass (MS/MS) technology in the newborn screening for inborn errors of metabolism. This technology allows screening of about 50 metabolic diseases by measuring more analytes simultaneously (amino acids and acylcarnitines). Therefore, over the years, MS/MS technology has contributed to the dramatic increase of the number of the diseases candidate for screening programs. On this regard, the possibility to detect T_4 from a filter paper blood spot using essentially the same method as MS/MS analysis of amino acids and acylcarnitine has been recently demonstrated [23]. This method may provide a cost-effective means of analyzing both T_4 and TSH by consolidating a T_4 analysis into the MS/MS panel. Therefore, when central CH is the clinical target of a newborn screening program for CH, MS/MS may help and facilitate the necessary dual analysis of TSH and T_4 by eliminating the need for a separate assay (T4). Laboratories utilizing only TSH as screening marker could add T_4 for minimal costs and resources if they are using MS/MS for acylcarnitines and amino acids.

5.4 Quality Assurance Policy in the Neonatal Screening Laboratory

Newborn screening represents one of the most important results of preventive medicine in childhood, as it provides useful information for the prevention of diseases characterized by high morbidity and mortality. This implies that reliability of the result of the screening test is guaranteed by newborn screening laboratories because the quality of future life of an affected baby depends on this result. To this end, criteria of good laboratory practice have been well defined. These include quality assurance policies [24] which can be classified into

- *External Quality Assessment* which allows laboratories to evaluate accuracy of their measurements on the basis of target values and to compare their performances with those of other laboratories (proficiency assay)
- *Internal Quality Assessment* which allows the daily monitoring of the test performance in terms of precision and accuracy *an*d the early identification of any trend of systematic errors before the analytical process is out of control

Newborn screening is a complex process which starts with a blood sample (DBS) and ends with a negative or a positive screening test. The latter case results in the identification of a baby with a high risk for the screened pathology in whom the confirmation of the diagnosis with further tests and clinical referral are needed [25]. Therefore, each phase of this multidisciplinary process needs specific quality assurance policies.

Newborn screening laboratories should have policies and procedures to address the time-sensitive issues of testing and the handling of varying conditions of the infants, including specimen collection for preterm or low birth weight infants, sick newborns, or those in need of special care. Written procedures addressing specimen-related issues (timing of specimen collection and submission, sample's quality, number and sources of samples) should be consistently applied. Again, newborn laboratories should continuously monitor the performance of their screening tests and determine the need for reevaluating performance specifications as new disease information or test performance data become available [26]. Guidelines and reference procedures are currently available [7, 14, 27, 28] and allow laboratories to define their quality assessment policy.

For what strictly concerns newborn screening for CH, the wide variability of reference intervals of TSH and thyroid hormones at birth, which may be affected by neonatal factors such as gestational age, low birth weight, sex, ethnicity, age at screening, and type of delivery, should be also taken into consideration . The definition of correct reference intervals for TSH in each category of newborns (preterm, at term, acutely ill newborns, etc.) is needed to define the cutoff values that will be used to select an affected baby. The reference interval is generally the nonparametric central 95 %. In a screening program,the cutoff value represents the "decision value" at which a result is considered positive. Therefore, each laboratory, to correctly determine the test's cutoff, should calculate the test's sensitivity and specificity and weigh increased detection of mild cases vs harm from recall of normal infants.

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Epidemiology of Congenital Hypothyroidism

6

Antonella Olivieri

6.1 Introduction

Congenital hypothyroidism (CH) is defined as thyroid hormone deficiency present at birth. This condition is the most common congenital endocrine disorder and one of the most common preventable causes of mental retardation [1].

Institution of newborn screening for CH since 1970 and its development have provided the opportunity for early detection and treatment of CH and prevention of its neurodevelopmental consequences [2–5]. Furthermore, institution of regional and nationwide newborn screening programs for CH has provided essential epidemiological data which have been helpful in better understanding the natural history of the disease and in appropriate management of babies with CH [1, 6, 7].

In this chapter, the most important issues regarding epidemiology of CH will be reviewed and discussed.

6.2 Primary Congenital Hypothyroidism

Primary CH is the most common form of CH. It occurs as a result of developmental defects of the thyroid gland (dysgenesis) or is due to disruption in thyroid hormone biosynthesis (dyshormonogenesis). This leads to goitrous hypothyroidism, although it is rarely seen in babies detected by newborn screening [8].

About two thirds of the CH cases are due to thyroid dysgenesis, such as the arrested migration of the embryonic thyroid (ectopic thyroid), the lack of one – in most cases the left – lobe of the thyroid (hemiagenesis), or a complete absence of

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thyroid tissue (athyreosis). About one third of babies diagnosed as having hypothyroidism at recall examination had a normally located gland [9, 10]. Ten to fifteen percent are due to autosomal recessively inherited defects in hormone synthesis, whereas the remaining cases (15–20 %) are due to mild functional disorders [11–14].

Thyroid dysgenesis is generally thought to be sporadic, although the possibility of a genetic component is supported by some studies. One study of all cases of thyroid dysgenesis found that 2 % were familial [15]. Additional studies also showed that 7.9 % of first-degree relatives of infants with CH had a thyroid developmental anomaly [16].

Nearly all screening programs report a female preponderance among babies with thyroid dysgenesis, with a female-to-male ratio of about 2:1 [17, 18]. A report from Quebec shows that this female preponderance occurs mostly with thyroid ectopy and less with agenesis [19]. On the other hand, a female-to-male ratio of about 1:1 is generally reported among babies with normally located gland [18].

CH may also frequently be associated with an increased risk of congenital extrathyroidal malformations [20]. In our previous study conducted on 1,420 CH babies recorded in the Italian National Registry of Infants with Congenital Hypothyroidism (INRICH), extrathyroidal congenital malformations had a prevalence of 8.4 % [21]. Given the availability of a population-based registry, it was possible to study a high number of CH infants with extrathyroidal malformations and compare these data with those of the International Clearinghouse for Birth Defects, the worldwide database collecting information on infants born with congenital malformations. By using this approach, it was possible to demonstrate that not all congenital extrathyroidal malformations but only anomalies of heart, nervous system, eyes (representing precocious structures in the developing embryo), and multiple congenital malformations were significantly associated to CH. These findings have strongly suggested a very early impairment in the first stages of embryo development with a consequent involvement of different organs and structures [21]. Moreover, it was demonstrated that the most frequent cardiac malformations in the CH population were represented by the atrial septal defects, differently from that found in the general population in which the most frequent cardiac anomalies are represented by ventricular septal defects. Other associated malformations include spiky hair, cleft palate, neurological abnormalities, and genitourinary malformations [20, 22, 23].

6.3 Incidence of Primary CH

Prior to the onset of newborn screening programs, the incidence of CH, as diagnosed after clinical manifestations, was in the range of 1:7,000 to 1:10,000 [24]. With the introduction of newborn screening, the incidence was reported in the range of 1:3,000 to 1:4,000 [25]. It remained relatively constant until 1990 when a progressive rise in the rate was reported in the USA, Europe, and other parts of the world. Currently, the CH incidence rate is reported in the range of 1:1,660 to 1:2,828 live births (Table 6.1) [13, 26–30]. These rates refer to babies with confirmed CH

Table 6.1 Modifications of incidence rates of primary congenital hypothyroidism (confirmed at birth) reported worldwide

Country and period of observation	Incidence of CH	TSH cutoff (mU/L)	Re-sampling strategy for at risk categories of neonates	Country and period of observation	Incidence of CH	TSH cutoff (mU/L)	Re-sampling strategy for at risk categories of neonates	Reference
Western Australia 1981–87	**1: 5745**	>25	No	Western Australia 1988–98	**1: 2828**	>15	No	Kurninczuk (2002) [26]
New Zealand 1993–2001	**1: 3846**	>15	No	New Zealand 1993–2010	**1: 2778**	>15	No	Albert (2012) [27]
Quebec 1990–2000	**1: 2898**	>15[a] >15[b]	No	Quebec 2001–2009	**1: 2450**	>15[a] >5[b]	No	Deladoey (2011) [13]
Italy 1987–1998	**1: 3000**	>20	No	Italy 1999–2008	**1: 1940**	>10	Yes	Olivieri (2015) [30]
Greece 1990–99	**1: 3300**	>20	No	Greece 2000–2009	**1: 1749**	>10	Yes	Mengreli (2010) [29]
Massachussets (USA) 1991–1994	**1: 3000**	Cut-off T4: 7.2–10 µg/dl TSH with the lowest 10 % of T4	No	Massachussets (USA) 2003–2010	**1: 1660**	Cut-off T4: 13 µg/dl TSH with the lowest 10 % of T4	Yes	Mitchell (2011) [28]

[a]TSH cutoff on the 1st NBS test
[b]TSH cutoff on the 2nd NBS test

requiring the start of the replacement therapy. Some case–control studies have been conducted in different parts of the world to investigate the risk factors for CH [31–33]. In these studies, a common set of risk factors for the disease was identified which included birth defects, female gender, maternal diabetes, twins, preterm deliveries, and gestational age > 40 weeks.

6.4 Causes Influencing the Increasing Incidence of Primary CH

Several factors have been proposed to explain the cause of this approximate doubling of the incidence rate. These include changing in screening strategies and cut-offs, increase in preterm survival, demographic changes, and environmental factors. However, the higher incidence rate observed in the last decades worldwide includes additional cases of mild CH.

6.4.1 Seasonality

There was some speculation on a possible seasonal variation in the incidence of CH. However, this topic is still under debate. Studies demonstrating an increase in CH in the winter months have been conducted in Japan, Finland, Iran, and USA [33–36], indicating this is an effect that is observed globally in geographic areas with varying climates. In Japan, sex-specific seasonal patterns of incidence have been also found [37]. However, similar variations have not been confirmed in other parts of the world [27, 38–40].

6.4.2 Changes in Screening Strategies and Lowering TSH Cutoff

The increase of the CH incidence has been attributed to the widespread shift from primary T4 to primary TSH screening strategies and to the reduction of TSH cutoff [41, 42]. With increasing accuracy of TSH measurements on the small volume of blood available in the dried blood specimens obtained for screening, several programs employing a primary T4 test switched to a primary TSH test. Currently, most newborn screening programs around the world employ a primary TSH strategy, the exceptions being some state programs in the United States, Israel, the Netherlands, and some programs in Japan measuring free T4 and TSH simultaneously [43].

Data from the literature have shown that the impact of lowering TSH cutoff on the incidence of CH has resulted in a significant rise of the incidence with an increased detection of milder cases of CH [44]. As shown in Table 6.1 when the TSH cutoff was lowered from a range of >20–25 mU/L to >10 mU/L (whole blood), a doubling of the incidence of CH confirmed at birth was generally observed. An exception was represented by Quebec where the CH incidence increased less. It was 1:2,850 in the period 1990–2000 and 1:2,450 in the period 2001–2009. This lower

increase can be at least partially explained by the fact that the reduction of the TSH cutoff (from 15 to 5 mU/L) only concerned the second (recall) test, which is performed when the result of the first screening test (at 3–5 days of life with a cutoff of 15 mU/L) is positive [13].

An important clinical question is whether these milder cases of CH are transient or require permanent treatment. In our previous study conducted on the data of the INRICH, we have demonstrated that in the period 2000–2006 21.6 % of the Italian population of babies with permanent CH had a milder increase of TSH at screening (≤15.0 mU/L whole blood), whereas in the group of infants with transient hypothyroidism this percentage was 54 % [45]. In another Italian study conducted on a group of 84 Italian children with eutopic thyroid glands and mild CH, results of the reevaluation of the diagnosis after the age of 3 years showed that 35 % of these children had abnormal TSH elevations after thyroxine withdrawal and 27 % had persistent hyperthyrotropinemia (TSH 5–10 mU/L). A minority of cases had mutations in the genes commonly linked to mild forms of CH [46]. Taken together, these data suggest that newborns with mild abnormalities on neonatal screening have a significant risk of permanent CH that may become more severe in the future. Furthermore, babies with thyroid dysgenesis may have a mild increase of TSH at screening. In our more recent study conducted on 4,195 babies with a diagnosis of CH confirmed at birth and recruited in the INRICH, it was found that 8.7 % of infants with thyroid dysgenesis had a TSH at screening <15.0 mU/L whole blood. This finding can explain the slight increase (+8.0 %) of CH incidence due to thyroid dysgenesis observed in our analysis (from 1:4,000 live-borns in the period 1987–90 to 1:3,300 in the period 2007–08) [30]. Such a result was not found in other studies conducted in countries where screening programs adopted higher TSH cutoff or used T4 as the primary screening test [13, 28].

6.4.3 Premature Birth and Multiple Pregnancy

Thyroid dysfunction is frequently observed among comorbidities associated with prematurity. The most common pattern of thyroid dysfunction seen in preterm infants is transient hypothyroxinemia of prematurity (low T4 with normal TSH) which is observed in up to 50 % of infants born before 28 weeks [47]. In addition to transient hypothyroxinemia of prematurity, preterm babies have a higher incidence of primary CH, mostly with eutopic thyroid [48]. It has been reported that VLBW babies have a risk of CH about 14-fold higher than that of normal birth weight babies (1:250) [49]. Moreover, about two thirds of VLBW infants with CH detected on newborn screening show a delayed TSH rise [49, 50]. The timing of this elevation generally occurs between 2 and 4 weeks of age. According to recent guidelines [6, 7], NBS programs in the USA and Europe now collect a second specimen at 2–4 weeks of life in special categories of at-risk neonates, including babies born preterm [28–30, 51, 52].

Preterm babies show a high risk of both permanent and transient CH. In the Italian population of infants with CH recorded in the INRICH between 1987 and

2008, the frequency of preterm babies was 12.4 % among infants with permanent CH and 30.7 % among babies with transient hypothyroidism ascertained by reevaluation of the diagnosis. These frequencies were significantly higher than the 6.5 % observed in the Italian newborn population in the same period [30]. Improvements in perinatal and neonatal care have increased the survival rate of a growing number of preterm babies [53]. Therefore, with improved survival rates more preterm and LBW infants, who would previously have died in the newborn period, now have a greater potential to be tested and confirmed to have CH. This fact has been confirmed by a recent analysis conducted on data of the INRICH demonstrating that about 50 % of the increased incidence of CH observed in Italy between 1987 and 2008 (from 1:3,000 to 1:1,940 live-borns) was attributable to preterm babies, including those with low TSH at birth who have been identified by means of a low TSH cutoff and special procedures for at-risk newborns (resampling at 2–4 weeks of life) [30]. Another factor which can affect the frequency of preterm babies with CH is the iodine nutritional status. Because the ability to escape from the Wolff-Chaikoff effect does not mature until 36 weeks, preterm babies are at risk of hypothyroidism from excess iodine exposure due to topical application of iodine antiseptics [41]. However, as preterm infants have lower iodine stores and greater iodine requirements than term infants, they are also at risk of thyroid hypofunction due to iodine deficiency. This risk is particularly high in hospitalized preterm infants. In fact, it has been demonstrated that a hypothetical 1 kg preterm infant would receive less than the recommended 30 μg/kg/day with any standard nutritional regimen (enteral and parenteral nutrition) [54].

As regards twins, recent decades have seen a major increase in multiple birth rates globally, given the increasing use of techniques of assisted reproduction and drugs inducing ovulation [55, 56]. In our previous study conducted on the INRICH data, a risk of CH occurrence was found threefold higher in twins than in single deliveries. The estimated CH incidence was 10.1 per 10,000 live births in multiple deliveries and 3.2 per 10,000 live births in single deliveries [57]. Moreover, the analysis of reevaluated infants with high suspicion of transient hypothyroidism recorded in the INRICH has also shown a twin prevalence of 1.9 % among infants who resulted affected by permanent CH and 13.2 % in those with final diagnosis of transient CH. Taken together, these findings have demonstrated an increased risk for both permanent and transient CH in multiple than in single pregnancies.

6.4.4 Demographic Changes

Changes in the ethnic composition of the population have been also reported as a potential cause of the worldwide rise in CH incidence, as different ethnic groups may have a different risk for CH. In the USA, white newborns have been reported to have a higher risk of CH than black infants. Moreover, between 1991 and 2000, the highest incidence rate of CH in the USA was found in Hispanic newborns, and this rate was associated with a high percentage of Hispanic births in that period [58]. In another study conducted in New Zealand, it was found that the rate of dyshormonogenesis in

hypothyroid cases was associated with higher birth rates among Asian and Pacific populations [27]. Beside the ethnicity and the fertility rate of a population, the prevalence of consanguinity among parents is an important factor that can help to explain certain differences in CH incidence rates. In our recent study conducted on the data of the INRICH between 1999 and 2008, consanguinity was found to be significantly higher among African (24 %), Asian (13 %), and Hispanic CH babies (9.0 %) than Caucasian CH babies (Italian 2.0 % and East-European 1.6 %). Moreover, in the same period, the group of babies born to consanguineous parents showed a significantly higher frequency of normal/hyperplastic thyroid than of thyroid dysgenesis (65 % vs 35 %, $P<0.05$), suggesting a high occurrence of genetically determined dyshormonogenesis among babies born to consanguineous parents [30].

6.5 Transient Forms of CH

Transient hypothyroidism may be caused by maternal or neonatal factors. Maternal factors include antithyroid medications [59], transplacental thyrotropin receptor blocking antibodies, which are relatively rare causing transient congenital hypothyroidism in approximately1:100,000 neonates [60], and exposure to iodine deficiency or excess. Neonatal factors include neonatal iodine deficiency or excess [5], congenital liver hemangiomas [61], and mutations in the genes encoding for DUOX and DUOXA2 [62, 63]. In an Italian case–control study, preterm delivery was described as being an independent risk factor for transient CH [31]. In another study conducted in Greece, a higher prevalence of transient hypothyroidsm was shown in premature compared with full-term infants [29]. However, the most common causes of transient CH still remain iodine deficiency or overload, particularly in premature newborns. According to iodine intake in the population, transient CH is found to be more common in Europe (1:100) than in the USA (1:50,000) [1]. In Italy, over a period of observation of 10 years (1999–2008), 58 % of CH cases reevaluated after a withdrawal of the replacement therapy at 2–3 years of age showed transient hypothyroidism [30].

6.6 Secondary (Central) Congenital Hypothyroidism

While most CH cases are due to CH of thyroidal origin (primary CH) manifesting as thyroid dysgenesis or thyroid hormone synthesis defects, a significant number of CH cases are due to inadequate thyroid-stimulating hormone (TSH) secretion from the anterior pituitary [64–66]. This category of CH cases is termed as CH of central origin (secondary CH). Congenital TSH deficiency may rarely be an isolated problem (caused by mutations in the TSH beta subunit gene), but most commonly it is associated with other pituitary hormone deficiencies, as part of congenital hypopituitarism [1].

Newborn screening programs for secondary CH are active only in few countries worldwide. Moreover, the incidence of this condition has been reported with a rate

ranging widely between 1:106,304 and 1:16,404 [67–69]. This variability can be essentially explained by different screening strategies and methods used in different countries. The Northwest Regional Newborn Screening Program in the USA, using a primary T4 test approach with a cutoff <10th percentile, reported a central CH incidence of 1:106,304 [67]. The Kanegawa prefecture of Japan, measuring free T4 in the dried blood spot with follow-up of infants with a free T4 < 0.7 ng/dl, reported an incidence of 1:30,833 [68]. The newborn screening program in the Netherlands, using a primary T4 test with TBG and TSH measured in samples from infants with a T4 < 5th percentile, detected central CH with an incidence of 1:16,404 [69].

Conclusion

Over the years, availability of epidemiological data from population-based and local surveillance systems have contributed to increase understanding on the incidence, cause, treatment, and outcome of CH. On the other hand, new data have raised new questions. Therefore, new epidemiological studies are needed to further improve knowledge on CH, to promote and orient future molecular studies to more precise targets, and to support clinical research.

Moreover, a recent epidemiological evaluation of the current status of screening programs for CH around the world estimated that approximately 71 % of babies worldwide are not born in an area with an established newborn screening program [43]. This implies that despite the existence of newborn screening for over five decades in developed countries, the majority of babies with CH worldwide are not detected and treated early. New efforts should be done to establish new screening programs for CH and to avoid that the economic burden of neuropsychological sequelae of the disease remains a significant public health problem in many countries.

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Congenital Hypothyroidism due to *PAX8* Mutations

7

Satoshi Narumi and Tomonobu Hasegawa

7.1 Introduction

A thyroid gland produces adequate amounts of thyroid hormones only if (1) the thyroid completes anatomical/histological development, (2) the thyroid expresses thyroid-specific molecules involved in thyroid hormone production (*e.g.*, enzymes and transporters), and (3) the thyroid is stimulated by TSH.

A thyroid-specific transcription factor paired-box 8 (PAX8) is required for normal thyroid development and for maintenance of hormone production in a developed gland. Thus, PAX8 can lead to, when mutated, not only a reduction of the net number of thyroid follicular cells ("quantitative" defect) but also defective tissue architecture and defective gene expression pattern ("qualitative" defect). In this review, an overview of the CH due to *PAX8* mutations is given, dealing with multiple levels of pathogenesis, including the molecular, cellular, tissue, and organ system levels.

7.2 Ontogeny/Physiology

In vertebrates, PAX gene family consists of nine paralogues (PAX1 to PAX9). Each PAX gene encodes a transcription factor protein that is hallmarked by the presence of DNA-binding "paired" domain in its N-terminus. The paired domain is composed of two helix-turn-helix subdomains (α1-3 helices and α4-6 helices), and the N-terminal subdomain contains two β sheets (Figs. 7.1 and 7.2). The two helix-turn-helix subdomains, which are connected with a linker sequence, bind to target DNA

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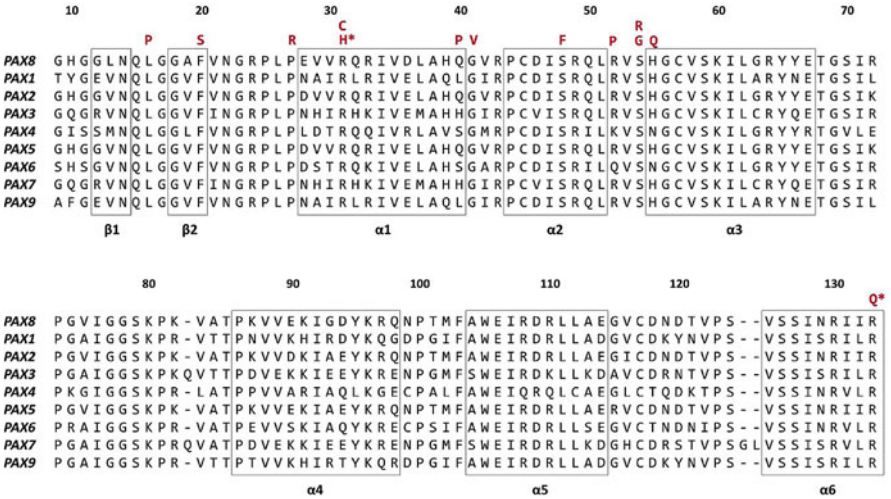

Fig. 7.1 Locations of missense *PAX8* mutations (secondary structure). Amino acid sequences of human *PAX* genes (*PAX1* to *PAX9*) are shown. The sequences correspond to the paired domain, which consists of two β sheets (β1 and β2) and six α helices (α1 to α6) (shown in boxes). Although the roles of *PAX* genes in development and physiology are distinct, the protein sequence are highly conserved among the gene family members. To date, thirteen *PAX8* mutations affecting eleven amino acid residues have been reported (shown in red letters). The majority of the mutations are located in α1 to α3 helices. Only two mutations have been observed in two or more unrelated families (p.R31H and p.R133Q; asterisks)

independently. The C-terminal regions of PAX transcription factors are supposed to act as a transcriptional activator, although precise mechanisms of action are largely unknown. PAX family genes are usually expressed from early embryogenesis in a tissue-specific fashion, working as a "molecular switch" to start or enhance the expression of tissue-specific molecules.

Murine PAX8 was first identified from mouse cDNA library in 1990 [1]. PAX8 is exclusively expressed in the thyroid from the time of specification of thyroid anlage to adulthood [1], suggesting its roles in both thyroid development and physiology. Expression of PAX8 is also observed in the kidney, where PAX2 (a closely related paralogue of PAX8) is expressed. PAX8 is essential for the activation of the thyroperoxidase (TPO) gene, thyroglobulin gene, and sodium/iodine symporter (NIS) gene *in vitro* [2, 3] . In a rat thyroid cell line model, loss of PAX8 expression, which is triggered by transformation with polyoma middle T-antigen, causes remarkable loss of thyroid-specific proteins such as thyroglobulin, TPO, and NIS [4]. These changes can be reversed by reinduction of PAX8.

In 1998, establishment and characterization of genetically engineered PAX8-deficient mice was reported (the paper was published "back to back" in *Nature Genetics* with reports of first human *PAX8* mutations)[5]. *Pax8* knockout mice are hypothyroid and die soon after weaning if not replaced with thyroid hormones. In the mice, thyroid diverticulum develops, but the cell population cannot expand. This proliferation defect in early organogenesis results in severe thyroid hypoplasia.

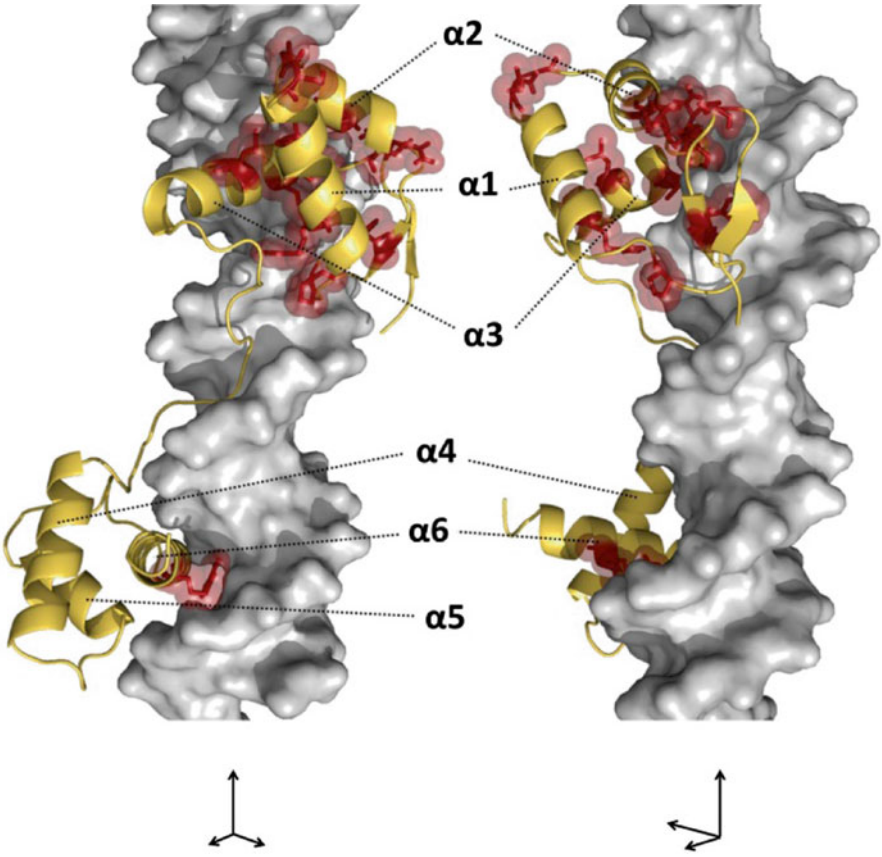

Fig. 7.2 Locations of missense *PAX8* mutations (three-dimensional structure). An image of three-dimensional structure of the PAX-DNA complex (PAX, gold; DNA, silver). The image was produced with PyMOL (www.pymol.org) based on the data of PAX6-DNA complex (protein data bank accession 6PAX; http://www.rcsb.org/). Side chains of the amino acid residues that are affected by human *PAX8* mutations are shown in red. As shown in Figs. 7.1 (secondary structure) and 2 (three-dimensional structure), most of human *PAX8* mutations are located in α1 to α3 helices

Development of calcitonin-producing tissues is not affected. The phenotype of *Pax8* knockout mice indicates that proliferation of thyroid precursor cells require at least one copy of PAX8, but specification of thyroid anlage does not in mice. This is why PAX8 is not regarded as the "master switch" of thyroid development. The situation is contrasting to PAX6, which acts as a master switch of eye development [6].

More recently, new insights into the role of PAX8 in early thyroid development were provided from a series of cell engineering experiments. Antonica and colleagues, who established the protocol to induce thyroid follicular cells from mouse embryonic stem cells (ESCs), reported that transient expressions of PAX8 and NKX2-1 (also known as thyroid transcription factor-1) were sufficient to induce

thyroid-specific molecules including thyroglobulin, TPO, NIS, and TSH receptor [7]. In the paper, transient expression of "PAX8 alone" or "NKX2-1 alone" was also tested as control experiments. Surprisingly, overexpression of PAX8 in mouse ESCs results in virtually no effect on abovementioned thyroid-specific molecules. Contrastingly, overexpression of NKX2-1 alone can induce those genes, although the mRNA expression levels were low as compared with PAX8/NKX2-1 coexpression. NKX2-1 is the most potent "molecular switch" of thyroid development known to date, but gene expression sufficient to initiate thyroid hormone production in ESC-derived cells requires simultaneous expression of PAX8 and NKX2-1. These observations agree well with previous *in vitro* observations that PAX8 and NKX2-1 synergistically transactivate thyroid-specific genes [8, 9]. NKX2-1 exhibits wider spatial expression pattern (*e.g.*, lung and brain) than PAX8, but thyroid-specific molecules are not expressed in these extrathyroidal tissues. The restricted expression patterns of NKX2-1 responsive thyroid-specific genes (*e.g.*, thyroglobulin, TPO, NIS, and TSHR) are likely defied by coexpressed transcriptional partner, PAX8.

7.3 Congenital Hypothyroidism due to *PAX8* Mutations in Humans

In 1998, Macchia and colleagues screened *PAX8* mutations in 145 Italian patients with thyroid dysgenesis and identified two heterozygous *PAX8* mutation carriers (p.R31H and p.R108X) [10]. These two patients were sporadic, and family analysis showed that the mutation occurred *de novo*. Macchia et al. also identified a dominantly inherited mutation (p.L62R) in a German family with autosomal dominant hypothyroidism (two children and their mother were affected).

After the first identification of *PAX8* mutation-carrying patients, more than 50 mutation carriers belonging to 23 families have been described in the literature [11–26]. These publications have revealed variable phenotypes of mutation carriers regarding thyroid morphology and hormone-producing capacity. About 80 % of patients that evaluated their thyroid morphology had thyroid hypoplasia. Therefore, thyroid hypoplasia is a typical radiological finding of *PAX8* mutation carriers. Only one patient with thyroid ectopy was reported [10], while no patients had thyroid aplasia. Though the majority of mutation carriers have a hypoplastic thyroid, thyroid morphology can be completely normal and can be variable even within a family [14, 16]. Decreased echogenicity of the thyroid has been described in several mutation carriers [24, 25]. This change is presumably due to the altered tissue architecture of mutation-carrying thyroid (described below).

As for thyroid functions, most mutation-carrying patients have severe permanent congenital hypothyroidism, with decreased serum-free thyroxine levels. Although patients are usually diagnosed in the frame of newborn screening for CH, three cases that had a negative newborn screen result have been described [17, 24]. These observations suggest that a minor subset of *PAX8* mutation carriers have delayed-onset CH.

Although extrathyroidal manifestation is rare among *PAX8* mutation carriers, at least four mutation carriers with congenital anomalies of the kidney and urinary tract (*e.g.*, unilateral kidney agenesis, congenital ureterocele, horseshoe kidney) have been reported [23]. Considering the expression of PAX8 in the developing kidney, these anomalies could be associated with the mutation.

7.4 Frequency of Human *PAX8* Mutations

As described above, PAX8 was first identified as a protein that is predominantly expressed in the developing thyroid of mice. The remarkable phenotype (severe thyroid hypoplasia) of the *Pax8* knockout mice also confirmed the important role of PAX8 in thyroid development. Therefore, early human mutation screening studies of *PAX8* have focused on CH with thyroid dysgenesis (thyroid aplasia, ectopia, or hypoplasia) [10, 13, 14]. In these early studies, *PAX8* mutations were found in 1.4–3.3 % of thyroid dysgenic CH subjects, confirming that *PAX8* is also involved in development of the human thyroid (Table 7.1). However, considering the wide clinical variability of human *PAX8* mutation carriers, these frequency data could have a potential bias resulting in either an underestimate or an overestimate of the true frequency. More recently, mutation screening has been extended to CH patients lacking radiological evidence of thyroid dysgenesis, finding that 0.7–2.0 % of total CH patients (with or without thyroid dysgenesis) have *PAX8* mutations [17, 21, 22, 25].

All the previous mutation screening study concluded that *PAX8* mutation is not the major cause of human CH. Nonetheless, *PAX8* mutation is still the most common genetic cause of dominantly inherited CH. We stress that clinicians should consider *PAX8* mutations when he/she sees CH patients with positive family history of CH.

Table 7.1 Summary of the results of *PAX8* mutation screening

Author	Year	Study site	Study subjects	Frequency of mutations	Ref.
Macchia et al.	1998	Italy	Thyroid dysgenesis	2 in 145 (1.4 %)	[10]
Vilain et al.	2001	Belgium	Thyroid dysgenesis	2 in 61 (3.3 %)	[13]
De Sanctis et al.	2004	Italy	Thyroid dysgenesis	1 in 54 (1.9 %)	[14]
Al Taji et al.	2007	Czech	Congenital or early-onset hypothyroidism (Thyroid dysgenesis, 65 %)	3 in 170 (1.8 %)	[17]
Narumi et al.	2010	Japan	Congenital hypothyroidism (Thyroid dysgenesis, 51 %)	2 in 103 (2.0 %)	[25]
Alcántara-Ortigoza et al.	2012	Mexico	Congenital hypothyroidism (Thyroid dysgenesis, 96 %)	1 in 100 (1.0 %)	[21]
Liu et al.	2012	China	Congenital hypothyroidism (Thyroid dysgenesis, 61 %)	2 in 300 (0.7 %)	[22]

7.5 Molecular Mechanisms of Human *PAX8* Mutations

To date, 19 distinct *PAX8* mutations affecting 17 amino acid residues have been reported. These include 15 missense mutations (p.L16P, p.F20S, p.P25R, p.R31C, p.R31H, p.Q40P, p.G41V, p.S48F, p.R52P, p.S54G, p.S54R, p.H55Q, p.C57Y, p.L62R, and p.R133Q) (Figs. 7.1 and 7.2), one small duplication mutation (p.K80_A84dup), and three truncating mutations (p.D46fs, p.R108X, and p.T277X). No gross deletion involving the *PAX8* locus has been reported.

Each reported mutation has been detected in only one family (*i.e.*, "private" mutations), except for two mutations (p.R31H and p.R133Q). The p.R31H and p.R133Q mutations have been identified in four and two unrelated families, respectively [10, 19, 21, 22, 24, 26]. The exceptional recurrence of mutations affecting Arg31 and Arg133 is presumably due to hypermutability of CpG dinucleotides [12].

Reported missense mutations are exclusively located in the paired domain (Figs. 7.1 and 7.2). These mutations are expected to have defective target DNA binding. In fact, a couple of mutations were shown to have the DNA binding defect *in vitro* with use of electrophoretic mobility shift assay [10, 11, 15, 17, 23–26]. Reported *PAX8* mutations commonly have loss of transactivating function of thyroid-specific gene(s), such as *TG*, *TPO,* and *SLC5A5* (encoding NIS). No dominant negative effect has been observed *in vitro*, except for one mutation (p.S48F). Considering the fact that an early truncating mutation (p,D46fs) causes CH [24], haploinsufficiency, wherein a single functional copy of a gene is insufficient to maintain normal function, is a major cause of the dominantly inherited CH among *PAX8* mutation carriers.

As described above, inactivation of one *PAX8* allele is sufficient to cause hypothyroidism in humans. This circumstance is contrasting to genetically engineered PAX8-deficient mice: heterozygous deletion of *Pax8* produces no phenotype in mice, whereas homozygous deletion causes early proliferation defect of thyroid precursor cells [5]. This incomplete recapitulation of the human disease necessitates in-depth analysis of human cases to understand the human disease. In this context, we investigated the thyroid histology of a *PAX8* mutation carrier [20]. The patient was a mother of two children affected by severe CH. We first detected a duplication mutation with the DNA binding defect (p.K80_A84dup) in the two children. The identical mutation was also detected in the mother. She had a history of right hemithyroidectomy due to a thyroid nodule at age 28 years but had had normal thyroid function before the surgery. Subsequent genetic analysis in her showed that the mutation was present as a somatic mosaic state, and this explained why her thyroid gland could produce hormones: her thyroid was a mixture of cells with or without the mutation. Histologically, she had hypercellular lesions surrounded by morphologically normal thyroid tissue (Fig. 7.3a). The hypercellular aggregates consisted of thyroid follicular cells with absent or very small follicles, resembling the fetal thyroid tissue in the follicular growth stage (Fig. 7.3b) [27]. Cell-type-specific mutation analysis with use of laser capture microdissection revealed that the fetal-like thyroid tissue had the *PAX8* mutation, whereas morphologically normal thyroid tissue did not. Despite the dramatic change in tissue architecture,

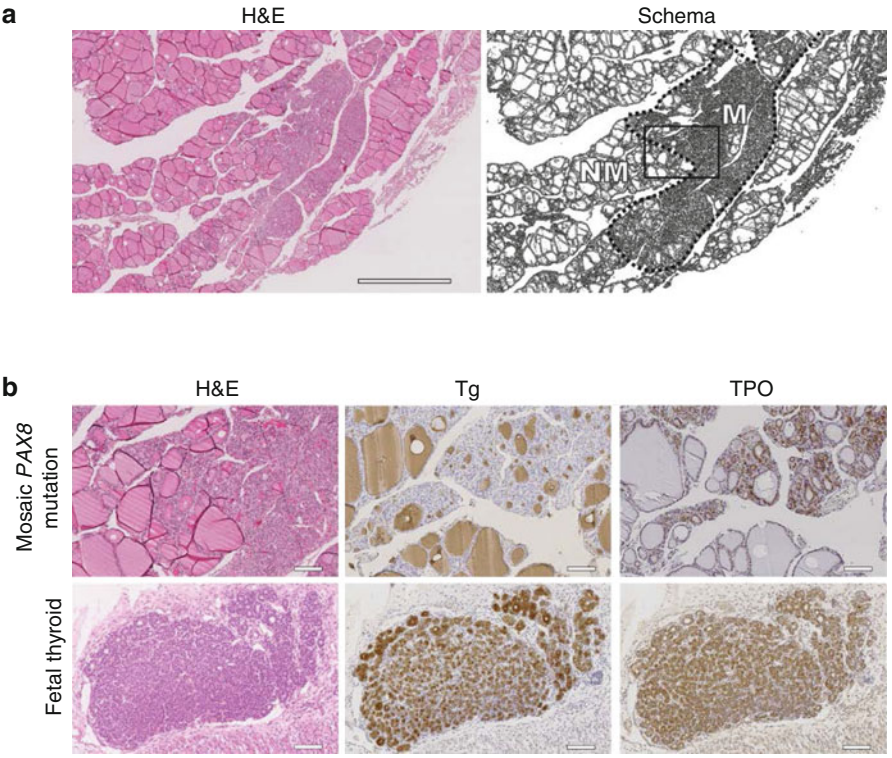

Fig. 7.3 Thyroid histology of a mosaic *PAX8* mutation carrier. Thyroid histology of a patient with somatic mosaic *PAX8* mutation (p.K80_A84dup) (Narumi et al. [20]). (**a**) Semimacroscopic appearance of the thyroid section (left, hematoxylin and eosin (H&E) stain, scale bar, 500 μm; right, schema of the section) are shown. Thyroid tissue with the mutation (indicated by M in the right panel) shows remarkably high cellularity, while non-mutated tissue (indicated by NM) has large follicles with abundant colloid. (**b**) Microscopic images of H&E staining and immunostaining (scale bars, 100 μm). The fields correspond to the box in panel **a**. Images of the *PAX8* mutation-carrying thyroid were obtained at the border of mutated and non-mutated tissues. Although the mutation-carrying tissue had absent or very small follicles, the colloid was immunoreactive for thyroglobulin as in the non-mutated tissue and normal fetal thyroid tissue (12 gestational weeks). TPO staining also showed comparable cytoplasmic immunoreactivity with the nonmutated tissue and normal fetal thyroid tissue

immunostaining for thyroglobulin and TPO showed comparable signals between the mutation-carrying tissue and nonmutated tissue (Fig. 7.3b). Several unique insights can be obtained from this peculiar case with a somatic inactivating *PAX8* mutation: (1) a *PAX8* mutation disturbs development of thyroid follicles in humans; (2) the mutation-carrying thyroid cells can complete early stages of histological differentiation and express comparable levels of thyroglobulin and TPO with nonmutated cells; (3) thyroid hypoplasia of *PAX8* mutation carriers is possibly due to a reduction in size of follicles ("qualitative" defect), as well as a reduction in number of cells ("quantitative" defect). The "qualitative defect" hypothesis is also supported

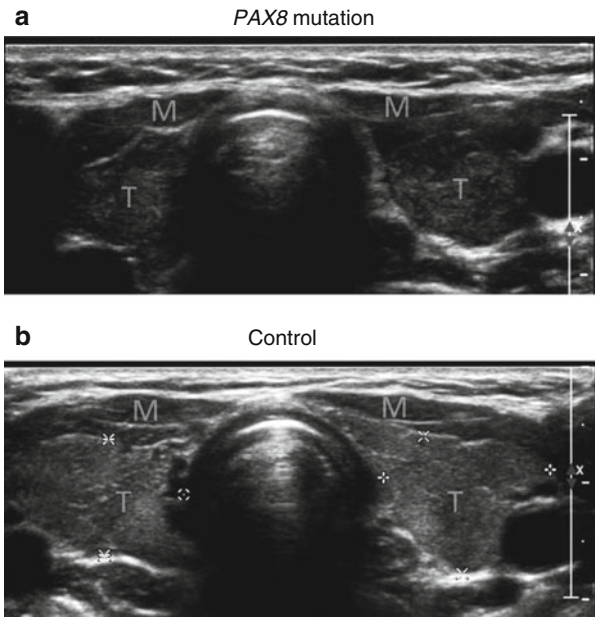

Fig. 7.4 Thyroid ultrasonography of a *PAX8* mutation carrier. *Upper panel*, an ultrasonographic image of a *PAX8* mutation carrier (p.K80_A84dup; Ref. [25]). The patient had a hypoplastic thyroid with low echogenicity. *Lower panel*, an ultrasonographic image of a control individual. Note that echogenicity of the thyroid gland (*T*) is higher than that of sternocleidomastoid muscle (*M*) in the control

by ultrasonographic images of a subset of mutation carriers showing thyroid with low echogenicity (Fig. 7.4) [24, 25]. It has been known that thyroid echogenicity is negatively correlated to the cellularity of the tissue. Therefore, low thyroid echogenicity observed in several mutation carriers might indicate the altered tissue architecture (small follicles; "qualitative" defect) among them.

Conclusions

PAX8 mutation is the most common transcription factor defect associated with human CH. *PAX8* mutation carriers show dominantly inherited CH with mild to severe thyroid hypoplasia in most cases. There is a well-established rodent disease model, *Pax8* knockout mice. However, significant species differences regarding the haplosensitivity and timing of onset of developmental abnormalities (later in humans) are the major limitations of the model. At present, careful observations on human mutation-carrying patients are mandatory to clarify the nature of CH due to *PAX8* mutations.

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Transient Neonatal Hypothyroidism

8

Silvana Caiulo, Maria Cristina Vigone,
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Transient neonatal hypothyroidism is defined as a temporary abnormality of the thyroid function discovered at birth, which later reverts to a normal status. It may or may not require replacement therapy [1]. Recovery to euthyroidism typically occurs in the first few months or years of life [2].

Transient hypothyroidism is much more common in preterm infants but may occur in apparently healthy term infants [3].

Causes of transient neonatal hypothyroidism include

- Maternal and neonatal iodine deficiency or excess
- Drugs
- Intrauterine exposure to maternal antithyroid drugs
- Transplacental passage of maternal TSH receptor blocking antibodies
- Genetic mutations
- Prematurity – critically ill newborn
- Congenital hemangioma/hemangioendothelioma

In many cases, an underlying etiology may not be determined.

8.1 Maternal and Neonatal Iodine Deficiency or Excess

Iodine is essential for the production of thyroid hormones.

Worldwide, *iodine deficiency* resulting in hypothyroidism is the most important preventable cause of cognitive impairment in children [4]. Iodine deficiency in the newborn is mainly due to *maternal iodine-deficient diets* [2].

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Iodine deficiency is more common in preterm infants than in term infants. Preterm infants, in fact, have low iodine and thyroid hormone stores [5] and require relatively more iodine than full-term infants and older children to maintain a positive iodine balance [6, 7] putting them at risk for deficiency without an adequate *iodine dietary intake*. The Committee on Clinical Practice Issues of the American Society of Clinical Nutrition recommended parenteral intakes of iodine of 1 μg/kg/day for the preterm infant, even though this might be below their requirements. It should be considered that in recent years exposition to iodine excess in obstetrics and neonatology units has been reduced. Therefore, iodine excess should not be considered anymore as a valuable source of iodine [8].

Studies on healthy preterm and full-term newborns lead to believe that the iodine intake required to maintain a positive balance is at least 15 μg/kg/day in full-term newborns and 30–60 μg/kg/day in preterm babies [8].

It has long been recognized that *iodine excess* leads to a paradoxical inhibition of the first step of thyroid hormone synthesis. Thyroid hormone synthesis decreases transiently over a period of 24 to 48 h as a result of an increased concentration of intrathyroidal iodine, "termed the acute *Wolff–Chaikoff effect.*" The mechanism for the acute Wolff–Chaikoff effect is not completely understood but is thought to be at least partially explained by the generation of several inhibitory substances on thyroid peroxidase activity [9]. In a few days, escape from the acute Wolff–Chaikoff effect occurs with resumption of normal thyroid function. Excess iodine results in hypothyroidism if the acute Wolff–Chaikoff effect persists. Newborns cannot adapt to iodine excess in the blood and can be affected with neonatal transient hypothyroidism related to the Wolff-Chaikoff effect [10]. Neonates are particularly sensitive to iodine excess because their skin is especially permeable, iodine trapping processing in the thyroid gland is very active, and iodine renal clearance is low. Premature babies are more susceptible, and lower iodine overload may impair their thyroid function [11].

Iodine exposure occurs because of the use of iodine-containing antiseptics, drugs such as amiodarone, or radiocontrast agents [12–14] in the mother or in the newborn. A recent study showed no abnormal thyroid functions in the infants of 21 mothers given iodide contrast during pregnancy, suggesting that thyroid dysfunction may be related to the type and duration of exposure [15].

Urinary iodine concentration is the recommended method to assess iodine status of a certain population in a specific time point; thyroglobulin may be a biomarker of iodine status [16, 17]. Further studies are required to confirm the utility of thyroglobulin as a biomarker of iodine status. Typically in case of iodine excess, the urinary iodine concentration is high. The raised urinary iodine levels confirm the exposure to iodine but are not always associated with greatly elevated TSH levels [18]. In the review by Aitken J [18], the incidence of transient hypothyroidism/hyperthyrotropinaemia in the exposed infants in the cohort studies ranged from 12 per 100 to 33 per 100.

The evaluation at birth of urinary iodine excretion would be helpful in detecting the transitory nature of TSH elevation since iodine excess causes transient hypothyroidism [19].

8.2 Drugs

Some anticonvulsant agents (carbamazepine and sodium valproate) can cause transient mild hypothyroidism.

Aminophylline and caffeine used as respiratory stimulants in preterm infants with recurrent apnea can transiently alter thyroid function. *Steroids and dopamine*, used in critically ill babies, can interfere with the hypothalamic-pituitary-thyroid axis, causing transient central hypothyroidism with low FT4 concentrations and a low or normal TSH level [1].

8.3 Intrauterine Exposure to Maternal Antithyroid Drugs

During pregnancy, the use of *antithyroid drugs* (ATDs) can temporarily alter the function of the thyroid gland in the fetus and newborn. Propylthiouracil, methimazole, and carbimazole all cross the placenta. The smallest possible dose of ATDs should be used in order to avoid a deleterious fetal impact [20]. Overtreatment should be avoided because of the possibility of inducing fetal goiter and/or fetal hypothyroidism [21]. ATDs are able to cross the placenta resulting in a blockade of thyroid function of the fetus, leading to fetal and neonatal transient hypothyroidism. The T4 and TSH values tend to return to normal within 1–3 weeks after birth without treatment [3].

8.4 Transplacental Passage of Maternal TSH Receptor Blocking Antibodies

Maternal autoimmune thyroid disease is a common disorder. Thyroid peroxidase antibodies (TPOAb) have been found in 10 % of women during or shortly after pregnancy [22].

Although antithyroglobulin (TGAb) and TPOAb apparently have no pathogenetic effect on fetal and neonatal hypothyroidism, transient mild elevation of serum TSH above the normal reference value for age is frequently observed in the first month of life in infants born from mothers affected by autoimmune thyroiditis [23].

Maternal autoimmune thyroid disease, in rare cases, may be associated with the production of *thyrotropin receptor blocking antibodies* (TRBAb).

The TRBAb can cross the placenta and block the TSH receptor in the neonatal thyroid, leading to transient congenital hypothyroidism. Scintigraphy may show no uptake despite the presence of a eutopic thyroid gland with maternal TRBAb. Hypothyroidism can last up to 3–6 months after birth as maternal antibody levels fall. In some cases, it might be necessary to start therapy with L-T4, planning a reevaluation of therapy at a later time.

The incidence of transient congenital hypothyroidism due to maternal TRBAb in North America is 1 in 180.000 healthy infants or approximately 2 % of babies with congenital hypothyroidism [24]. As autoimmune thyroiditis characterized by the

presence of TRBAb is a relatively rare disorder, Rastogi and LaFranchi only recommend TRBAb determinations in a case where a previous child has had transient congenital hypothyroidism and the mother has a diagnosed autoimmune thyroid disease and is pregnant again [2].

The newborn screening program for congenital hypothyroidism of Lombardy region, Italy, considers newborns of mothers affected by thyroid disease a special risk category and provides a second sampling. The resampling is collected between days 15 and 30 of life. If neonatal screening for congenital hypothyroidism is positive, serum TSH and FT4 are sampled. In this case, we also suggest testing TGAb, TPOAb, and TSH receptor antibodies.

8.5 Genetic Mutations

Genetic mutations, mostly in heterozygosity, may contribute to the development of a transient thyroid dysfunction detected by neonatal screening.

Mutations in the genes *dual oxidase 2* (DUOX2) and *dual oxidase maturation factor 2* (DUOXA2), involved in the etiology of dyshormonogenesis, can lead to transient or permanent congenital hypothyroidism, with a high intra- and interfamilial phenotypic variability [25, 26]. Heterozygous mutations in DUOX2 usually lead to transient congenital hypothyroidism [27].

The possible hypotheses to explain the variability of the DUOX2/A2 phenotype are the existence of other H(2)0(2) generating systems, the different requirements for thyroid hormones according to age, the ethnicity, and the iodine intake [25].

The most significant features to select patients for the DUOX2 analysis are goiter, partial iodide organification defect, low free T4, and high TSH concentrations at the first postnatal serum sampling, despite borderline blood spot TSH [28].

A defect in *thyroid peroxidase* (TPO) is one of the causes of dyshormonogenesis of the thyroid gland.

Niu et al. suggested that the presence of heterozygous TPO gene mutations contributes to development of neonatal transient hypothyroidism [29]. The authors suggest as possible pathogenetic explanations the effect of the stress of extrauterine adaptation during labor on an immature pituitary-thyroid axis in genetically predisposed individuals, combined with environmental triggers.

Subjects who are heterozygous for *TSHR gene mutations* can show various phenotypes, from mild hypothyroidism to the euthyroid condition [30–33]; they are not always identified during neonatal screening. The use of a low TSH spot threshold allows the detection of more cases with mild thyroid dysfunction that are generally associated with monoallelic defects [34].

The thyroid dysfunction caused by monoallelic defects of TSHR gene is usually a permanent condition; however, as TSH values fluctuate from mild hypothyroidism to euthyroidism, this condition may erroneously suggest a transient hypothyroidism.

The need for therapy with L-T4 in patients with partial TSH resistance is still a matter of debate.

8.6 Prematurity – Critically Ill Newborn

Children born prematurely compared with children born at term have a greater spectrum of thyroid dysfunction. *Premature infants* are characterized by hypothalamic-pituitary immaturity, a premature loss of the contribution of transplacental T4 and iodine, limited thyroid gland reserve, immaturity of the mechanism of thermogenesis mediated by brown adipose tissue, a persistent fetal thyroid hormone metabolism, and a high morbidity predisposing to euthyroid sick syndrome [35]. As a consequence, premature babies may face multiple variations of thyroid function, such as transient hypothyroxinemia of prematurity, persistent hyperthyrotropinemia, and congenital hypothyroidism, which can occur with delayed TSH rise and euthyroid sick syndrome. In addition, *very low birth weight infants* usually have various systemic diseases and are administered drugs that can alter the hypothalamic-pituitary-thyroidal axis, such as dopamine, morphine, and caffeine [36].

The European Society for Paediatric Endocrinology suggests a *strategy of second screening* in preterms, low birth weight and very low birth weight neonates, and ill and preterm neonates admitted to neonatal intensive care unit.

In our previous study from Lombardy region, Italy, on 24 preterm infants affected by congenital hypothyroidism and treated with L-thyroxine, we found that only 23,8 % of patients with gland in situ at reevaluation showed permanent hypothyroidism requiring therapy reintroduction. There do not appear to be any obvious clinical or laboratory features that predict which infants will or will not recover normal thyroid function [37].

8.7 Congenital Hemangioma/Hemangioendothelioma

The majority of *hemangiomas* are small and require no therapy. It is likely that only patients with both high levels of type 3 iodothyronine deiodinase activity and large tumor burdens are at risk for hypothyroidism. Congenital liver hemangiomas can produce large amounts of the enzyme type 3 iodothyronine deiodinase, producing a consumptive type of hypothyroidism in which high doses of thyroxine are required to maintain euthyroidism. Serum T4 levels are low, TSH is elevated, and reverse T3 levels are also increased. Hypothyrodism resolves as the tumor involutes or is treated [38, 39].

8.8 Starting Treatment with L-T4 and Reevaluation of the Thyroid Axis

Since the transient nature of the hypothyroidism will not be recognized clinically or through laboratory tests in some infants, initial treatment will be similar to that in any infant with permanent congenital hypothyroidism [3].

If TSH concentration remains between 6 and 20 mU/L with an FT4 concentration within the normal limits for age for more than 3–4 weeks, it can be decided

in discussion with the family either to start L-T4 supplementation immediately and retesting, off treatment, at a later stage, or retest 2 weeks later without treatment [40].

It is not known whether or not babies with mild, transient hypothyroidism do or do not benefit from *thyroid hormone treatment*. Until these data are available, it might be prudent to treat infants with this atypical form of hypothyroidism, with reevaluation of thyroid function after age 2 years [41]. In these cases, it is important to distinguish at some later point between permanent and transient congenital hypothyroidism.

Reevaluation of the thyroid axis is recommended [40]

- When no etiological diagnostic assessment was carried out during early infancy and/or when treatment was started in the context of the infant being ill (e.g., preterm)
- When initial evaluation has shown a normally located gland with or without goiter
- In neonates with positive thyroid antibodies
- In children who have required no increase in L-T4 dose since infancy
- In children in whom no enzyme defect has been identified (either because no molecular genetic investigations have been carried out or because investigations have proved negative for all mutations tested)

Reevaluation should be performed in case of athyreosis diagnosed on the basis of isotope scanning alone when there is a condition of

- Excess iodine exposure
- Maternal antibodies blocking the TSH receptor
- Iodine transport defects

Reevaluation should be performed in case of DUOX2 mutation.

Diagnostic reevaluation consists of a trial off therapy of 4 weeks followed by measuring TSH and FT4 levels.

According to ESPE guidelines [40], reevaluation of the thyroid axis, off treatment, should normally take place after the age of 3 years. *Earlier reevaluation* (from 1 year of age) can be considered if transient increases in TSH concentration are likely.

8.9 Transient Neonatal Hyperthyrotropinemia and Thyroid Function in Childhood

Leonardi et al. [42] studied the *long-term outcome* of thyroid function in children with very short-lasting neonatal hyperthyrotropinemia ("false positive" at neonatal screening) in an observational, prospective study. Thyroid function and morphology were evaluated in 44 "false-positive" children up to 8 years of age. In these children, a high prevalence (50 %) of subclinical hypothyroidism in early childhood

(2.8 ± 0.5 years) had already been described [43]. At an average of 5.3 years, subclinical hypothyroidism persisted in 43.2 % of children, and at 8 years of age, subclinical hypothyroidism persisted in 31.8 % of children. This study confirms that newborns who resulted "false positive" at neonatal screening have a high risk to develop persistent subclinical hypothyroidism. *Thyroid morphology abnormalities* found at ultrasound evaluation, during more advanced childhood, were frequent. Thyroid morphology abnormalities were present both in children with normal serum TSH in childhood and in children with slightly elevated TSH but were more frequent in the latter group. Common TPO and TSHR polymorphism were present with similar frequency in the two groups. The authors conclude that a "false-positive" result at screening allows to identify subjects at risk for subsequent subclinical hypothyroidism.

According to the review by Monzani et al. [44], subclinical hypothyroidism in children is usually a remitting process with a low risk of evolution toward overt hypothyroidism.

Conclusion

The pathogenesis of neonatal transient hypothyroidism includes both *environmental and genetic factors*.

In the future, further studies might better explain if the presence of mutations in heterozygosity of genes involved in the synthesis of thyroid hormones during critical periods such as extrauterine adaptation can cause neonatal transient hypothyroidism.

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Central Hypothyroidism

9

Luca Persani and Marco Bonomi

9.1 Definition and Epidemiology

Central hypothyroidism (CeH) is a disease characterized by a defective thyroid hormone production originating from an insufficient stimulation of an otherwise normal thyroid gland. This condition is the consequence of anatomic or functional disorders of the pituitary gland or the hypothalamus causing a defective TSH secretion [1, 2].

Though an isolated failure of thyrotrope cells is possible, the TSH defect is more frequently part of *combined pituitary hormone deficiencies* (CPHDs), which indeed complicate both diagnosis and clinical management of CeH. Diagnosis is usually made biochemically with low circulating free T4 (FT4) concentrations associated with low/normal serum TSH levels. Therefore, CeH represents the major false-negative result of the "reflex TSH strategy," a worldwide diffuse method to screen thyroid function by the first-line TSH test [3]. CeH can affect patients of all ages and severely affect their quality of life. Therefore, the existence of mild forms of CeH should always be suspected in patients with *hypothalamic-pituitary disorders* or in those with suggestive clinical manifestations after the exclusion of a primary thyroid disease.

CeH most frequently occurs as a sporadic form of hypothyroidism, and differently from primary hypothyroidism, there is no female prevalence. It apparently accounts for about 1 out of 1,000 hypothyroid patients as its prevalence was estimated to range from 1:16,000 to about 1:100,000 in the general adult or neonatal populations [3–5]. Such variable prevalence is probably depending upon several factors, including ethnicity and diagnostic strategies.

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Table 9.1 Known causes of central hypothyroidism (CeH) in children

Main causes	
Inheritable defects	CPHDs Pituitary transcription factor defects *LEPR* or *PROKR2* mutations Isolated CH *TSHβ*, *TRHR* or *IGSF1* mutations
Invasive or compressive lesions	Craniopharyngiomas Pituitary macroadenomas Meningiomas or gliomas Rathke cleft cysts Empty sella
Iatrogenic causes	Cranial surgery or irradiation Drugs (anti-cancer treatments, RXR agonists, dopamine)
Injuries	Head traumas Traumatic delivery
Unusual causes	
Vascular accidents	Pituitary infarction Subarachnoid haemorrhage
Autoimmune diseases	Lymphocytic hypophysitis Polyglandular autoimmune diseases
Infiltrative lesions	Iron overload Sarcoidosis or Histiocytosis X
Infective diseases	Tuberculosis or Mycoses

The mechanisms underlying CeH pathogenesis variably involve both hypothalamic and pituitary cells but are still undetermined in several cases. The major causes of CeH are listed in Table 9.1.

9.2 CeH Diagnosis in Children

9.2.1 Inheritable CeH Forms

The various genes so far linked to CeH are illustrated in Fig. 9.1.

In the neonates, CeH can be identified only by screening programs based on concomitant TSH and total T4 measurements in the dry blood spot [4–8]. CeH confirmation by serum FT4 and abnormal TSH response to TRH testing may reveal the risk of CPHDs and impending adrenal crisis [8]. CPHDs represent the more frequent forms of neonatal CeH. They can be the consequence of mutations in genes encoding for various *pituitary transcription factors*, such as *PROP1*, *POU1F1* (or PIT1), *HESX1*, *LHX3,* or *LHX4*, or for hormone receptors relevant for several hypothalamic functions, such as *LEPR* or *PROKR2* [1, 2, 9, 10]. In pituitary transcription factor defects, CeH can also have a delayed onset and be associated with hypoglycemia, craniofacial or pituitary abnormalities, and severity of growth retardation [1, 2, 9, 10]. The complex phenotypes associated with these defects are summarized in Table 9.2.

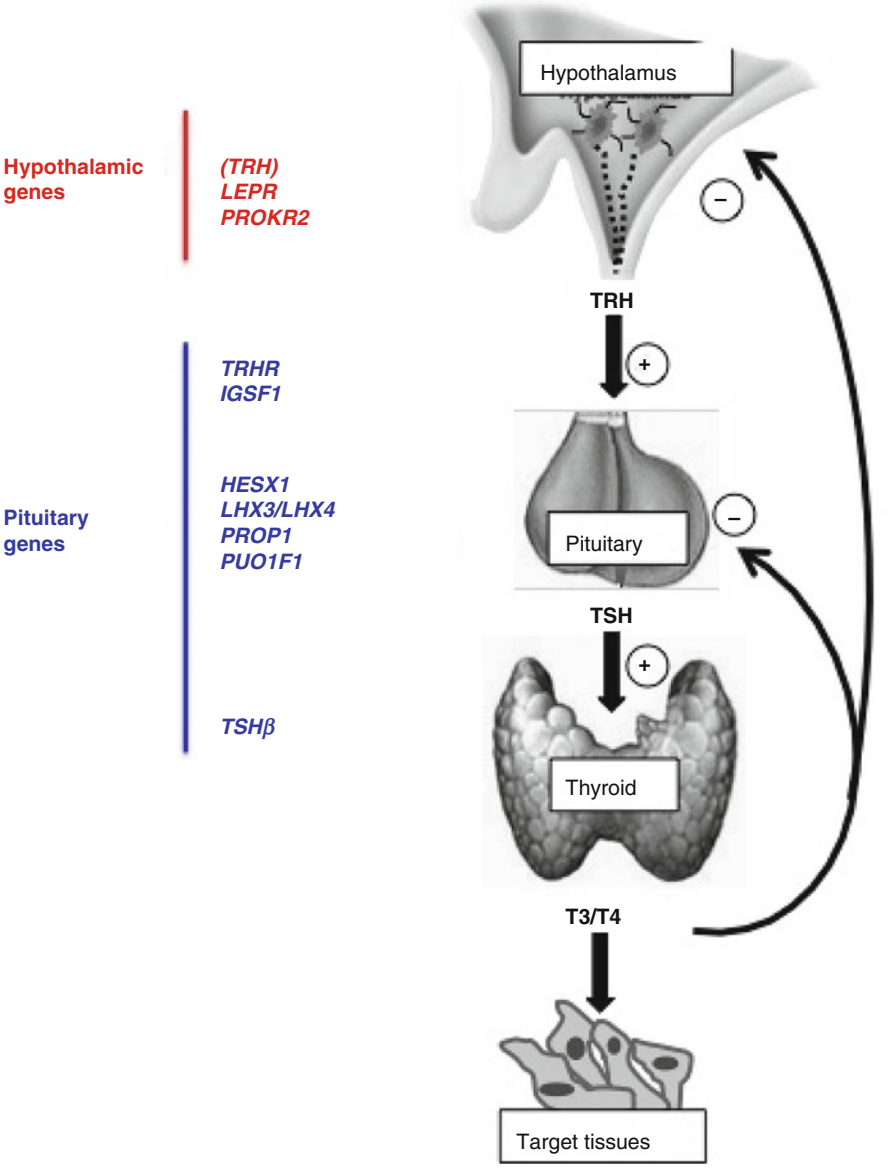

Fig. 9.1 Schematic illustration of the hypothalamic and pituitary genes so far associated with the pathogenesis of CeH. Though the involvement of TRH gene seems obvious, no variants have been so far detected in humans with CeH

Inheritable forms of CeH due to biallelic *TSHβ mutations* are frequently associated with severe neonatal onset and characterized by the typical manifestations of congenital hypothyroidism (macroglossia, coarse cry, jaundice, failure to thrive and retarded growth, umbilical hernia, hypotonia, etc.). If untreated with l-thyroxine

Table 9.2 Phenotypes associated with the inheritable forms of CeH

Genes (OMIM *gene number)	Associated phenotype and transmission (OMIM #disease number)
TSHβ (*188540)	Recessively inherited severe isolated CeH of neonatal onset with high α-GSU, pituitary hyperplasia (#275100)
TRH-R (*188545)	Recessively inherited isolated CeH with blunted TSH/PRL response to TRH and apparently uneventful infantile development, and with childhood (growth retardation) to adulthood onset
IGSF1 (*300137)	X-linked CeH associated with low PRL, variable partial GH deficiency, and macrorchidism
POU1F1 (*173110)	Moderate/severe CeH (dominant or recessive inheritance) of neonatal to infantile onset combined with GH and PRL defects, prominent forehead, mid face hypoplasia, depressed nose (#613038)
PROP1 (*601538)	Recessively inherited moderate/severe CeH of neonatal to infantile onset, combined with GH, PRL, LH/FSH defects, and delayed ACTH deficiency, pituitary hypo-/hyperplasia (#262600)
HESX1 (*601802)	Dominantly or recessively inherited panhypopituitarism associated with septo-optical dysplasia (SOD), supernumerary/hypoplastic digits (#182230)
LHX3 (*600577)	Recessively inherited hypopituitarism with conserved ACTH function and associated with pituitary hypo- or hyperplasia, short/rigid cervical spine and variable deafness (#221750)
LHX4 (*602146)	Dominantly inherited CPHD associated with abnormalities of cerebellum and small sella turcica (#262700)
PROKR2 (*607123)	Variable CPHD associated with SOD or pituitary stalk interruption (SIP) (variable inheritance)
LEPR (*601007)	Recessively inherited severe obesity and hyperphagia combined with delayed puberty and mild thyrotropin defect

within 6 weeks of life, these patients generally develop cretinism [1, 6, 11, 12]. The association of CeH with high α-GSU levels in an infant is invariably indicative of a TSHβ defect [11].

The TRH knockout mice have a typical CeH phenotype [6], but no *TRH* gene defect has been documented so far in humans. However, a defective TRH action due to natural mutations in the *TRHR* gene has been so far described in two families [13, 14]. We reported a family with a complete TRH receptor defect caused by an early stop codon potentially leading to the translation of a truncated protein lacking all the seven transmembrane and intracellular domains [14]. The probands of this family represent a natural *TRHR* knockout model: a unique opportunity to understand the role of *TRHR* in humans. The early development of patients with *complete TRH resistance* appeared uneventful. The diagnosis in the male proband with homozygous TRHR mutations was reached because of delayed growth accompanied by

fatigue at 11 years of age. The presence of this defect can be suggested by the blunted responses of TSH and PRL to TRH stimulation [13, 14] (Table 9.2). Unexpectedly, the same diagnosis was reached in the 33-year-old sister by genetic testing, during her second pregnancy. This woman with complete TRH resistance had reached her target height and normal IQ and has presently delivered three heterozygous babies with normal pre- and postnatal growth. In none of these cases, she experienced any lactating defect. Interestingly, when a hypothyroid questionnaire was administered to this woman before the start of l-thyroxine during the second gestation, she responded positively to only 1 question out of 12. Nevertheless, when the therapy was withdrawn for 6 weeks during her puerperium, the number of positive responses rose up to 10/12, thus indicating that thyroid hormone replacement had certain subjective beneficial effects that were unexpected a priori. Therefore, this study showed that the hypothalamic hormone is required to set the *pituitary feedback mechanism* at a level adequate to maintain free thyroxine levels in the normal range. In addition, the conservation of a significant *nocturnal TSH surge* in this condition indicates that TRH action influences the amplitude, but additional sleep-related factors account for the determination of the circadian oscillation. Interestingly, though *TRH* is also expressed in the pancreatic islets, we could not demonstrate any defect of glucose homeostasis in these patients [14].

Very recently, several familial cases of *X-linked* CeH from the Netherlands, the UK, and Italy have been reported to be associated with genetic defects in *IGSF1* [15, 16]. This gene encodes a membrane protein containing immunoglobulin-like motifs but of still unclear biological functions that is expressed in the pituitary and testes. IGSF1 defects are associated with a novel syndrome including CeH and *macroorchidism* and seldom GH deficiency. CeH in these cases is associated with blunted TSH and PRL responses to TRH testing consistent with the finding of a reduced Trh-r expression in the pituitaries of IGSF1 knockout mice. Accordingly, IGSF1 transcripts were found in Pit1-dependent lineages (thyrotrope, lactotrope, and somatotrope). Igsf1-deficient male mice show low pituitary and serum TSH concentrations, decreased thyroxine and triiodothyronine concentrations, and increased body mass [15].

9.2.2 Acquired CeH Forms

The hypothyroid state is mild to moderate in most patients with acquired CeH, as the pituitary TSH reserve is infrequently depleted. Although manifestations of CeH are similar to those of primary hypothyroidism, they can be masked by symptoms of CPHDs [1, 2]. CeH represents a major false-negative result of the "*reflex TSH strategy*" for the diagnosis of thyroid dysfunction [1, 5–7]. Therefore, acquired CeH should be suspected in all subjects with known hypothalamic/pituitary lesions (e.g., *craniopharyngiomas*, pituitary macroadenomas) or in those with clinical and biochemical manifestations (e.g., growth retardation, fatigue, cholesterol elevation) suggestive of hypothyroidism despite normal/low circulating TSH. On serum samples, the diagnosis of CeH is usually suggested by the finding of low FT4

Table 9.3 Conditions that can be associated with diminished FT4 serum levels and aberrantly low/normal TSH

Central hypothyroidism (hypothalamic hypothyroidism may seldom be associated with TSH values above the upper normal limit)
Severe non-thyroidal illness (e.g., anorexia)
L-T4 withdrawal syndrome
Recovery from thyrotoxicosis
Drugs inhibiting TSH secretion
Allan-Herndon-Dudley syndrome (MCT8 mutations)
TRα1 mutations

concentrations, associated with low/normal TSH levels [1, 2, 17, 18]. Nevertheless, some CeH patients with a predominant hypothalamic defect have high serum immunoreactive TSH levels but devoid of full biological activity. In these cases, TSH elevations are similar to those generally found in subclinical or mild primary hypothyroidism and may lead to the misdiagnosis [1, 2]. In Table 9.3, the conditions associated with low/normal TSH and low FT4 levels and that could come into differential diagnosis with CeH are listed.

When a low FT4 is combined with a normal TSH value, the diagnostic workup for the confirmation of CeH should include the exclusion of interference in FT4 or TSH measurements [1, 2]. In general, automated FT4 assays are less reliable than the equilibrium dialysis, which is however not compatible with the routine work. If interference is suspected, this should be explored by using a "two-step" assay or by mass spectrometry. If the problem persists, hormone measurement following equilibrium dialysis remains the gold standard for eliminating FT4 assay interference. Less frequently, TSH immunometric measurement can be interfered by the presence of heterophile antibodies in a patient's serum, if directed against the same species as the assay antibodies: thus, a heterophile antibody that blocks TSH binding to either capture or detection antibodies will cause a falsely low TSH readout potentially indicating a central instead of a primary hypothyroidism. Though most of the manufacturers are nowadays providing reagents including the preimmune serum from the source animal, heterophile antibodies may still interfere in the TSH determination on some instances. If interference is suspected, the discordant TSH concentration should be checked (a) by means of an immunoassay using a different antibody pair, (b) after immunosubtraction by treatment with polyethylene glycol (PEG) or protein G, or (c) by dilution or recovery tests [1, 2].

Once the interference is excluded, the finding of "low FT4" combined with an abnormally "low TSH" outlines the diagnosis of overt forms of CeH, but the diagnosis of milder defects, characterized by FT4 levels still within the normal range, remains unsolved. An indication on how these cases can be disclosed comes from studies on children surviving cancer disease [19–21]. Cranial irradiation can indeed cause hypothalamic defects with TRH secretory abnormalities resulting in either *hidden CeH* (CeH with FT4 values included in the normal range that can be recognized only by the demonstration of abnormal circadian or stimulated TSH secretory kinetics) or *manifest CeH* (most frequently associated with low TSH and FT4).

Since mild CeH may be associated with a decreased growth velocity in children surviving cancer disease, several groups investigated the possible solutions for the diagnosis of mild or hidden CeH [19–21]. Though abnormalities in *circadian TSH secretion* may not correlate with FT4 levels, the lack of a nocturnal TSH rise can be useful in the diagnosis of CeH but can be evaluated only in hospitalized patients [19–21]. TRH is not available in the USA, but *TRH test* may confirm the suspect of mild CeH and may be of help in the differential diagnosis between tertiary (hypothalamic) and secondary (pituitary) hypothyroidism as the two defects may be associated with exaggerated/delayed/prolonged or blunted TSH responses, respectively [1, 2, 19–23]. However, it must be underscored that a significant portion of patients with CeH may still have a normal TSH increase after TRH stimulation, and a clear distinction between the two forms of CeH may be difficult, as both sites are affected in most patients. The practical utility of TRH testing is therefore to be limited to the patients with uncertain diagnosis, in whom the abnormal TSH response to TRH may confirm the CeH [22, 23].

Interestingly, time-related decreases in circulating FT4 concentrations larger than 20 % *versus* the initial FT4 determination were reported to support the diagnosis of CeH in patients with different pituitary diseases followed up for several years [1, 18]. This cutoff value was set on the basis of a 10 % variation over time of T4 levels in normal individuals [24]. Provided that FT4 determination is repeatedly performed in the same laboratory, this approach would then allow the diagnosis and treatment of mild or hidden hypothyroid states of central origin.

The indexes of peripheral thyroid hormone action, such as sex hormone-binding globulin (SHBG), bone markers, serum lipids, and others, lack sufficient sensitivity and specificity for the diagnosis of mild or subclinical hypothyroidism, especially in patients who present with CPHDs, which may *per se* affect the levels of these indexes.

In the presence of low thyroid hormone levels, the exclusion of a primary thyroid defect may be required either because CeH may sometimes result from an *intermittent thyrotoxic state* (Table 9.3) or because hypothalamic hypothyroidism may be associated with slightly raised TSH concentrations at immunoassay. Indeed, the exclusion of primary thyroid disease by biochemical testing and/or ultrasound examination is the main objective in this differential diagnosis. Conversely, a family history of CeH or the clinical history (e.g., *head trauma*) or manifestations (e.g., headaches or visual field defects) may be suggestive of the presence of hypothalamic-pituitary defects, and the MRI imaging generally confirms the central origin of hypothyroidism. It is worth to note that some drugs have been associated with an increased risk of CeH [25], including the use of dopamine in dystocic delivery.

Severe and chronic *nonthyroidal illness* (NTI) are associated with values of thyroid function tests that largely overlap with those of CeH patients [1, 2]; therefore, the presence of concomitant diseases at the time of blood sampling should always be excluded before suspecting "true" CeH.

Allan-Herndon-Dudley syndrome, an X-linked form of mental retardation associated with tissue-specific resistance to thyroid hormones, can be associated with low FT4 and normal or slightly elevated TSH levels [26]. This disease is caused by mutations in the *MCT8* gene encoding a membrane thyroid hormone transporter.

These patients can be distinguished from those with CeH by the severe clinical phenotype, including cognitive and psychomotor retardation, and the typical elevation of T3 circulating levels that are usually two- to threefold higher than in normal subjects. Similar biochemical findings can be found also in patients with *thyroid hormone action defects* (THAD) [27] due to heterozygous mutations in *THRA gene*, encoding the thyroid hormone receptor α1 (TRα1). Severe constipation, defective and disharmonic growth, mental retardation, and delayed bone development appear as distinct features of this disease [28, 29].

9.3 CeH Replacement Therapy

As in primary hypothyroidism, treatment of CeH should restore appropriate serum concentrations of thyroid hormones. As for other forms of hypothyroidism, the daily administration of l-thyroxine is the preferred treatment of CeH [1, 2, 30, 31]. Several studies in children and adult primary hypothyroid patients did not find a superiority of combined LT4 plus triiodothyronine (LT3) treatment [31]; therefore, the LT4 + LT3 combinations should still be considered as an experimental treatment modality for patients that do not reach well-being on an adequate l-thyroxine regimen.

Because of the risk to induce an *adrenal crisis*, if a combined corticotrope deficiency has not been excluded, a prophylactic steroid treatment should be administered before the start of thyroxine therapy.

In normal infants and children, the thyroid hormone levels are higher than in adults [1, 2, 32]. Therefore, higher l-thyroxine doses are recommended in hypothyroid pediatric patients, and treatment should be started at full replacement doses, especially in patients with neonatal onset due to TSHß mutations in order to rapidly reach adequate circulating FT4 levels and promptly support neurological development [11, 33]. Guidelines recommend to initiate treatment of neonatal disorders with 10–15 μg/kg of l-thyroxine and to adjust doses on the basis of FT4 measurements every 2–4 weeks [34]. *L-T4 treatment* has been reported to promote an acceleration of growth velocity allowing reaching the target height [1, 14, 34]. Progressively lower doses are required in childhood and in *transition to adulthood* [35].

The target range should be that observed in normal children. Since TSH values do not correlate with thyroid function in CeH, the decision to modify the replacement regimen should be primarily taken on the basis of the clinical manifestations. In addition, several evidences, reviewed in ref. 1, indicate that *TSH determination* is not completely devoid of significance during L-T4 treatment in CeH. In summary, one should suspect undertreatment in all the conditions listed here below:

- Serum TSH above 0.5 mU/L, in particular if associated with serum FT4 values below the lower tertile of normal range
- Fall of serum FT4 values below the lower tertile of normal range
- Introduction of *GH replacement therapy*
- Introduction of *estrogen replacement* therapy or oral contraceptives
- Introduction of treatments impacting LT4 absorption or thyroid hormone metabolism

Conversely, one should suspect overtreatment in the presence of clinical manifestations suggestive of thyrotoxicosis, when associated with one of the following conditions:

- Serum values of FT4 and/or FT3 above the upper tertile of normal range
- Withdrawal of GH or estrogen replacement therapy
- Withdrawal of oral contraceptives or estrogen
- Withdrawal of treatments impacting LT4 absorption or thyroid hormone metabolism

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Syndromes of Resistance to Thyroid Hormone Action

10

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10.1 Mechanisms of Thyroid Hormone Action on Target Tissues

The biological actions of TH in target tissues are regulated by a number of variables such as the cell surface transporters, the deiodinase system controlling the metabolism of TH, and the nuclear TH receptors that in concert with specific cofactors mediate the TH actions [1–4]. In principle, defects affecting all these components may be associated with an alteration/reduction of the sensitivity to thyroid hormone action. Accordingly, the nomenclature and classification of the already described and still potential defects of TH action or metabolism have recently been revised [5]. Here, we provide a brief illustration of these different molecular mechanisms.

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10.1.1 Cell Surface Transporters

Since TH essentially exerts its effects inside the cells, an efficient mechanism of transport of iodothyronines across the plasma membrane is required.

Up to now, several TH transporters have been identified, such as the Na+/taurocholate cotransporting polypeptide (SLC10A1), multidrug resistance-associated proteins, the heterodimeric L-type amino acid transporters (LAT1 and LAT2), the organic anion-transporting polypeptide (OATP) family, and the MCT family. Most of these transporters are not specific for T3, since they bind to different ligands, with the exception of MCT8 (SLC16A2), MCT10 (SLC16A10), and OATP1C1 (SLCO1C1) [4, 7].

MCT8 is expressed in the heart, liver, kidney, adrenal glands, and thyroid, but its function appears to be critical especially in the brain: in fact, genetic defects of this transporter are involved in the pathogenesis of the Allan-Herndon-Dudley syndrome and of the Pelizaeus-Merzbacher-like disease, which are associated with a severe neurological phenotype. Data about the role of MCT10 and OATP1C1 in human physiology are limited [4]. However, T3 concentrations in the CNS are roughly 20 % of serum levels [6]. In order to reach the brain, the THs need to cross the blood–brain barrier (BBB), which is composed of endothelial cells of brain capillaries surrounded by the astrocyte processes, separating the blood from the brain extracellular fluid.

THs enter neurons by two mechanisms, mediated by OATP1C1 and MTC8, respectively. In the first case, TH uptake occurs from the endothelial cells into the astrocytes, with a greater efficiency for T4 than T3. Thereafter, DIO2 converts T4 locally to T3, which enters the neurons by binding to MCT8. Alternatively, TH may also enter directly in the neurons through gaps in the astrocyte processes via MTC8, which exhibits a higher affinity for T3 compared to T4 [7].

10.1.2 Deiodination System

The intracellular concentrations of T3 are regulated by three deiodinases, selenocysteine-containing enzymes, which activate or inactivate the iodothyronines through the removal of iodide from thyroxine and its metabolites.

The incorporation of the selenium (Se) in the selenoproteins is mediated by a multiprotein complex; genetic mutations in proteins belonging to this system, such as the selenocysteine insertion sequence-binding protein 2 (SECIBP2), cause a deficient production of selenoprotein associated with an abnormal thyroid hormone metabolism [8].

Type 1 deiodinase (DIO1) deiodinates T4 (3,5,3′,5′-tetraiodothyronine) into T3 (3,5,3′-triiodothyronine) and T3 into T2 (3,3′-diiodothyronine); DIO1 is highly expressed in liver and kidney and contributes to the production of plasma T3 by deiodination of T4. The type 2 deiodinase (DIO2) converts T4 to T3 intracellularly; it is highly expressed in the thyroid, being responsible for the increased intrathyroidal production of T3 in Graves' disease and toxic thyroid nodules.

Finally, type 3 deiodinase (DIO3) degrades T4 to rT3 and T3 to 3,3′-diiodothyronine (T2), thus downregulating the local T3 production and protecting tissues from TH excess.

In the CNS, DIO2 is mainly expressed in glial-derived cells, such as the astrocytes and the tanycytes, while DIO3 is primarily found in neurons. In order to maintain adequate levels of T3 in neural tissues, the deiodinase function is finely regulated. In particular, recent data suggest that astrocytes produce active T3, which enters neurons via MCT-8. In these cells, DIO3 controls the local bioavailability of T3 by the production of rT3 and T2 from T4 and T3, respectively [6].

10.1.3 Nuclear Receptors

Thyroid hormone receptors belong to the nuclear receptor superfamily. They bind as heterodimers with retinoid X receptor (RXR), or less frequently as homodimers, to regulatory DNA sequences, known as thyroid response elements (TRE) located in the promoter of target genes. A number of cofactors (proteins acting as coactivators and corepressors) are involved in TH receptor signaling.

On positively regulated genes, in the absence of T3, the hetero/homodimers are associated to corepressors and bind to TREs repressing transcription. The arrival of T3 and its binding to TRs results in dissociation of corepressors, recruitment of coactivators, and transcriptional activation.

Conversely, negatively regulated TH target genes show transcriptional activation in the absence of TH and repression in the presence of the ligand T3.

The two major corepressors, the nuclear receptor corepressor (NCoR) and the silencing mediator of retinoic acid and thyroid hormone receptors (SMRT), are crucial regulators of nuclear receptor signaling [9]. They form a complex with other repressors, such as Sin 3, and histone deacetylases [10], thus leading to shut down basal transcription. Several coactivators interact with TRs, such as the steroid receptor coactivator complex (SRC) and the vitamin D receptor interacting protein–TR associated protein complex (DRIP–TRAP), which enhance the T3-dependent transcription. SRC complex interacts with CREB-binding protein (CBP), responsible for cAMP-stimulated transcription, interacting with the phosphorylated form of CREB (cAMP-regulated enhancer binding protein) and with the related protein p300. CBP/P300 interacts with P/CAF (p300/CBP-associated factor), which has an intrinsic histone acetyltransferase (HAT) activity, and with the RNA pol II [11]. Several interacting components associate with RNA Pol II, thus connecting nuclear receptors to the basal transcriptional machinery [12].

Two receptors (TRα and TRβ) mediate the TH effects at the nuclear level. They are encoded by two separate genes, on human chromosomes 17 and 3, respectively. Two TRβ isoforms have been identified: TRβ2 is mainly expressed in the hypothalamus, pituitary, retina, and inner ear; conversely, TRβ1 is the principal isoform in the liver and kidney and is also widely expressed in the brain [13–15]. The TRα1 predominates in the CNS, skeleton, intestine, and cardiac muscle. The TRα2 isoform differs for the TRα1 in the C-terminus and is unable to bind T3 but retains DNA-binding properties and is considered a modulator of TH action.

The TRα1 is the isoform that accounts for about 80 % of the TH receptors expressed in the brain. Compared to TRα, the TRβ is expressed at a later stage of brain development. In addition, the TRβ1 and TRβ2 isoforms are expressed in a neurogenic subpopulation, located in the hippocampus apparently involved in the proliferation of neuronal progenitors [16]. In particular, it has been suggested that unliganded TRβ isoforms may exert an inhibitory effect on hippocampal cellular growth [17]. A similar role has been hypothesized for unliganded TRα1 in the cerebellum [18, 19] and hippocampus [20].

10.2 Resistance to Thyroid Hormones Syndrome due to Mutations in THRB Gene (RTH β)

10.2.1 General Clinical Features

Resistance to thyroid hormones syndrome (RTH β) is a rare condition, and more than 3,000 cases have been published from about 1,200 different families with a wide geographic and ethnic distribution [1, 2, 5, 21]. The prevalence of the disease is indefinite, since the routine screening programs for congenital hypothyroidism are based on the sole TSH determination which is typically normal in this condition. A limited survey in a cohort of 80,000 newborns found one case among 40,000 live births.

The majority of the cases (nearly 85 %) are associated with heterozygous mutations in the TRβ gene, and the condition is inherited in an autosomal dominant fashion [2].

This inheritance depends on the dominant negative effect, due to the inhibition of the activity of the wild type β- and α-receptors, by the mutant TR-beta. These mutant receptors display either a reduced affinity for T_3 or an impaired interaction with the cofactors (coactivators and corepressors), thus losing its ability to modulate target gene expression in different tissues.

Different mechanisms have been evoked to explain this dominant negative effect [21]:

1. Formation of inactive dimers between mutant TRs and wild-type TRs
2. Competition between mutant and wild-type receptors for essential cofactors
3. Competition between mutant TR and wild-type TR for DNA-binding sites.

In the original RTH β family, in which a deletion of exons 4–10 resulted in the abolition of the dimerization and DNA-binding properties of TRβ, the disease segregated as an autosomal recessive trait. The homozygous patients had goiter and deaf-mutism together with high TH levels; conversely, the heterozygous subjects were phenotypically normal, supporting the hypothesis that reduced amount of TRβ does not produce haploinsufficiency [2, 22] and that the mutant receptor must conserve its DNA-binding and dimerization properties, in order to cause the biochemical phenotype of RTH β, i.e., high free TH in the presence of unsuppressed TSH levels.

The TRβ mutations are distributed in the carboxyl terminus of the TRβ. Typically, three CpG-rich "hot spot" regions are located in the ligand-binding domain and in the contiguous hinge domain of the protein.

In contrast to what is observed for other nuclear receptors (such as vitamin D, androgen receptor, or PPARγ), no mutations have been identified in the DNA-binding domain or in other regions of the receptor.

In about 10–15 % of the cases with the biochemical phenotype of RTH β, no mutation could be found in the TRβ gene, and this situation is defined as "non-TR – RTH." It is speculated that these patients may have an abnormality in one of the cofactors or TH transporters into the cells. However, screening of several families with non-TR-RTH excluded the involvement of coactivators (SRC-1/NcoA-1; and NcoA-3/SRC-3/AIB1/RAC-3), two corepressors (NCoR and SMRT), and two coregulators (RXRγ and TRIP1) as well as the cell transporter LST-1 (OATP1B1) [23].

The clinical picture of RTH β ranges from thyrotoxic manifestations to the absence of any signs of TH excess. Differences in the degree of hormonal resistance are probably due to the different TRβ and TRα expression in different tissues.

TRβ mainly is expressed in the hypothalamus, kidney, liver, anterior pituitary gland, hypothalamus, retina, and cochlea, whereas TRα predominates in the skeletal and cardiac muscle, brain, brown fat, intestine, spleen, and vascular endothelial cells. Consequently, symptoms of TH deficiency and excess could coexist in the different tissues of one subject. As an example, hypercholesterolemia, delayed bone maturation, growth retardation, and learning disabilities (suggestive of hypothyroidism) may coexist with weight loss, heat intolerance, hyperactivity, and tachycardia (typical of thyrotoxicosis).

Classically, RTH β subjects have been classified into two subgroups according to the absence or presence of symptoms of thyrotoxicosis, selective pituitary resistance (PRTH), and generalized thyroid hormone resistance (GRTH), respectively. Patients with PRTH display variable symptoms of hyperthyroidism [24, 25]. Conversely, subjects with GRTH exhibit a sort of "compensated hypothyroidism," being the genetic defect of TH responsiveness balanced by the high circulating TH concentrations; the efficiency of this compensatory mechanism is variable in each individual, in different tissues, as well as in different periods of life.

In addition, TRβ mutations found in both GRTH and PRTH may be the same, and patients of the same family may present with either form. Indeed, PRTH patients have normal levels of sex-hormone-binding globulin, a marker of peripheral thyroid hormone action, elevated in the case of hyperthyroidism, thus suggesting that insensitivity to TH action is present not only in the hypothalamic-pituitary region but also in the liver [25]. Therefore, this clinical distinction may be loose and more theoretical than actual.

The main clinical features of patients with RTH β are summarized in the following paragraphs and in Fig. 10.1.

10.2.1.1 Goiter

Diffuse or multinodular goiter is a common finding in RTH β, independently from the presence of clinical symptoms. An increased biological activity of circulating TSH molecules may favor the formation of goiter in RTH β subjects with normal

TSH levels [26]. In RTH β patients treated by surgical ablation, the goiter commonly relapses with nodular alterations and gross asymmetries, requiring additional surgery or radioiodine.

10.2.1.2 Cardiovascular Symptoms

Approximately 75 % of RTH β patients exhibit palpitations and tachycardia at rest. Predominance of TRα may explain the presence of partially hyperthyroid response in the heart, as the dominant negative effect exerted by mutant TRβs on the normal receptors should be weaker than in other tissues. The finding that some indices of cardiac systolic and diastolic function (e.g., heart rate, stroke volume, cardiac output, diastolic filling, maximal aortic flow velocity) showed values that are intermediate between normal and hyperthyroid subjects supports this hypothesis. However, the normal values of other parameters (e.g., ejection and shortening fractions of the left ventricle, systolic diameter, and left ventricle wall thickness) suggest an incomplete response of the heart to the high TH concentrations. In addition, systemic vascular resistance and arterial stiffness are increased in RTH β, as seen in subclinical hypothyroidism, thus indicating a more complex derangement of cardiovascular function. A reduced insulin sensitivity and dyslipidemia have been documented in a number of patients, suggesting an increased cardiovascular risk in RTH β [27–30].

10.2.1.3 Skeletal Abnormalities

Similarly to the cardiovascular system, also the bone is affected by a mix of hypothyroid and thyrotoxic manifestations in RTH β. Studies performed in animal models suggest that skeletal thyrotoxicosis, due to elevated circulating thyroid hormone levels which overstimulate the intact TRα1 signaling pathway, may be responsible for bone abnormalities in RTH β [31].

In humans, dysmorphic skeletal features, such as "stippled epiphyses," dysmorphic facies, and winged scapulae, have been documented only in the cases harboring complete TRβ resistance due to homozygous deletion of TRβ gene.

Delayed bone maturation and growth are present in about one third of children with RTH β; however, the final adult height seems unaffected.

A decreased bone mineral density and increased risk of fractures have been reported in adult RTH β. Conversely, the normal levels of the markers of bone turnover may imply a reduced bone formation rate resulting in a low peak bone mass similar to that observed in childhood hypothyroidism.

10.2.1.4 Metabolism

Low body mass index (BMI) is reported in about 30 % of RTH β children, in spite of the hyperphagia and the enhanced energy intake.

Basal metabolic rate (BMR) has been found normal or even increased. Indirect calorimetry assessment showed enhanced resting energy expenditure (REE), either in adults or children with TRβ mutations. This increase was intermediate between euthyroid and thyrotoxic subjects. Skeletal muscle and myocardium, in which the TRα isoform expression is prevalent, seem responsible for increased energy expenditure, as suggested by the correlation between mean heart rate and REE in both

RTH and thyrotoxicosis. In both these conditions, TH excess was associated with uncoupling between tricarboxylic acid cycle activity and ATP synthesis *in vivo*, as measured by magnetic resonance spectroscopy [30].

10.2.1.5 Immune System

An increased frequency of respiratory infections (pulmonitis and infections of the upper respiratory tract) has been reported in RTH β patients, compared to their unaffected relatives. This susceptibility has been related to reduced immunoglobulin concentrations but may also derive from an abnormal regulation of granulocytes and lymphocytes that express TH receptors.

10.2.1.6 Neurological System

It has been hypothesized that in RTH β an uncompensated hypothyroidism at an early stage may be responsible for defects of neuroanatomical development.

Few data are available about the brain anatomical abnormalities associated with RTH β. A single MRI study in 43 RTH β patients found, in male patients, an increased frequency of cerebral anomalies of the left hemisphere, particularly an extra or missing gyrus in the parietal bank of the Sylvian fissure or multiple Heschl's transverse gyri in the primary auditory cortex when compared to unaffected relatives. No patent abnormalities were found in female patients [32].

Although severe mental retardation (IQ <60) is uncommon (only 3 %), about 30 % of affected subjects display a mild learning disability (IQ <85). In particular, either the verbal or the performance component were impaired compared with controls [33]. Some authors have reported in their RTH β cohort a high frequency of attention deficit hyperactivity disorder (ADHD). This finding has not been confirmed by other groups, but it is possible that the low IQ may be responsible for ADHD manifestations, more than RTH β *per se*. In addition, an increased frequency of delayed developmental milestones and language disorders has been found in RTH β patients, compared to their unaffected relatives [33–36]. The association with Tourette syndrome has been also described [63] (Fig. 10.1).

The neuroanatomical regions involved in attention and vigilance are located in the right lateral prefrontal cortex, in the parietal lobe, and in anterior cingulate. Consistently, Matochik et al. found a severe impairment on an attention auditory discrimination task in adults with RTH β compared to controls. The PET scan performed during this task demonstrated the presence of an increased metabolic activation of the anterior cingulate in RTH β. The reduction of the functional activity in this brain area and the subsequent activation of other structures, such as the frontal cortex, are required for an efficient performance on complex attention tasks. However, it is not clear whether these functional anomalies are related to a defect in brain development or may be a consequence of the elevated levels of thyroid hormones via overstimulation of the TR-α [37].

Patients with homozygous deletion of THRB display a phenotype characterized by deaf-mutism due to sensorineural hearing loss, delayed bone maturation, stippled epiphyses, goiter, and high levels of circulating thyroid hormone in the presence of a normal TSH [2, 22].

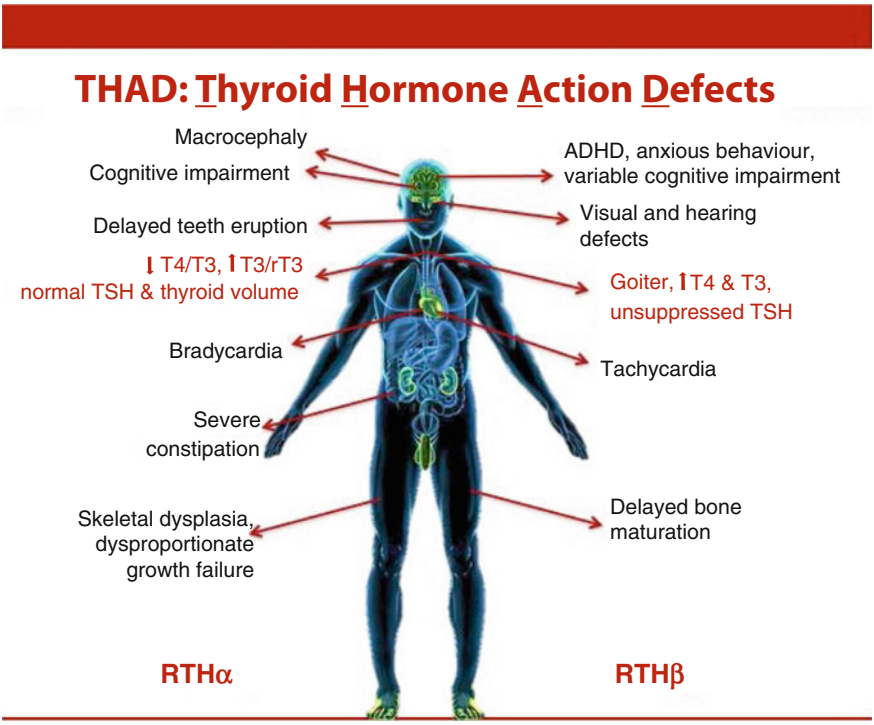

Fig. 10.1 The main clinical characteristics of the patients with TH action defects, RTHα or RTHß. *ADHD* attention-deficit, hyperactivity disorder

Interestingly, these patients with deletions do not display growth delay, mental retardation, or cognitive impairment, while the five cases, homozygous for missense mutations of THRB, are invariably associated with a mild to severe intellectual impairment, neuropsychomotor retardation, goiter, hyperactivity, tachycardia, and hearing loss as the extreme manifestations of resistance [38–40].

"Conventional" heterozygous mutations, resulting in a premature stop codon with the consequent production of a TR-β lacking a number of residues in the C-terminal, also display a strong dominant-negative effect in vitro and are often associated with a severe clinical phenotype, including mental retardation [41–43].

10.2.1.7 Visual System

In animal models, the deletion of the TRβ2 isoform produces a selective loss of M cone photoreceptors resulting in abnormal color vision. In particular, during embryogenesis, TR-β seems responsible for the photoreceptor distribution in the retina, inhibiting the S-opsin and committing the differentiation of M-opsin photoreceptor. However, no abnormalities of color sensitiveness have been identified in "conventional" RTH β patients with heterozygous TRβ mutations. Patients with homozygous deletion of THRB gene [2, 22] are color blind, while in one patient

with compound heterozygous mutation (R338W in exon 9 and R429W in exon 10 of THRB gene), an abnormal electroretinographic pattern was found, characterized by a normal scotopic response and a reduced photopic response. In particular, this patient showed a small-amplitude b-wave to a red flash and a larger-amplitude b-wave to the blue flash, similar to what is commonly described in the enhanced S cone syndrome [44].

10.2.1.8 Hearing System

An increased incidence of conductive or sensorineural hearing impairment, which may contribute to the defective speech development, has been reported in some RTH β children. The pathophysiology of these abnormalities is composite, being the conductive defect due to the higher susceptibility to upper airway infection of RTH β children, whereas the defective TRβ expression may be responsible for the cochlear dysfunction [45].

Noteworthy, mice with targeted disruption of the TRβ locus develop profound sensorineural hearing loss, thus suggesting an important role of TH in the development of the hearing system.

10.2.1.9 Other Features

In mothers affected with RTH β, there is a higher rate of miscarriage and intrauterine growth retardation of unaffected offspring, thus suggesting that intrauterine exposure to high TH levels does have adverse effects on the fetus.

There is only one patient, homozygous for TRβ mutation, in whom RTH β may have contributed to death: this patient had resting pulse of 190 beats/min and died from cardiogenic shock complicated by septicemia.

Coexistence of TSH-secreting pituitary adenomas (TSHomas) and RTH β has been suggested in only two cases. The impaired TH feedback in the pituitary may lead to a continuous stimulus to thyrotropes to synthesize and secrete TSH molecules, which may play a role in the development of pituitary tumors. However, the pituitary lesions associated to RTH β appear to be pituitary "incidentalomas" [46]. Interestingly, somatic mutations of TR-beta have been found in two TSH-secreting pituitary adenomas [47, 48] but never on the germinal DNA of patients with TSHomas.

Occasionally, RTH β occurs in association with autoimmune thyroid disorders, such as Graves' disease or Hashimoto's thyroiditis. The occurrence of anti-TPO or anti-TSH receptor autoantibodies in RTH subjects has been described. Recent data suggest that the individuals with RTH β due to TRβ gene mutations have an increased likelihood of AITD compared to unaffected relatives [49]. The reason for this association seems related with the hyperstimulation, via TR-alpha, of the cells of the immune system.

The RTH patients, who develop Graves' disease, undergo a progressive increase in goiter size along with frank symptoms of thyrotoxicosis. The further elevation of TH levels causes TSH secretion to be totally inhibited. Conversely, hypothyroidism may occur in the presence of normal serum TH concentrations, as a consequence of Hashimoto's thyroiditis.

10.2.2 Differential Diagnosis of RTH β

RTH β shares the same biochemical features of patients with TSH-omas, and occasionally with Familial Dysalbuminemic Hyperthyrotoxinemia (FDH) (Table 10.1). Since these two diseases have completely different therapeutic and management approaches, their differential diagnosis is mandatory [46]. The presence of the same abnormal biochemical pattern of thyroid function in other first-degree relatives supports the diagnosis of RTH β, since familiar cases of TSHoma have never been reported (except for four families in a setting of multiple endocrine neoplasia 1). In these cases, molecular analysis of the THRB gene makes a definitive diagnosis in 85–90 % of cases of RTH β.

Although different clinical parameters have been proposed (basal metabolic rate, systolic time intervals, Achilles reflex time) in order to discriminate among these two conditions, the clinical presentation of patients with RTH β may be similar to those with TSHoma [46], though the onset of central hyperthyroidism generally occurs beyond 30 years of age in the latter condition.

In patients with TSH-omas, serum levels of glycoprotein hormone α-subunit (α-GSU) and α-GSU/TSH molar ratio are elevated, whereas in RTH β patients both indices are in the normal range.

To assess the degree of resistance in specific target tissues, different in vitro parameters have been proposed. Particularly, SHBG and ICTP are in the hyperthyroid range in patients with TSH-oma and within the normal range in RTH β. The sensitivity and specificity of these tests is improved, when assessed after T3 suppression test, performed with oral administration of supraphysiological doses of T3 (50 μg/day for 3 days, followed by 100 μg/day for another 3 days and then 200 μg/day for another 3 days) [7]. In RTH β patients, the increase of peripheral markers of TH actions and heart rate is blunted in comparison to normal subjects, thus definitively confirming the presence of resistance to TH action.

The TRH test (IV injection of TRH 200 μg) has been also widely used: in the majority of patients affected with TSH-oma, TSH and α-GSU levels do not increase after TRH injection, whereas RTH β subjects show normal response of TSH.

T3 inhibitory test, performed as reported above or administering T3 for 8–10 days at the dose of 80–100 μg/day, may show a full inhibition of TSH levels in RTH β patients but persistent TSH response to TRH, carried out at the end of T3 administration. Since none of these tests have a clear diagnostic cutoff value, the combination of them, if possible, increases the specificity and sensitivity of the diagnostic process.

The administration of long-acting somatostatin analogues (e.g., long-acting Octreotide-LAR 30 mg intramuscularly every 28 days) for at least 2 months can be useful in the differential diagnosis in problematic cases of central hyperthyroidism. Chronic administration of long-acting somatostatin analogues in patients with central hyperthyroidism caused a marked decrease of FT3 and FT4 levels in patients with TSH-oma (>30 % of pretreatment values), while patients with PRTH did not respond at all.

Pituitary MRI is required in case of not univocal results with other tests; however, the detection of pituitary lesions does not definitely rule out the diagnosis of

RTH β. In fact, pituitary lesions are quite a common finding (20–25 % of MRI performed for other reasons) in the general population. These lesions are usually considered as "pituitary incidentalomas," especially when a hypothalamic-pituitary dysfunction has been excluded. The presence of a microadenoma in combination with lack of TSH response to dynamic tests and high levels of α-GSU or α-GSU/TSH molar ratio strongly sustains the diagnosis of a TSH-oma.

10.2.3 Therapy

There is currently no definite therapy to correct the molecular defect causing RTH β, and in most patients a specific treatment is not even necessary, as goiter may be the only sign of the disease. The high levels of circulating free TH may be able to compensate for the resistance in several of the peripheral tissues but may create a thyrotoxic state in several others.

Patients with tachycardia and palpitations at rest may benefit by the use of cardioselective β-blockers (atenolol or others). In the event of severe thyrotoxic symptoms, not responding to β-blockers, a reduction of thyroid hormone levels may be beneficial. This can't be achieved using antithyroid drugs, because the consequent increase of TSH levels may determine goiter enlargement. The treatment of choice in such cases is the administration of thyromimetic compounds, such as 3,5,3′-triiodothyroacetic acid (TRIAC), which through the feedback mechanism reduces TSH secretion and causes a slight decrease of circulating T4 levels (values of T3 are unreliable as TRIAC cross-reacts in T3 measurement methods). As a consequence of its weaker effects on peripheral tissues, TRIAC reduces the thyrotoxic signs and symptoms, particularly at the heart level. TRIAC has been shown to be beneficial in both children and adult patients with RTH β at the dose of 1.4–2.8 mg/day, fractionated in two or three administrations [50].

The use of dopaminergic drugs and somatostatin analogues has limited success because TSH secretion rapidly escapes the inhibitory effects of both drugs, as the T4 reduction triggers the much more potent stimulatory effect of TH negative feedback mechanism.

Although controversial, in children with signs of growth or mental retardation, the administration of supraphysiological doses of L-T4 to overcome the high degree of resistance present in some tissues can be beneficial. Supraphysiological doses of thyroid hormones are also necessary in patients treated with total thyroidectomy for a missed diagnosis of RTH β. The use of high doses of L-T4 requires a careful monitoring of patients, assessing the indices of peripheral thyroid hormone action.

Recently, TRβ selective agonists (GC1, eprotirome) have been developed and could be beneficial for some abnormalities (dyslipidemia) found in RTH β. Unfortunately, the development program on this drug has been discontinued after the evidence of cartilage damage after 12 months administration in dogs. In addition, there is evidence that eprotirome may induce liver injury in humans [51].

10.3 Resistance to Thyroid Hormones due to THRA Mutations

10.3.1 General Features of RTH α

Recently, the first three families with TH resistance due to TR-alpha (RTH α) have been described [5, 52–55]. Similar to that described in animal models [56, 31], these subjects present variable features of hypothyroidism associated with normal TSH levels (Fig. 10.1).

The clinical presentation of RTH α is characterized by abnormalities in tissues in which the TRα is the major isoform expressed. Free T4 levels were described at the lower limit of the normal range or slightly below, while free T3 levels were above the upper level of normal, resulting in a reduced FT4/ FT3 ratio (Table 10.1). Interestingly, the four affected individuals of the three families showed a truncated form of the receptor (E403X, F397fs406X, and Ala382ProfsX7) with a premature stop codon located in exon 9, thus affecting the only TRα1 isoform and not the other transcript (TRα2, Rev-erbα) generated from the THRA locus. All these mutations showed in vitro a reduced transcriptional activity and a strong dominant negative effect on wild-type receptor. More recently, a missense mutation involving a domain common to TRα1 and TRα2 has also been described. The biochemical and clinical features did not differ from those described in the other cases [57].

Common features of this syndrome are growth retardation, which transiently improves after L-T4 administration, disproportionate short stature characterized by femoral epiphyseal dysgenesis, macrocephaly due to delayed closure of skull sutures, together with delayed tooth eruption, hypotension, subnormal heart, and basal metabolic rate. Several of these manifestations had been also reported in mice with TRα1-PV mutation [31, 52, 56].

Constipation due to delayed intestinal transit is present in all the cases described up to now; however, in the family with the TRα1-F397fs406X, the administration of L-T4 improved the intestinal manifestations in both father and affected daughter [54]. Interestingly in all the affected patients, low or low-normal levels of IGF-1 were found. One patient was treated with hr-GH, without a significant improvement of the growth retardation [54]. Also, the L-T4 administration was only transiently beneficial on the growth delay.

In summary, these patients retain normal hormone responsiveness in the hypothalamic–pituitary axis and liver, but they display manifestations due to neurological, skeletal, gastrointestinal, and myocardial resistance (Fig. 10.1).

10.3.2 Neurological and Cognitive Impairment in RTH α

In the first pediatric case described, the cognitive deficits were consistent with a congenital hypothyroidism [52]. She was inappropriately placid; her speech was slow and monotonous. A neuropsychological assessment showed selective cognitive deficits in the adaptive behavior, in the short-term memory, and in the visuoperceptual function, while the verbal comprehension was in the normal range. In addition, she experienced motor dyspraxia associated with difficulties in fine motor coordination

Table 10.1 Genetic disorders characterized by increased serum thyroid hormones levels and detectable TSH concentrations

	GENE	Free T4	Free T3	TSH	Total reverse T3	SHBG
Familial dysalbuminemic hyperthyroxinemia (FDH)	*ALB*	N[a]	N[a]	N	↑	N
Resistance to thyroid hormone (RTH β)	*THRB*	↑	↑	N or slightly ↑	↑	N
Defect of THRA gene (RTH α)	*THRA*	borderline or slightly↓	↑	N	↓	↑
Defect of thyroid hormones transport (Allan-Herndon-Dudley syndrome)	*MCT8*	slightly ↓	↑	N or slightly ↑	↓	↑
Defect of thyroid hormones metabolism (SBP2 deficiency)	*SBP2*	↑	N or slightly ↓	N or slightly ↑	↑	N

SHBG sex hormone-binding globulin

[a]As measured by equilibrium dialysis or direct "two-step" measurement methods. Interferences leading to spuriously high levels of FT4 and/or FT3 may be present by using other methods

resulting in the inability to write or draw. In addition, she had a broad-based ataxic gait. Finally, muscular hypotonia, but not weakness, was present.

A similar phenotype, thus associated with a more severe cognitive impairment, has been described in another female patient harboring the Ala382ProfsX7 mutation. The patient was unable to read, and her IQ was around 52. In addition, this patient was affected with epilepsy, confirmed by the electroencephalographic demonstration of bilateral theta waves during hyperventilation; seizures decreased in frequency with sodium valproate administration [55].

The proband of the second family (TRα1-F397fs406X) and her affected father had a mild cognitive deficit with an IQ of 90 and 85, respectively [53]. The observed neurocognitive deficits seem associated with structural abnormalities such as reduced cerebellar and hippocampal volume, diminished white matter density, and accord with the known developmental actions of TH and substantiate the critical role of TRα1 in CNS (Moran C, 2014 BES meeting, personal communication).

The visual and the hearing systems do not seem affected by the THRA mutation.

10.3.3 Therapy

The administration of L-T4 resulted in normalization of FT4, with a further increase of FT3 and suppressed TSH levels, suggesting a conserved negative feedback of TH on TSH secretion.

In the patient with E403X mutant, the levels of IGF-1 normalized, without a real improvement in growth velocity; growth rate and intestinal transit time did not change significantly. Heart rate and blood pressure did not improve. In the second

family, L-T4 treatment caused an improvement of constipation with a persistence of growth retardation in both subjects.

Higher-dose thyroxine therapy or the use of TRα-selective thyromimetic agents may be necessary to avoid hyperthyroidism in TRβ-expressing tissues [54]. The design of novel TH analogues targeting the TRα mutations may open novel therapeutic perspectives in these subjects.

10.4 Disorders of Thyroid Hormone Metabolism

10.4.1 General Features

Iodothyronine deiodinases (DIOs) are a family of selenocysteine-containing enzymes, required for activation or inactivation of thyroid hormones.

Although the most common alterations of TH metabolism are acquired, such as the "low T3 syndrome" of nonthyroidal illness, genetic conditions associated with defective function of deiodinases have been recently described. Mutations in SECIS-binding protein 2 (SBP2), a key protein that allows the incorporation of selenium in selenoproteins, cause defective production of DIOs. Selenoproteins being ubiquitous and multifunctional, those individuals manifest a complex phenotype alongside the abnormal thyroid function [8, 58–61].

The main laboratory finding is an abnormal pattern of thyroid function characterized by high free T4, low free T3, and raised reverse T3, associated with normal or slightly elevated TSH levels (Table 10.1).

Up to now, only six families exhibiting reduced TH sensitivity due to a disorder of thyroid hormone metabolism have been described. The defect is inherited in an autosomal recessive fashion and is caused by homozygous and compound heterozygous mutations in the SBP2 gene.

Selenoproteins are a family of about 25 proteins with wide functions, which include metabolism of thyroid hormones (deiodinases), removal of cellular reactive oxygen species, reduction of oxidized methionines in proteins, and transport and delivery of selenium to peripheral tissues. The inclusion of the rare amino acid, selenocysteine (Sec), is critical for their enzymatic activity.

A multiprotein complex including SBP2 is responsible for the incorporation of selenium into selenoprotein. A specific stem-loop (called SECIS element) in the 3′-UTR region of selenoprotein mRNAs interacts with and leads to selenocysteine incorporation at UGA codons [7, 58]. Defects in this machinery result in miscoding of the UGA as a signal to stop synthesis, and the transcript may undergo decay. The affinity of SBP2 for SECIS elements of different mRNA is variable, and this contributes to hierarchy in selenoprotein production in case of defective function of SBP2 or Se deficiency. In mice, complete disruption of SBP2 is embrionically lethal. In humans, the SBP2 mutations described up to now cause a severe reduction of selenoprotein but not a total depletion. This is probably due to the highly complex architecture of SBP2, with internal methionine residues capable of starting the synthesis of shorter protein isoforms.

Besides the abnormalities in TH metabolism, the phenotype of affected individuals is highly variable ranging from milder to more severely affected individuals.

Deficiencies of multiple selenoproteins have been documented in all cases: glutathione peroxidases are markedly reduced, and circulating levels of hepatic selenoprotein P are low, accounting for the low serum selenium levels recorded in these families. Childhood growth retardation is a common feature in all the families described [8, 58–61]. The only adult subject described was azoospermic, with reduced levels of testis-enriched selenoproteins that cause spermatogenic arrest. In addition, he was markedly photosensitive, with a dermal deficiency of antioxidant selenoenzymes causing increased cellular reactive oxygen species, membrane lipid peroxidation, and oxidative DNA damage. Reduction of antioxidant enzymes in immune cells was associated with impaired T-cell proliferation and shortened telomeric DNA. The latter was associated with anemia and lymphopenia, similar to that observed in aplastic anemia found in telomerase deficiency. Increased adipose mass and increased insulin sensitivity have been described in two families, and eosinophilic colitis was found in one individual.

The first three families described did not display a specific neurological phenotype [58, 59], while the affected individuals recently described showed a more severe impairment. A 12-year-old girl presented with hypotonia and weakness early in her life [60]. She had motor coordination disorder with delayed motor and intellectual milestones (walking at age 2 years and speaking at 3 years); later in childhood, she developed a symmetrical peripheral sensitive neuropathy confirmed by electroneuromyography and somatosensory evoked potential test, characterized by a slow progression. Brain MRI was normal while the audiometric test showed bilateral sensorineural loss. At the age of 11 years, she was mentally retarded and suffered for a progressive peripheral myopathy, similarly to that observed in patients with mutations in the SEPN1 gene.

Mild bilateral high-frequency hearing loss was also found in a male child presenting at age 2 years with failure to thrive, developmental delay, and short stature and in an adult patient presenting at 35 years for infertility and fatigue. Similarly to that observed in the previous family, they both showed delayed motor and speech development and myopathy resulting in muscle weakness [8]. In two patients, an increased frequency of exudative otitis media and rotary vertigo was found [8, 61]. A 10-year-old Japanese boy and an 11-year-old Turkish girl showed a mild mental retardation [61, 21].

10.4.2 Therapy

Clinical trials with oral selenium supplementation showed raised circulating selenium concentrations, without improving the thyroid abnormalities [61, 62]. T3 treatment was clearly beneficial for growth in two children. Tocopherols, lycopene, and other antioxidant agents may be beneficial in reducing the oxidative damage, as suggested by preliminary *in vitro* experiments. In one Japanese case, treatment with rhGH combined with T3 improved both longitudinal bone growth and maturation [61].

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Management of Congenital Hypothyroidism

11

Cavallo Luciano

Infants with CH have to be treated with L-T4 as soon as possible, hopefully within the second week of life without any delay due to aetiological evaluation. It is recommended to refer the patients to a paediatric endocrinologist. A complete interview about family, pregnancy course, maternal drugs and medications during foetal life, physical examination and clinical evaluation with particular attention to the presence of any extra-thyroidal disease should be performed to evaluate any cause of CH. Confirmatory measurement of TSH and fT4 is always needed before starting L-T4 administration. In case of maternal autoimmune thyroid disorder or a previously affected infant, measurement of anti-TSH blocking receptors and anti-thyroid antibodies in the mother and the infant may identify a transient form of CH.

Thyroid ultrasound and iodine 123 (123I) or sodium technetium 99 m pertechnetate (99mTc) thyroid uptake to identify thyroid tissue should be performed before initiating the replacement therapy. The risk-benefit ratio of early thyroid scanning of infants with suspected hypothyroidism is still debated; however, the benefits can be summarized as follows:

1. When radioiodine uptake is absent but ultrasonographic examination reveals a normal gland, a defect of TSH receptor or of iodine transport or the maternal transfer of anti-TSH receptor antibodies should be suspected.
2. Normal scan findings (or a goitre) indicate a functioning thyroid gland with regard to iodine uptake and consistent with a probable defect in T4 synthesis. In these patients, serum thyroglobulin assay may help to separate thyroglobulin synthetic defects from other causes of CH [1]. Infants born from mothers with anti-TSH receptor antibodies or exposed to substances such as antithyroid drugs may have a similar ultrasound picture. The identification of a genetic defect is

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important for families planning on having additional children and to assess the permanent course of CH.
3. Infants with normal scan findings at birth who do not fall into one of the above categories may have a transient form of hypothyroidism. These infants should undergo a careful follow-up evaluation after 3 years of age, when it is safe to discontinue treatment temporarily.

Treatment has not to be delayed to perform the scan. A thyroid scan can be performed within the first few days of treatment, because the elevated TSH found in patients with permanent CH rarely normalizes within this period. A serum TSH measurement should be obtained at the time of the scan. The scan can be performed after the child is 3 years of age, when thyroid hormone replacement treatment can be interrupted without any danger to the developing central nervous system.

11.1 CH Severity

CH severity can be assessed [2]

1. From a clinical point of view, on the basis of symptoms of hypothyroidism such as jaundice, macroglossia, hypotonia, etc.
2. From a biological point of view, as severe, moderate or mild on the basis of serum fT4 levels of <5, 5 to <10, and 10 to 15 pmol/l, respectively
3. From a radiological point of view, on the basis of delayed epiphyseal maturation on knee X-ray
4. From an aetiological point of view, on the basis of the cause of CH

11.2 Treatment

An optimal cognitive and auxological outcome depends on both the adequacy and timing of postnatal therapy, particularly in biologically severe CH. All infants with hypothyroidism should be rendered euthyroid as promptly as possible by the replacement therapy, with normalization of T4/fT4 within 2 weeks and TSH within 1 month [3–6]. In the first weeks of treatment, some infants may have serum TSH concentrations above the normal range, despite T4/fT4 concentrations in the upper half of the reference range. Rarely, the elevated TSH relative to the fT4 value is hypothesized to result from in utero hypothyroidism, producing a resetting of the pituitary-thyroid feedback threshold with relative pituitary resistance. In such cases, characterized by a normal or increased serum T4/fT4 and an inappropriately high TSH concentration, the T4/fT4 value has to be used to titrate the appropriate L-T4 dose.

L-T4 alone, orally given, is recommended as the medication of choice. Although T3 is the most biologically active thyroid hormone, most brain T3 is derived from

local mono-deiodination of T4. There is no evidence that combined T4 and T3 therapy is more effective than T4 alone. The tablets may be crushed and administered via a small spoon, in few drops of water or also milk some 20–30 minutes before the feed or food ingestion. Liquid preparations (drops) are available and require a shorter period of time between drug administration and food intake than the tablets. L-T4 intestinal uptake may be inhibited by specific foods (soy, fibre), medications (iron, calcium), or malabsorption. Increased degradation may be caused by anticonvulsants and large haemangiomas with high deiodinase activity.

An initial dosage of 10–15 μg/kg of L-T4 is recommended. Patients with severe CH should be treated with higher initial dose than infants with moderate CH, who should be treated with lower dose. In newborn patients with cardiac insufficiency, the starting L-T4 dose should be at 50 % of the target replacement dose and should be further increased in accordance with fT4 levels after 2 weeks. Usually, when a higher initial dose of L-T4 is used, the serum T4/fT4 normalizes in 3 days and the TSH returns to the target range by 2 weeks of therapy [6]. The L-T4 dose should be adjusted according to the infant's clinical response and serum fT4 and TSH concentrations. The fT4, rather than the total T4, has to be measured periodically to assess the concentration of the biologically relevant unbound or free form of circulating T4 [7]. During therapy, the serum total T4/fT4 should be kept in the upper half of the reference range during the first 3 years of life with a low/normal serum TSH. Afterwards, it is important that the patients regularly take the L-T4 dose to keep TSH and thyroid hormones within the age-appropriate target values, which are different from those for adults [8]. During the follow-up, patients with in situ thyroid require lower dose of L-T4 than those with ectopic gland or athyreosis. Figures 11.1a, b display the daily L-T4 requirement until the age of 12 years on the basis of body surface area and of body weight, respectively, in CH (personal data on 216 patients).

A proper initial and ongoing counselling of parents is very important to optimize the adherence to the replacement therapy, which has to be promoted throughout life. An optimal cognitive outcome depends on the adequacy and timing of postnatal therapy. Poor compliance to the treatment is the most common cause of persistent TSH elevation, accounting for the risk of major sequelae. These episodes are usually caused by poor parental compliance or impaired T4 bioavailability. In patients with episodes of insufficiently suppressed TSH after the age of 6 years, school delay in childhood and a subtle decrease in health-related quality of life in young adulthood may be detected.

11.3 Follow-up

Clinical examination, including assessment of growth and development, should be performed every few months during the first 3 years of life and then less frequently (see Table 11.1). Infants with CH appear to be at increased risk of other congenital anomalies (approximately 10 % of infants with CH). Cardiovascular anomalies (i.e. pulmonary stenosis, atrial septal defect, ventricular septal defect) are the most

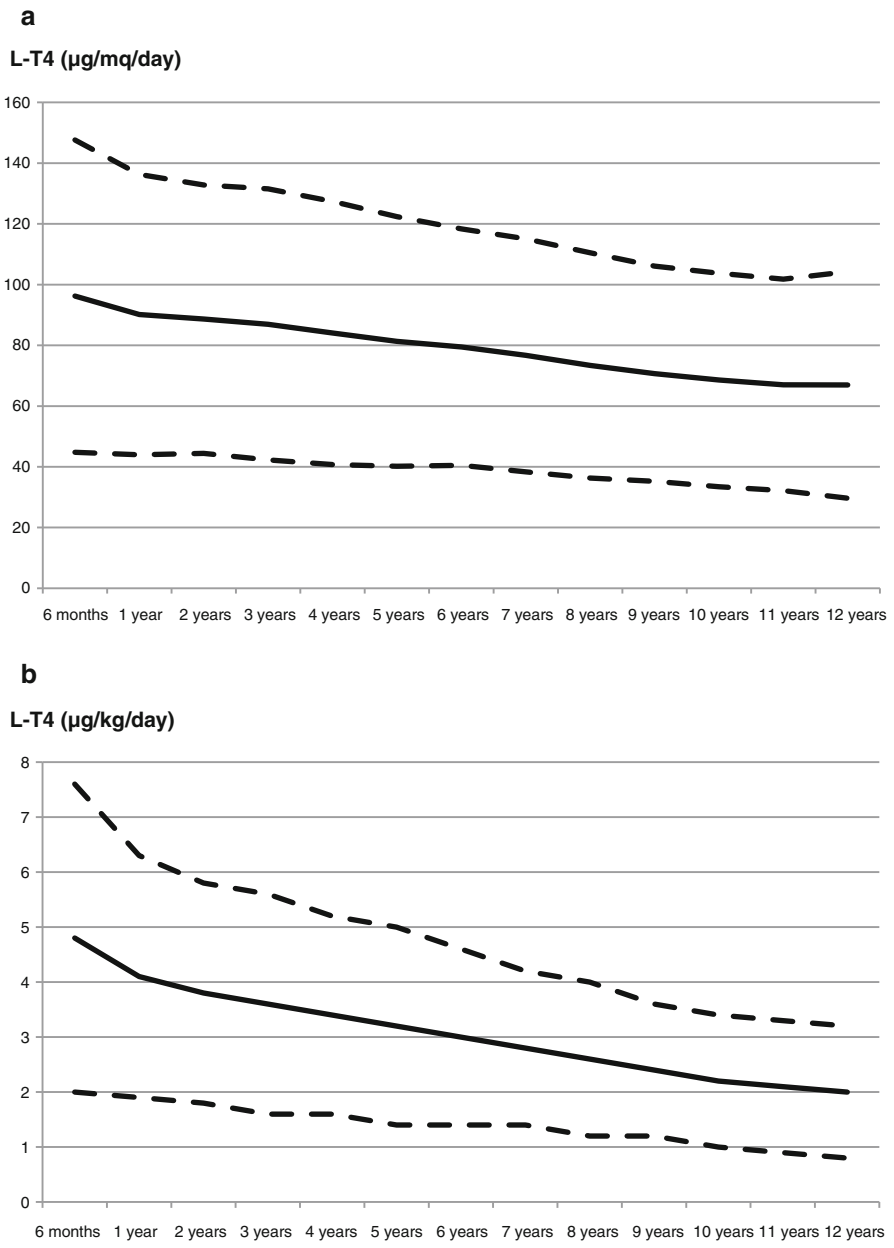

Fig. 11.1 The figure displays the mean (*full line*), the -2 SDS and +2 SDS (*dotted lines*) L-T4 requirement in 216 CH patients diagnosed with neonatal screening from 6 months to 12 years of age (personal data). On the basis of body surface area (**a**) and body weight (**b**)

Table 11.1 Summary of the suggestion during the follow up

Biochemical and auxological assessment	1. 2 and 4 weeks after the initiation of L-T4; 2. Every 1–2 months during the first 6 months; 3. Every 3–4 months between 6 months and 3 years; 4. Every 6–12 months until growth is completed.
L-T4 change because of abnormal values	Check hormones values after 4–6 weeks
Re-evaluation of the thyroid axis	After the age of 3 years in selected patients
Neurodevelopmental outcome	To be monitored at each visit; keep attention to patients with severe CH at diagnosis
Hearing test	Before school age
Visual development	If clinically indicated
Speech evaluation	By 3 years of age
Genetic counselling	Suggested in case of recurrence of CH and extra-thyroidal diseases (heart, palate, pituitary, kidney etc malformation).

common [9]. Serum T4/fT4 and TSH measurements should be performed as reported in the table. It is recommended to perform the thyroid function tests more frequently when the adherence to the treatment appears low and abnormal values occur. When the daily dose is changed, fT4 and TSH measurements should be repeated by 4–6 weeks.

The aim of therapy is to ensure normal growth and development by maintaining the serum total T4/fT4 concentration in the upper half and serum TSH in the lower half of the reference range.

In the first months of life, serum TSH may be above the normal values despite T4/fT4 concentrations in the reference range. Excessive dose of L-T4 may cause hyperthyroidism with adverse effects, such as tachycardia, shortening of sleeping and overall premature craniosynostosis.

In patients properly treated, growth, puberty and fertility are normal [10–12]. There are only minor differences in IQ score, school achievement and neuropsychological tests in patients with CH as compared to control groups of classmates and siblings [13–17]. These defects may be more evident in patients with severe CH at diagnosis. Other defects may be impaired visuospatial processing and selective memory and sensorimotor defects. It is still debated whether these minor differences are preventable by further optimizing postnatal therapy. The prognosis of developmental outcome is worsened in patients with delayed diagnosis and treatment.

It must be noted that the L-T4 replacement treatment used today is more aggressive in targeting early correction of TSH than the regimens used 20 or even 10 years ago. As a result, newborn infants with CH today may have an even better intellectual and neurological prognosis than those described in literature.

It is recommended to have optimal thyroid hormones for women with CH who are planning a pregnancy. In newly pregnant patients, we recommend an immediate increase in L-T4 dose by 25–30 % as soon as the pregnancy begins. Pregnant women with TSH out of range present a fourfold increased risk of miscarriage.

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Neurodevelopmental Outcomes in Children with Congenital Hypothyroidism

12

Mariacarolina Salerno, Lucia De Martino, and Manuela Cerbone

12.1 Introduction

Congenital hypothyroidism (CH) is the most common preventable cause of mental retardation in children. The introduction of neonatal screening programs and early levothyroxine (l-T4) replacement therapy have drastically changed the neurodevelopmental outcomes of CH children.

Hypothyroidism, including its association to mental retardation, was first mentioned by Paracelsus, a physician and alchemist of the sixteenth century [1]. In *Appletons' Popular Science Monthly*, William Jay Youmans described children affected by hypothyroidism as little cretin dwarfs: "Cretins are always short, and may never grow taller than a normal child of 2 or 3 years. They never attain a high degree of intelligence, and most commonly are idiots with only the power to comprehend the simplest things of daily life, and with a vocabulary limited to a few words" [2].

Thyroid hormone (TH) plays an essential role in brain development during fetal and postnatal life since it acts on neuronal migration and differentiation, myelination, and synaptogenesis [3]. Despite considerable progresses in recent years in deciphering TH signaling pathways, the molecular mechanisms by which TH exerts its cell-specific effects remains to be elucidated [3].

It is well known that TH deficiency in utero may affect visual attention and processing, memory, and motor function. Moreover, even in the postnatal period, hypothyroidism may result in cognitive impairment mainly in language, verbal, attention, and memory skills [4].

Intellectual disabilities were a common finding in CH children before the newborn screening era. Early cross-sectional and longitudinal studies showed an inverse

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relationship between the age at diagnosis of CH and the cognitive deficit [5]. Indeed, mean intelligence quotient (IQ) dropped from 89 in infants diagnosed by the age of 3 months to 54 when the diagnosis was delayed beyond 6 months of age [6].

The introduction of neonatal screening program for CH and early replacement therapy has significantly reduced the prevalence of intellectual disabilities in high-income countries from 8–28 % to 1 % or less, and the mean global IQ is now 10–30 points higher in these patients than in the prescreening era [7].

12.2 Neurocognitive Development

Although neonatal screening has dramatically improved the prognosis for children affected by CH, this does not necessarily imply that early and appropriate l-T4 replacement therapy results in the same health outcomes of unaffected children.

In a large cohort of 361 children from the UK national register of children with CH detected by neonatal screening, Tillotson et al. [8] reported a normal IQ at the age of 5 years. However, compared to controls, IQs were 10.3 points lower in patients with severe CH as defined by low serum T4 at diagnosis (T4 < 24.8 nmol/L) [8]. Similar results were reported by Rovet et al., who observed an IQ 8 points lower in children with CH with respect to their unaffected siblings at the age of 6 years [9]. Salerno et al. reported subnormal IQ in 13 out of 40 patients with CH aged 12 years as compared to their sibs, with lower IQ scores being associated with lower serum T4 concentrations at diagnosis and poor treatment compliance during follow-up [10]. In 49 young adults with CH aged 20 years, although within normal range, IQs were found to be 9 points lower than their siblings (total IQ 102.4 vs 111.4) [11]. Moreover, Kempers et al. reported a mean IQ 10 points lower (IQ 91.3 vs 101.3) in adult patients with severe CH (initial T4 < 30 nmol/L) compared with mildly affected patients (T4 ≥ 60 nmol/L) [12].

Because most children exhibiting subtle neurocognitive impairment started treatment with relatively low doses of l-T4, in the 1990s CH treatment guidelines were revised to recommend an increase of l-T4 starting dose from 6–8 to 10–15 mcg/kg per day in order to faster normalize circulating thyroid hormone and reduce the postnatal period of TH insufficiency [13, 14].

Indeed, high-dose l-T4 treatment (10–15 mcg/kg per day) resulted in a rapid normalization of thyroid hormone levels, within the first 2 weeks after therapy initiation, and in a better intellectual outcome [15–22].

In 44 CH children evaluated at a mean age of 8 years, high-dose l-T4 treatment followed by a rapid postnatal normalization of thyroid function resulted in IQ scores similar to their siblings. Moreover, the proportion of lower IQ (i.e., <85) was similar in both groups (27 % in CH children vs 26 % in their siblings) [22].

However, despite optimized treatment regimens improving neurodevelopmental outcomes, some children with CH are still reported as having subtle cognitive deficits. Indeed, Dimitropulos et al. evaluated intellectual outcome at a median age of 14 years in 63 children with CH treated early and with high l- T4 dose and reported an IQ within normal range but almost 10 points lower than controls, with patients

with athyreosis being more severely affected [23]. These deficits may reflect a prenatal brain injury due to thyroid hormone insufficiency in utero not completely reverted by postnatal treatment. Even though transplacental supply of maternal T4 may protect fetal brain from severe neurological impairment, it may not be sufficient to protect from severe fetal hypothyroidism [24].

In conclusion, neurodevelopmental outcome in CH patients is affected by several factors such as severity of CH at diagnosis, etiology, delayed bone maturation at diagnosis, delayed age at initiation of replacement l-T4 therapy, low initial l-T4 dose, later time of thyroid function normalization, poor compliance to treatment, and sociodemographic factors [10, 11, 18, 19, 25–31].

Although allowing better neurocognitive outcome, high-dose l-T4 therapy may lead to an increased risk of supraphysiological free T4 (fT4) levels, which have been associated with attention and behavioral problems in childhood [29]. Indeed, in the study by Alvarez et al., the number of episodes of overtreatment during the first 6 months was a stronger predictor of alertness deficit during the school age than the initial l-T4 dose and the etiology of CH [32].

Recently, Bongers-Sckokking et al. found that the episodes of overtreatment during the first 2 years of therapy were associated with a lower IQ at the age of 11 years in comparison to CH patients with no episodes of overtreatment [33].

Therefore, beyond l-T4 starting therapy, a further concern involves optimal testing frequency. The American Academy of Pediatrics recommends to assess thyroid function 2 and 4 weeks after the start of therapy, then 1–2 monthly till 6 months of age, 3–4 monthly from 6 months to 3 years, and every 6–12 months till the end of growth [34]. However, better outcomes may be achieved with more frequent tests in order to prevent iatrogenic hyperthyroidism [35, 36].

Moreover, different l-T4 initial doses and thyroid hormone monitoring based on etiology of CH have been suggested as a possible strategy to avoid prolonged period of supraphysiological FT4 levels. Indeed, children with athyreosis would require higher and more frequent monitoring [37]. Using a different initial dose strategy according to the etiology of CH, Mathai et al. [36] reported a rapid normalization of thyroid function without prolonged secondary hyperthyroidism. Thus, they recommend starting CH treatment with high but variable l-T4 doses based on etiology, in conjunction with close monitoring of thyroid function [36].

12.3 Sensorimotor Problems

Several studies on children and adults with CH detected by neonatal screening reported decreased motor skills with fine motor and balance function impairment with respect to controls, more pronounced in children with severe CH at diagnosis [38–40]. Indeed, children with athyreosis aged 7–14 years performed significantly worse in motor functioning than children with thyroid dysgenesis, despite early and high-dose l-T4 treatment [30]. However, data are still controversial; indeed, Albert et al. recently observed that early and high-dose treatment, followed by a rapid postnatal normalization of thyroid function, resulted in visual perception and

motor coordination similar between children with CH at a mean age of 9 years and their siblings [22].

12.4 Hearing Problems

TH plays a role in cochlear and auditory function development.

Mild hearing impairment and abnormal auditory brainstem-evoked potential tracings have been reported in children with CH [41, 42].

A recent questionnaire-based report from France has shown a higher prevalence of sensorineural hearing loss as well as conductive deficits in young adults with CH (particularly those with a severe form) with respect to the general population. Hearing loss in CH children is mostly bilateral, mild to moderate, and in some cases requires hearing aids [43–46].

Therefore, early and regular evaluation of hearing acuity through childhood should be considered in patients with CH to avoid substantial adverse effects on speech development, school performance, and social interactions that may occur in case of undiagnosed hearing impairment [47].

12.5 Behavior and Schooling Problems

Children with CH may display behavioral problems such as inattention, distractibility, hyperactivity, and restlessness [48–50]. Introversion and motor clumsiness have also been described, particularly in those with thyroid agenesis [51]. Even suboptimal initial treatment has been associated with attention problems and aggression [21]. Attention deficits were also associated with late normalization of TH after treatment initiation as well as with higher circulating fT4 levels at the time of testing [52, 53].

Even young adults with CH, aged 20 years, exhibited increased scores on anxiety and somatic complaint subscales [54]. They also experienced difficulties in arithmetic, and a greater percentage of them, particularly those who received lower l-T4 starting dose, did not finish high school [11].

Schooling aspects are strictly related to neurodevelopmental outcomes, and many authors focused on educational performances in children with CH with contrasting results [50, 54–56]. Generalized learning disorders have been reported in up to 20 % of CH children [57]. These children were particularly defective in symbol copy, geometric copy, phrase repetition, and spontaneous writing.

In young adults with CH born up to 1988, educational attainment was associated with CH severity and treatment adequacy [45]. Moreover, initially low l-T4 dosage (below vs ≥7 mcg/kg/day), absence of near normalization of thyroid hormone levels after 15 days of treatment, and poor compliance to the treatment throughout childhood were associated with an increased risk of school delay [58].

Primary school age seems to be the period of life when CH children are more likely to develop behavioral problems, thus at this age special attention should be paid to parental worries and anxiety [59].

12.6 Memory Problems

During the last decade, the mechanisms involved in memory processes have been extensively studied in children with CH. It is well known that the hippocampus, which is essential for learning and memory, starts to develop early in gestation and early maternal hypothyroidism affects offspring hippocampal development and memory. TH is a modulator of memory processes through mechanisms which are still not completely understood [60–62]. Animal models of gestational hypothyroidism have shown that the rodent hippocampus is particularly vulnerable to TH loss from early gestation to the end of infancy [4, 63, 64].

Children born from women diagnosed with hypothyroidism prior or during pregnancy exhibited significantly smaller right and left hippocampal volumes on magnetic resonance imaging (MRI) despite l-T4 treatment. Moreover, the hippocampal volumes were negatively correlated with maternal third-trimester TSH levels and positively correlated with third-trimester fT4 [65]. Even later in life, children and adolescents with CH exhibited poorer recall on memory tasks compared to controls (although in the average range for population norms) and an increased number of everyday memory problems correlated with hippocampal volumes. Moreover, on functional MRI, adolescents with CH showed increased magnitude of hippocampal activation which was also correlated with the severity of the hypothyroidism early in life [65, 66]. These findings suggest that perinatal deprivation of TH has long-standing effects on hippocampal function and may account for memory problems experienced by adolescents with CH. Thus, despite newborn screening and prompt treatment, early TH deficiency seems to have long-lasting effects on the hippocampus and its relationship to memory functioning [65, 66].

12.7 Emotional Aspects and Quality of Life

Besides the neurocognitive outcomes, other aspects of social and everyday life such as emotions, self-esteem, and quality of life (QoL) have been extensively assessed in children with CH. The results of these studies are difficult to interpret considering the multitude of factors influencing these psychological components even in healthy people. Moreover, the existence of a chronic disease *per se*, regardless of its severity, may represent a risk factor for psychological disorders such as anxiety, depression, affective problems, and aggressive behavior [67, 68].

Patients growing up with CH, diagnosed by neonatal screening, are more often at risk for health-related QoL impairment, lower self-esteem, and delayed developmental milestones on the domain of social development. In addition, the cognitive and motor problems of CH patients, when present, may also affect their social life, self-esteem, and emotional functioning [69]. Consequently, the focus during follow-up should shift to school performances, social-emotional functioning, and supporting the patients.

Studies on QoL and living conditions in adolescents and young adults with CH detected on newborn screening yielded contrasting results [45, 70].

Compared with their peers, fewer CH patients attained secondary or tertiary education, the highest socioeconomic category, or were in full-time employment, which seemed to be mainly dependent on a greater CH severity at diagnosis, a worse treatment adequacy, and the presence of associated chronic conditions. - However, from a public health standpoint, the subtle impairment reported seemed to have just a little impact since most patients were well integrated into society, in education, or had at least some employment [45].

How much of the emotional aspects and QoL of these patients depends on specific factors related to CH (i.e., severity of neonatal CH, etiology, timing and dose of treatment, monitoring of thyroid function, parental attitude, etc.) or on the fact of living with a chronic condition remains to be further defined. However, the improvement in the management of children with CH during the last years, with early, high-dose l-T4 treatment, and close monitoring of thyroid function will probably result in better outcomes in the majority of affected patients.

12.8 Recommendations

Psychomotor development and school progression should be periodically monitored in all children with CH, particularly in those with severe CH at diagnosis, athyreosis, and poor control during the first year. Hearing tests and screening for visual processing problems and speech delay are recommended prior to entering school. Adherence to treatment should also be regularly and carefully monitored [71].

Neuropsychological evaluation in CH children is useful from the time of diagnosis up to the age of 7 years in order to early identify subtle deficits or intellectual retardation, particularly in at-risk cases (athyreosis, very low FT4 and very high TSH concentrations at diagnosis, absent knee epiphyses at term, delayed normalization of TSH, poor control during the first year) or if developmental delay is suspected [72].

A neurocognitive evaluation by a specialized team can be scheduled as follows:

- Evaluation of intersubjectivity and interaction skills at 12, 18, and 24 months
- Evaluation of language and fine motor development at 36 months
- Evaluation of intellectual ability and requirements for reading and writing at 5 years
- Evaluation of intellectual ability, specific learning abilities, and assessment of attention at 7 years

Further neuropsychiatric evaluation may be required in subjects with detected abnormalities or with new cognitive or behavioral symptoms [72].

Conclusions

Although the neurocognitive outcome of CH children has significantly improved after the newborn screening program, there is nevertheless evidence for subtle

neurological and cognitive differences with unaffected subjects, despite early and appropriate treatment. Thus, neurodevelopmental aspects should be periodically monitored in all children with CH, and when identified, eventual deficits should be addressed appropriately.

Several factors have been documented to negatively affect neurodevelopmental outcome of children with CH. To which extent each of them impacts on the outcome and how this knowledge could help the clinician to further optimize the management of these children require additional studies.

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Iodine Deficiency from Pregnancy to Childhood

13

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13.1 Introduction

13.1.1 Iodine Sources and Metabolism

Iodine is a micronutrient essential for mammalian life because it is critical for the synthesis of thyroid hormones, thyroxine (T_4), and triiodothyronine (T_3), which contain in their molecules four and three atoms of iodine, respectively. Thyroid hormones (TH) are important for the growth and development of different tissues, especially the central nervous system and the skeleton, for the cardiac and gastrointestinal function, and for the regulation of the energy homeostasis throughout life. Disturbances in TH availability during early embryonic development, as in maternal iodine deficiency, cause severe neurological abnormalities in the newborns [1]. The World Health Organization (WHO) considers iodine deficiency to be "the single most important preventable cause of brain damage" worldwide [2]. Despite the great improvements in global iodine nutrition in the last century, it is currently estimated that iodine deficiency still affects 241 million school-aged children [3].

The only source of iodine is the diet. While most iodine is found in the oceans, its amount in potable water and vegetables and then in animal food is poor in many areas, where it was removed by the surface soil because of wide glaciations. Only foods of marine origin have high iodine content because marine plants and animals

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are able to concentrate iodine from seawater. Therefore, in the absence of iodine prophylaxis programs, iodine intake may be insufficient. More commonly affected by iodine deficiency are the extraurban populations, especially rural, that eat non-industrial local foods, poor in iodine. Indeed, the economic status more than the geographic location is the main determinant of the quality of the food and of its iodine content. In many countries, salt, bread, and milk are fortified with iodine, as an effort to eradicate iodine deficiency. Other sources of iodine are compounds used by industry and agriculture as well as supplements and disinfectants and medicines.

The iodine contained in the food is absorbed by about 90 % in the stomach and duodenum [4]. The absorbed iodine and that resulting from the peripheral metabolism of thyroid hormones and iodothyronines constitute the extrathyroidal pool of inorganic iodine, which is in equilibrium with the thyroid and the kidneys. The human body contains a small amount of iodine, 70–80 % of which is concentrated by the thyroid [5] through the sodium iodide symporter (NIS) that is located on the basolateral surface of thyrocytes [6]. On the apical surface of the thyrocytes, the thyroperoxidase (TPO) catalyzes the synthesis of monoiodotyrosine (MIT) and diiodotyrosine (DIT) and then the coupling of two DITs to produce T_4 or of a MIT and a DIT to produce T_3. A normal adult utilizes about 80 μg/day of iodine to produce thyroid hormones, 55 μg of which come from the diet and 25 μg from the peripheral metabolism of TH. Ninety percent of the plasma iodine is excreted by the kidney and only a small amount in the feces. When iodine intake is slightly insufficient (i.e., <100 mg/day), TSH induces a higher NIS expression with an increase of thyroid iodine uptake and preferential synthesis of T_3, thus allowing a normal content of intrathyroidal iodine. In chronic iodine deficiency, the thyroid content of iodine progressively decreases, the metabolic balance of iodine becomes negative, and goiter and hypothyroidism with its sequelae ensue.

The thyroid content in iodine of fetal thyroid changes with the gestational age: it is very low during the first stages of development, increases quickly after the 15th week of gestation, when the thyroid starts to actively concentrate the iodine, and reaches a plateau at the end of gestation. The total content in iodine of the thyroid of full-term newborns depends on the dietary iodine intake. Compared to adults, newborns and infants have less effective adaptative mechanisms to iodine deficiency.

13.1.2 Modification of Thyroid Function and Iodine Metabolism During Pregnancy

An adequate iodine intake is particularly important during pregnancy for the possible consequences of iodine deficiency (ID) both on the mother and fetus. Pregnancy is associated with relevant changes in thyroid physiology [7] (Fig. 13.1). During early gestation, serum thyroxine-binding globulin (TBG) increases markedly, under the influence of elevated estrogen concentration, and the clearance of plasma iodine increases as a consequence of the higher glomerular filtration. ID induces a relative hypothyroxinemia (low T4 levels) in pregnancy, which in turn stimulates TSH secretion, enhances thyroid

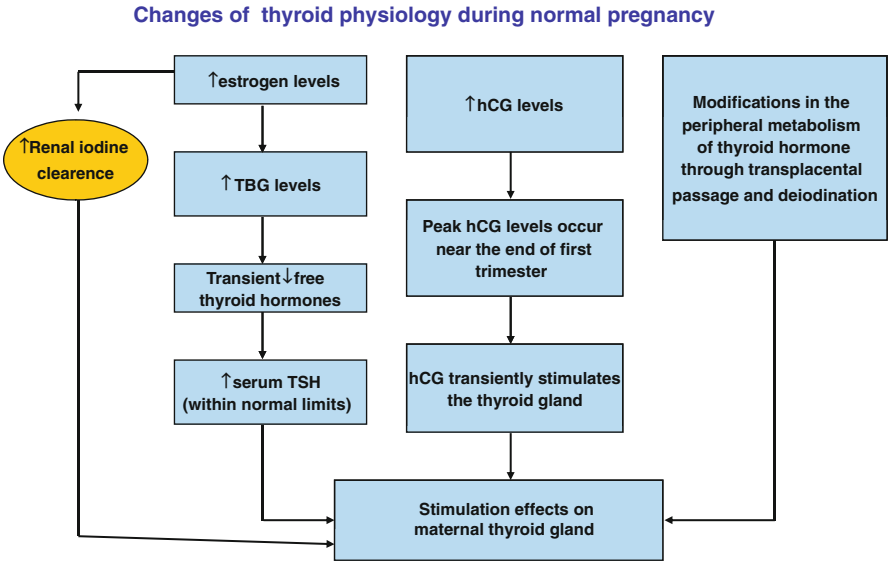

Fig. 13.1 The scheme shows the mechanisms which concur in the stimulation of maternal thyroid function during a normal pregnancy (Modified from Ref. [7])

stimulation, and increases thyroid volume in both the mother and the fetus. At the end of the first trimester, there is a transient thyroid stimulation by high levels of human chorionic gonadotropin (hCG) while during the second half of the gestation the placental type 3 iodothyronine deiodinase increases the metabolism of maternal T_4. As a consequence, the maternal thyroid gland is required to increase its hormonal production, through an increase of iodine uptake and a depletion of intrathyroidal stores. Later in gestation, the passage of iodine to the fetal-placental unit is another cause of deprivation of maternal iodine. For these reasons, as described in European areas with mild iodine deficiency, there is an increase of goiter formation during pregnancy that is only partially reversible postpartum. In addition, iodine deficiency correlates with a larger thyroid volume in newborns, and therefore goiter formation may start during the fetal thyroid development [7]. In presence of severe maternal iodine deficiency during pregnancy, there is a reduction in maternal thyroxine production, inadequate placental transfer of maternal thyroxine, and impairment of fetal neurological development.

13.1.3 Iodine Requirements

Although with some limitations, several studies have established the iodine requirement at different age and physiological conditions.

The WHO recommends 90 μg of iodine daily for infants and children up to 5 years, 120 μg for children 6–12 years, 150 μg daily for children ≥12 years and

Table 13.1 WHO recommendations for iodine intake by age or population group (Ref. [2])

Age or population group	Iodine intake (μg/die)
Children 0–5 years	90
Children 6–12 years	120
Children ≥12 years/ adults	150
Pregnancy	250
Lactation	250

adults, and 250 μg daily during pregnancy and lactation [2] (Table 13.1). The US Institute of Medicine–recommended minimum daily intake of iodine is similar: 90 μg daily for children 1–8 years old, 120 μg for children 9–13 years, 150 μg daily for older adolescents and nonpregnant adults, 220 μg for pregnant women, and 290 μg for lactating women [8].

The iodine requirements are higher in pregnant women because of the above-listed changes in thyroid physiology.

13.1.4 Assessment of Iodine Intake

The methods for the assessment of iodine nutrition in the populations are goiter prevalence, urinary iodine concentration (UIC), serum TSH in newborns, and serum thyroglobulin (Tg). The urinary iodine concentration indicates current iodine nutrition, while thyroid size and the serum thyroglobulin concentration reflect iodine nutrition over a period of months or years.

Goiter goiter can be measured by neck inspection and palpation or by thyroid ultrasonography. According to WHO/ICCIDD, grade 0 is a thyroid that is not palpable or visible, grade 1 is a goiter that is palpable but not visible when the neck is in the normal position, and grade 2 is a goiter that is clearly visible when the neck is in a normal position. Thyroid ultrasound is more sensitive and specific than palpation but requires valid references of thyroid volume data. Goiter surveys as indicators of iodine sufficiency are usually done in school-age children because they are easily recruitable and hopefully reflect the actual impact on humans of iodine deficiency, although enlarged thyroids in children who were iodine deficient during the first years of life may not regress completely after introduction of salt iodization [9]. The WHO has established total goiter rate in schoolchildren to define severity of iodine deficiency in populations: below 5.0 % indicates iodine sufficiency, 5.0–19.9 % mild deficiency, 20.0–29.9 % moderate deficiency, and above 30.0 % severe deficiency. In addition, a reduction of goiter rate by ultrasound indicates that iodine deficiency has disappeared, and therefore a frequency of goiter under 5 % in schoolchildren must be considered as a parameter of iodine sufficiency [10].

Urinary iodine concentration because >90 % of dietary iodine eventually appears in the urine, the UIC is an excellent indicator of recent iodine intake. It is measured in spot urine specimens from a representative sample of a population and expressed as the median, in μg/L. The median urinary iodine concentration in a population has been

Table 13.2 Epidemiological criteria from WHO for assessment of iodine nutrition in school-aged children, pregnant and lactating women, and infants based on median or range of UI (Ref. [2])

UI (μg/L)	Iodine intake	Iodine nutrition
School-aged children		
<20	Insufficient	Severe iodine deficiency
20–49	Insufficient	Moderate iodine deficiency
50–99	Insufficient	Mild iodine deficiency
100–199	Adequate	Optimum
>200	More than adequate	Risk of iodine induced hyperthyroidism
>300	Excessive	Risk of adverse health consequences
Pregnant women		
<150	Insufficient	
150–249	Adequate	
250–499	More than adequate	
≥500	Excessive	
Lactating women		
<100	Insufficient	
≥100	Adequate	
Children less than 2 years		
<100	Insufficient	
≥100	Adequate	

used to develop a system for classifying iodine deficiency and sufficiency (Table 13.2) [2]. The most usual survey group is school-age children, but their nutrition must reflect that of the community in order for the data to be meaningful. Mild iodine deficiency is defined as a median urinary iodine concentration of 50–99 μg/L, moderate deficiency as 20–49 μg/L, and severe deficiency as <20 μg/L [11]. Pregnant women require special attention because their renal threshold for iodine is lower, the needs of the fetus are greater, and dietary salt (including iodized salt) is often restricted [12, 13]. In pregnant women, urinary iodine concentrations of 150–249 μg/L are considered adequate [14].

TSH TSH concentration obtained during the neonatal screening of congenital hypothyroidism (CH) is useful to assess iodine nutrition, because the increase of fetal TSH is an adaptative mechanism to iodine deficiency. Compared with the adult, the newborn thyroid contains less iodine but has higher rates of iodine turnover. When iodine supply is low, maintaining high iodine turnover requires increased TSH stimulation. Therefore, iodine deficiency causes a shift toward higher TSH values in the neonatal screening of CH. A TSH value >5 mU/L in whole blood collected 3–4 days after birth that lasts for few weeks in more than 3 % of newborns indicates iodine deficiency in the population [2]. Studies suggest also that newborn TSH is a sensitive indicator of iodine nutrition during pregnancy along with determination of the median UIC [15].

Serum Tg Tg is the most abundant intrathyroidal protein. Serum Tg is higher in iodine-deficient than in iodine-sufficient areas as a consequence of the TSH

stimulation and the higher rate of goiter, and its concentrations fall quickly with the implementation of iodine prophylaxis. In iodine-deficient infants and children, serum Tg concentrations are high more often than are serum TSH concentrations. Although a nonspecific test, since any type of thyroid stimulation or injury can raise the serum Tg concentrations, the values correlate well with the severity of iodine deficiency. Tg has also been shown to be a sensitive measure of excess iodine intake in school-age children [16]. Moreover, in one study the serum Tg level was better than thyroid volume measurement by ultrasound as an indicator of iodine nutrition [17].

13.2 Consequences of Iodine Deficiency

The clinical consequences of inadequate iodine intake are collectively termed "the iodine deficiency disorders" (IDD) (Table 13.3) [18]. When severe iodine deficiency occurs during pregnancy, it is associated with cretinism and increased neonatal and infant mortality. Mild iodine deficiency is associated with thyroid enlargement and learning disabilities in children. All these consequences of iodine deficiency stem from the associated hypothyroidism.

Additional factors that can exacerbate the effects of iodine deficiency include coexistent deficiencies of iron, selenium, and vitamin A [19] and the ingestion of foods such as cassava or millet containing goitrogenic substances.

Goiter is the most frequent manifestation of iodine deficiency and can affect individuals of all ages. It represents a compensatory response to iodine deficiency. Low iodine intake leads to reduced T_4 and T_3 production, which results in increased TSH secretion in an attempt to restore iodothyronine production to normal. TSH also stimulates thyroid growth. The goiter is initially diffuse but eventually becomes nodular because the cells in some thyroid follicles proliferate more than others.

Table 13.3 Iodine deficiency disorders (IDD) by age group

Age groups	Consequences of iodine deficiency
All ages	Goiter
Fetus-neonate	Abortion
	Stillbirth
	Congenital anomalies
	Perinatal and infant mortality
	Endemic cretinism
	Overt or subclinical hypothyroidism
Infant-child/ adolescent	Impaired mental function
	Delayed physical development
Adult	Toxic nodular goiter
	Iodine induced hyperthyroidism
	Hypothyroidism
	Endemic mental retardation
	Decreased fertility rate

Therefore, in regions of iodine deficiency, children and adolescents generally have diffuse goiters, while adults who lived in conditions of long-standing iodine deficiency have nodular goiter.

This chapter will focus on the consequences of iodine deficiency from fetal life to childhood.

13.2.1 Consequences of Iodine Deficiency During Pregnancy and Infancy

13.2.1.1 Neurological Development

Goiter is the most common clinical manifestation of iodine deficiency, but another important consequence is a defective development of central nervous system because brain development depends on thyroid hormones during fetal and early postnatal life.

THs have no influence on very early stages of neurological development but regulate its later processes, which include neurogenesis, myelination, dentrite proliferation, and synapse formation [20, 21]. In particular, three stages of thyroid hormone–dependent neurological development can be recognized. The first occurs before the onset of fetal thyroid hormone synthesis, which occurs at 16–20 weeks post conception in humans. During this period, TH exposure comes only from maternal hormones [1, 22] and influences proliferation and migration of neurones in the cerebral cortex, hippocampus, and medial ganglionic eminence [23, 24]. The second stage occurs during the remainder of pregnancy after the onset of fetal thyroid function when the developing brain derives its supply of TH from both the fetus and the mother [1, 22]. During this period, thyroid hormone regulates neurogenesis, neuron migration, axonal outgrowth, dendritic branching, and synaptogenesis, together with the initiation of glial cell differentiation and migration and the onset of myelination [25]. The third stage occurs in the neonatal and postnatal period when thyroid hormone supplies to the brain entirely derive from the child and are critical for continuing maturation. During this period, while continuing gliogenesis and myelination, THs regulate migration of granule cells in the hippocampus and cerebellum, pyramidal cells in the cortex, and Purkinje cells in the cerebellum [25].

The frequency and severity of the neurological impairment are proportional to the magnitude of iodine deficiency. In areas of severe chronic iodine deficiency, maternal and fetal hypothyroxinemia can occur from early gestation onward.

13.2.1.2 Endemic Cretinism

The clinical manifestations caused by chronic severe iodine deficiency are referred to as endemic cretinism. In its classical description, endemic cretinism includes a neurological and a myxedematous form [26].

The neurological cretinism presents with severe mental retardation with squint, deaf-mutism, motor spasticity, and goiter. The mental deficiency is characterized by a marked impairment of abstract thought, whereas autonomic and vegetative functions and memory are relatively well preserved, except in the most severe cases. The

motor disorder is characterized by proximal rigidity of both lower and upper extremities and the trunk, whereas spastic involvement of the feet and hands is unusual, therefore most cretins can walk.

The myxedematous form has less severe mental retardation and more pronounced hypothyroid features, including severe growth retardation, incomplete maturation of the facial skeleton with naso-orbital configuration abnormalities and atrophy of mandibles, puffy features, dry and thickened skin, dry and rare hair, and delayed sexual maturation. In this form, goiter is usually absent, and the thyroid is usually atrophic.

In many instances, cretinism may present as a mixed form with features of both. Therefore, it can be often difficult to differentiate the two forms [27].

Studies suggest that selenium deficiency combined with severe iodine deficiency can more specifically induce forms of atrophic rather than goitrous hypothyroidism and therefore of myxedematous rather than neurological cretinism [28]. Selenium is normally present in high concentrations in the normal thyroid and is essential for the synthesis of selenoproteins such as glutathione peroxidase (GPX), which acts as antioxidant, and type I 5'-deiodinase, which metabolizes thyroid hormones. The mechanism would be the following: iodine deficiency causes thyroid hyperstimulation by TSH that leads to increased production of H_2O_2 within the thyroid follicular cells; selenium deficiency also results in accumulation of H_2O_2 due to GPX deficit. Excess of H_2O_2 can induce thyroid cell destruction and myxedematous cretinism. On the other hand, deficiency of the selenoenzyme 5′-deiodinase causes decreased catabolism of T_4–T_3 with increased availability of T_4 for the fetal brain and prevention of neurological deficits; T_4, in fact, crosses the brain–blood barrier more easily than T_3.

Cases of overt myxedematous, neurological, or mixed endemic cretinism are reported in areas of severe iodine deficiency such as Africa and Asia.

The only way to prevent neurological cretinism is by administration of iodine to women early in gestation or even before they become pregnant. In a randomized trial and several population-based studies of women living in severely iodine-deficient regions, iodine supplementation to women prior to conception or during early pregnancy was associated with substantially better neurological and developmental outcomes in children [29–31].

13.2.1.3 Subclinical Neurological Defects

Severe iodine deficiency has been almost eradicated through extensive iodine prophylaxis programs worldwide. As a consequence, new cases of cretinism have disappeared. However, several regions of mild to moderate iodine deficiency still exist [32].

Several reports have described cases of neurological deficits or minor neuropsychological impairments also in children born to mothers exposed to mild to moderate iodine deficiency during pregnancy. These defects may be detected by appropriate neuropsychological tests [33]. In Tuscany, neuropsychological performance was tested in 107 children living in a village characterized by mild iodine deficiency (UIC = 64 μg/L) by a block design subtest of the Wechsler Intelligence Scale for

Children and simple reaction times to visual stimuli. The results obtained in these children were compared with those obtained in children born and living in an iodine-sufficient area. The block design test was not different between the two groups of children, while reaction times were significantly delayed in children living in the iodine-deficient village. These data indicate that mild iodine deficiency may impair the rate of motor response to perceptive stimuli even in the absence of general cognitive defects. Mild to moderate iodine deficiency was also shown to be associated with minor neurological deficits in Sicily [34].

However, randomized trials of iodine supplementation to pregnant women with mild to moderate iodine deficiency have reported mixed results in terms of improvements of thyroid function parameters, which may be considered as surrogate markers of future infant development [35]. In some but not in all trials [36, 37], iodine supplementation resulted in smaller thyroid volumes and lower Tg concentrations in mothers and/or newborns compared with controls. Indeed, there was no effect on maternal or neonatal T_4 concentrations in the majority of the trials. Moreover, no final conclusion can be drawn from these studies because child cognitive outcomes were not measured.

An increased auditory threshold may be another clinical manifestation of iodine deficiency. As an example, in a study of 150 school-age children in Spain, 38 % had a goiter [38]. In this subset, there was an inverse relationship between auditory threshold and urinary iodine excretion (i.e., the more iodine deficient, the higher the auditory threshold).

The potential adverse effects of mild to moderate iodine deficiency during pregnancy on cognitive and/or neurological function of the offsprings are still uncertain.

Two prospective case–control studies have reported even mild thyroid dysfunction during pregnancy may impair cognitive development of the offspring [39, 40]. Children exposed to maternal hypothyroxinemia presented reduced IQ scores, subtle deficits in cognition, memory, visuospatial ability [39], and delayed mental and motor function [40]. Animal models confirmed that maternal hypothyroxinemia induced in a critical period of active neurogenesis resulted in alteration in cell migration and cytoarchitecture of the cortex and hyppocampus in the 40-day-old progeny [23, 41]. The limitation of these previous studies is that they were conducted in iodine-sufficient areas.

In two following observational studies of women with mild to moderate iodine deficiency and mild hypothyroxinemia, neurodevelopmental outcomes were better in children whose mothers received iodine supplementation (200–300 μg potassium iodide daily) early in pregnancy (prior to the 10th week of gestation) compared with children whose mothers did not [42, 43]. The better outcomes noted in these studies may be related to improvement in maternal hypothyroxinemia. Both mild and severe maternal hypothyroxinemia have reportedly been associated with a higher risk of expressive language delay in newborns [44]. Severe maternal hypothyroxinemia also predicted a higher risk of nonverbal cognitive delay. It is possible that iodine supplementation in women with iodine deficiency severe enough to cause maternal hypothyroxinemia may improve neurodevelopmental outcomes, but this has not been assessed in randomized trials.

13.2.1.4 Birth Weight and Infant Growth

There is evidence that correction of iodine deficiency during pregnancy in severely iodine-deficient areas determines improvements of head circumference and birth weight of offspring.

In an area of western China, iodine repletion of pregnant women reduced the prevalence of microcephaly from 27 to 11 % [29]. In studies conducted in Algeria and Zaire, treatment of women with oral iodized oil just before conception or early in pregnancy resulted in respectively 6.25 % and 3.7 % higher birth weight compared with offspring of untreated mothers [45, 46]. (For the relationship between iodine deficiency and somatic growth, see Sect. 13.2.2.2.)

13.2.1.5 Neonatal and Infant Mortality

Severe iodine deficiency increases neonatal and infant mortality, an effect that can be reduced by up to 50 % with correction of severe iodine deficiency [47]. The mechanism of this benefit is not known, but multiple factors are probably involved. Hypothyroid or retarded infants may suffer more birth trauma and be more prone to infectious diseases and nutritional deficiencies typical of the poor rural communities in which iodine deficiency is so prominent.

13.2.2 Consequences of Iodine Deficiency in Childhood

13.2.2.1 Intellectual Disability

Iodine deficiency appears also to have adverse effects on growth and development in the postnatal period. Children and adolescents in regions of iodine deficiency are at risk for some degree of intellectual disability and fine motor skill abnormalities compared to children in iodine-sufficient areas.

A meta-analysis of 21 observational and experimental studies relating iodine deficiency to cognitive development suggested that iodine deficiency alone caused an average loss of 13.5 IQ points in affected subjects [48]. This evidence is suggestive, although the developmental studies in iodine-deficient regions have many limitations; among these are the inability to distinguish between the persistent effects of fetal iodine deficiency and the ongoing effects of iodine deficiency in childhood and adolescence and the presence of other environmental factors which may affect child development (i.e., socioeconomic status, accessibility and quality of education and health).

Even in developed countries, marginal iodine sufficiency may lead to intellectual compromise [49]. In Jaen province, in Spain, schoolchildren with UI below 100 μg/L had lower IQ scores: 96.4 versus 99.0 in schoolchildren with UI greater than 100 μg/L [50]. In Australia, children born to mothers with urinary iodine concentrations during pregnancy of less than 150 μg/L compared with ≥150 μg/L had reductions in spelling, grammar, and English-literacy standardized test scores at age 9 years [51]. The children grew up in a region considered to be iodine replete (median UI 108 μg/L), and therefore, the results reflect the effects of fetal rather than childhood iodine insufficiency.

Intellectual disability resulting from the effects of iodine deficiency on the central nervous system during fetal development is not reversible. In contrast, the additional impairment caused by continuing postnatal hypothyroidism and/or iodine deficiency may improve with appropriate thyroid hormone replacement and/or iodine supplementation [52]. In a double-blind intervention trial of iodine supplementation or placebo in 310 10- to 12-year-old children in Albania, iodine supplementation with 400 mg of iodine as oral iodized oil significantly improved thyroid function (prevalence of hypothyroxinemia was reduced from approximately 30 to <1 %) and performance on cognitive testing evaluating information processing, fine motor skills, and visual problem solving [53].

13.2.2.2 Somatic Growth

It is known that severe iodine deficiency in womb causes cretinism and dwarfism, while iodized oil given during pregnancy in areas of moderate iodine deficiency increases birth weight by 100–200 g [45]. More controversial is the relationship between iodine deficiency and postnatal growth. However, most of the cross-sectional studies on iodine intake and child growth showed modest positive correlation [35].

Iodine status may influence growth through its effects on thyroid function. Thyroid hormone promotes growth hormone (GH) secretion and modulates the effects of GH at receptor level [54, 55]. Insulin-like growth factor (IGF)-1 and IGF-binding protein (IGFBP)-3 are also dependent on thyroid status. Indeed, hypothyroidism decreases circulating IGF-I and IGFBP-3 levels, whereas thyroid hormone replacement increases them [56, 57]. A controlled study including 10- to 14-year-old children from Morocco, Albania, and South Africa who were given iodine as iodized salt or oil and placebo showed that the increase in median UI to >100 μg/L was associated to an increase in IGF-1 and IGFBP-3 concentrations and an improvement in somatic growth [58].

13.2.2.3 Subclinical Hypothyroidism

Compared with the adult, the child thyroid contains less iodine but has higher rates of iodine turnover. Therefore, chronic iodine deficiency causes in children more than in adults an increase in TSH concentrations and a thyroid hormone pattern consistent with subclinical hypothyroidism (SH). Because SH is associated with cardiovascular disease risk factors [59], such as abnormalities in the lipid profile, correction of iodine deficiency may be beneficial also in reducing these risks. This effect of iodine supplementation has been reported in a recent controlled study, in which treatment of moderately iodine-deficient children affected by SH due to iodine deficiency improved their lipide profile and reduced insulin levels compared with controls [60]. However, more studies are needed to confirm the findings.

13.3 Prophylaxis and Treatment of Iodine Deficiency

An optimal correction of iodine deficiency should be carried out at the level of the community rather than the individual.

13.3.1 Salt Fortification with Iodine and Other Options

Iodization of salt is the preferred method of increasing iodine intake in a community, because salt is consumed by everyone, it is technically easy to produce, does not change salt taste, and the cost is relatively low.

However, the iodization of all salt for human and livestock consumption (Universal Salt Iodization – USI) is not commonly achieved. The usual dose for salt fortification is between 20 and 40 mg of iodine/kg of salt (sodium chloride) as potassium iodide or iodate. The optimal amount to be added for a particular country or region can be calculated from the daily per capita salt consumption, the amount of iodine consumed from other sources, and any losses of iodine between production and consumption.

Alternatives are needed when salt iodization is impractical or delayed. In these cases, effective options are iodized oil (Lipiodol), iodized water, and iodine tablets or drops. Lipiodol, developed as a radiographic contrast agent, contains 480 mg iodine/mL. A single oral dose of 0.5–1.0 mL provides an adequate amount of iodine for 6 months to 1 year; intramuscular administration of the same dose provides an adequate amount for 2–3 years [61]. Iodized oil is more expensive than salt iodization and requires direct administration to each subject. If given intramuscularly, it requires skilled administration and has a risk of infection if improper technique is used. Its main advantage is that it can be implemented promptly. It has been especially valuable for women of childbearing age and children in regions of severe iodine deficiency.

Water is another occasional iodization vehicle because it is a daily necessity like salt.

Other methods of iodide administration include oral administration of potassium iodide solution every 2–4 weeks and daily administration of tablets containing from 100 to 300 μg potassium iodide. The latter is particularly recommended to meet the increased needs for iodine during pregnancy and lactation, and it can be routinely incorporated into prenatal vitamin/mineral preparations. In addition, alternative methods of food iodine enrichment are currently under study. Hydroponic experiments were carried out to investigate the possibility of enriching the iodine uptake by spinach [62] or other vegetables such as tomatoes and potatoes. A recent study [63] tested the efficiency of vegetables (potatoes, cherry tomatoes, carrots, and green salad) fortified with iodine in a group of 50 adult healthy volunteers. A daily intake of 100 g of vegetables containing 45 μg of iodine (30 % of the Recommended Daily Allowance), increased after 2 weeks the UIC by about 20 %, showing that the biofortification of vegetables with iodine can determine a mild but significative increase in UI concentration and, along with the habitual use of iodized salt, may contribute to improve the iodine nutritional status of the population without risks of iodine excess.

13.3.2 Iodine Needs During Pregnancy and Lactation

In regions where <90 % of households use iodized salt and the median UIC in children is <100 μg/L, the WHO recommends iodine supplementation in pregnancy and

lactation (250 μg daily) [14]. In pregnant women, urinary iodine concentrations of 150–249 μg/L indicate adequate iodine intake.

In the USA, women who do not consume dairy products or iodized salt may have lower urinary iodine concentrations [64]. The American Thyroid Association recommends that women from the USA receive a supplement of 150 μg of iodine (in the form of potassium iodide) daily during pregnancy and lactation, which is the dose included in the majority of prenatal vitamins marketed in the USA [13]. The Institute of Medicine recommended minimum daily intake is somewhat higher: 220 μg for pregnant women and 290 μg for lactating women .

13.3.3 Adverse Effects

Iodine repletion in the doses used for iodization of salt and in prenatal supplements has few adverse effects. Iodine administration may result in hyperthyroidism in patients with endemic goiter or in patients with nodular goiters containing autonomously functioning tissue. In contrast, iodine administration may induce or exacerbate hypothyroidism in patients with underlying autoimmune thyroiditis. In regions of iodine deficiency, both hyperthyroidism and hypothyroidism have been reported after the introduction of iodine [65, 66].

Excessive iodine ingestion during pregnancy may have adverse effects on fetal thyroid function. Indeed, a sudden exposure to excess serum iodine inhibits organification of iodine through the Wolff-Chaikoff effect [67]. The fetal thyroid gland, which has not full functioning mechanisms of escape from the Wolff-Chaikoff effect, is particularly susceptible to the inhibitory effects of excess iodine, and this can result in a prolonged inhibition of thyroid hormone synthesis, an increase in TSH, and fetal goiter.

The tolerable upper intake amount for iodine, as established by European and US expert committees, ranges from 600 to 1100 μg daily for pregnant women >19 years of age [68]. For adolescents 15–17 years, it ranges from 500 to 900 μg daily and for younger children 200–450 μg/day.

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Thyroid Enlargement from Newborn to Adolescent

14

Alessandra Cassio, Milva O. Bal, Ilaria Bettocchi, and Federico Baronio

14.1 Introduction

A *thyroid enlargement*, i.e., a *goiter*, may be due to many causes both congenital and acquired and could be detected at any age during childhood and adolescence. Goiters can be diffuse or nodular and associated with normal or altered thyroid function. The thyroid enlargement may be caused by increased *TSH* secretion acting as growth factor, by *TSH receptor stimulating antibodies* or by a TSH-independent process, such as inflammation or neoplastic or infiltrative disease. The relative frequency of the various forms varies according to the age [1]. Table 14.1 shows goiter's classification according to congenital or acquired causes.

14.2 Thyroid Volume (TV) in Childhood and Adolescence: Normative Data

The availability of normative data is essential for goiter diagnosis, in particular in epidemiological studies to establish the goiter prevalence in school-age children as an indicator of iodine intake in a country [2]. Today, the measurement of TV by ultrasound is a validated technique used for grading goiter. Nevertheless also with this method, it is hard to establish globally valid reference intervals for TV due to differences in genetic and environmental factors. In 2004, the WHO and ICCIDD proposed new international reference values for TV by ultrasound in 6–12-year-old children living in six areas of long-term iodine sufficiency on five continents [2]. The median TV increased with age from 1.6 to 3.3 ml, but the differences found between regions suggested that population-specific references in different countries

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Table 14.1 Causes of goiter

Congenital goiters
Dyshormonogenesis
Iodine trapping defect (NIS-gene mutation)
Iodine organification defects (TPO, DUOX.DUOX2 gene muattions)
Pendred syndrome
Thyroglobulin biosynthesis defects
Iodotyrosine deiodinase defects (DEHAL1-gene mutation)
Activating mutations of the TSH-receptor
Activating mutations of the G-protein α subunit (McCune-Albright syndrome)
Thyroid hemiagenesis
Thyroglossal duct cysts
Acquired goiters
Neonatal goiters (maternal/environmental factors)
Transplacentar passage of TSH-receptor activating antibodies
Transplacentar passage of TSH-receptor blocking antibodies
Transplacentar passage of antithyroid drugs/other goitrogens
Severe iodine deficiency
Goiters in children and adolescents
Cronic autoimmune thyroiditis
Colloid goiter
Iodine deficiency goiter
Goitrogens
Graves' disease
Infectious (Subacute thyroiditis and acute suppurative thyroiditis)
Nodular goiter
Cysts, adenomas or carcinomas (solitary nodule or multinodular goiter)

may be more accurate. TV increases significantly with other anthropometric variables, in particular body surface area (BSA). In an extensive study on 859 prepubertal Danish children from an iodine-sufficient area, the GH/IGF1 axis was found positively correlated with thyroid size, suggesting a role in the regulation of thyroid growth [3]. In a cross-sectional survey of 937 Dutch schoolchildren aged 6–18 years, TV increased with age, but a steep increase has been observed at different ages in girls and boys coinciding with pubertal peak of height velocity [4]. In newborns, it is even more difficult to establish normative data, due to both greater technical difficulties and possible influence of maternal factors (iodine intake, smoking in pregnancy). The few studies in the literature are conflicting, with mean TV value varying from the Belgian value of 0.84 to the Scottish value of 1.6 ml [5].

14.3 Congenital Goiters

The causes of congenital goiters are sometimes hereditary, and usually only the most severe forms may be evident at birth. Different disorders lead to congenital goiters (Table 14.1).

14.3.1 Dyshormonogenesis

These genetic defects in each step of synthesis of thyroid hormones (TH) are inherited as autosomal recessive traits. Clinical manifestations comprise a variable degree of congenital hypothyroidism (CH), with increased secretion of TSH and goiter. Occasionally, these disorders can be identified prenatally, when a fetus with goitrous nonimmune hypothyroidism is diagnosed in a euthyroid mother. Intra-amniotic injections of L-thyroxine have been reported to decrease the size of the fetal thyroid gland. However, experience with this procedure is limited, and the risk of provoking premature labor or infections should be evaluated with care by multidisciplinary specialist teams [1, 6–8]

Actually, these defects may be detected by newborn screening and include

- *Iodide transport defects* (ITD), caused by impaired Na(+)/I(−) symporter (NIS)-mediated active iodide accumulation into thyroid follicular cells. Low to absent radioiodide uptake at scintigraphy represents the diagnostic tool. Hereditary molecular defects in NIS have been shown to cause ITD [9].
- *Iodide organification defects* (IOD) due to thyroid peroxidase (TPO), dual oxidase 2 (DUOX2), and the DUOX maturation factor 2 (DUOXA2) gene inactivating mutations. TPO is a heme-binding protein localized on the apical membrane of the thyrocyte, and TPO enzymatic activity is essential for thyroid hormonogenesis. Currently, 61 properly annotated mutations in the TPO gene have been reported, of which the majority are missense mutations [10]. Hydrogen peroxide (H2O2) is the substrate used by TPO for oxidation and incorporation of iodine into thyroglobulin (Tg), a process known as organification. The main enzymes composing the H2O2-generating system are DUOX2 and DUOXA2. Affected patients show a positive perchlorate discharge test and high phenotypic variability, ranging from transient to permanent forms of CH. Up to now, only two cases of CH due to DUOXA2 defects have been published. The phenotypic expression is probably influenced by genetic background and environmental factors. DUOX and DUOXA constitute a redundant system in which DUOX1/DUOXA1 can at least partially replace the function of DUOX2/DUOXA2. Furthermore, increased nutritional iodide could ensure a better use of H2O2 provided by DUOX1 [11]. To identify IOD, a ^{123}I scintiscan with KClO4 discharge test should be performed. A ^{123}I discharge >90 % of the basal uptake measured 2 h after ^{123}I administration is typical of total IOD, while discharge ranging 10–90 % indicates partial IOD [12].
- *Pendred Syndrome* characterized by sensorineural deafness, goiter, and a partial defect in iodide organification. The degree of goiter and hypothyroidism varies and appears to depend on nutritional iodide intake. Pendred syndrome is caused

by biallelic mutations in the SLC26A4 gene, which encodes pendrin, a multifunctional anion exchanger. Pendrin is mainly expressed in the thyroid, the inner ear, and the kidney. In the thyroid, pendrin localizes to the apical membrane of thyrocytes, where it may be involved in mediating iodide efflux. Loss-of-function mutations in the SLC26A4 gene are associated with a partial iodide organification defect, presumably because of a reduced iodide efflux into the follicular lumen. In the kidney, pendrin functions as a chloride/bicarbonate exchanger. In the inner ear, pendrin is important in the maintenance of a normal anion transport and the endocochlear potential [13].

- *Thyroglobulin* (*Tg*) *biosynthesis defects*. Impaired Tg synthesis is one of the putative causes for dyshormonogenesis of the thyroid gland. This type of hypothyroidism is characterized by intact iodide trapping, normal organification of iodide, and usually low or undetectable Tg levels in relation to high TSH [14]. When untreated, the patients develop goiter.
- *Iodotyrosine deiodinase defects* (*ITDD*) result from mutations in the DEHAL1 gene that encodes for the thyroidal enzyme that deiodinates mono- and diiodotyrosines (MIT, DIT). In fact, MIT and DIT are by-products formed in excess during TH synthesis, and this enzymatic activity represents an efficient system for iodide recycling within the thyroid gland [15]. ITDDs are characterized by hypothyroidism, compressive goiter, and variable mental retardation, whose diagnostic hallmark is the elevation of iodotyrosines in serum and urine. However, the specific diagnosis of this type of hypothyroidism is not routinely performed, due to technical and practical difficulties in iodotyrosine determinations [16].

14.3.2 Activating Mutations of the TSH Receptor

Germline mutations of the TSH receptor gene that result in constitutive activation of the receptor are a rare cause of diffuse goiter and not autoimmune hyperthyroidism, which may be present at birth or become evident years or even decades later. These mutations are inherited as autosomal dominant traits; as a result, there may be a family history of hyperthyroidism and goiter [17].

14.3.3 Activating Mutations of the G-Protein α Subunit

These somatic mutations are present in the thyroid gland in infants and children with the McCune-Albright syndrome and result in thyroid hyperplasia or formation of nodules and, ultimately, in toxic nodular goiters [18]. Other features of the syndrome, such as cafè au lait skin pigmentation, precocious puberty, and polyostotic fibrous dysplasia, are usually present and provide clues to the underlying diagnosis. The hyperthyroidism is permanent, and in some cases thyroid ablation could be needed.

14.3.4 Thyroid Hemiagenesis

Thyroid hemiagenesis may cause unilateral goiter in neonates or, more often, in children because of compensatory hypertrophy of the contralateral lobe. Pathology that can be associated in the remnant thyroid lobe includes adenocarcinoma, adenoma, multinodular goiter, and chronic thyroiditis [19].

14.3.5 Thyroglossal Duct Cyst

The thyroglossal duct cysts form along the pathway of the gland in fetal life from the base of the tongue to the neck. Normally, the duct is obliterated at birth, but cysts can form within it. Most are located in the midline between the hyoid bone and the isthmus of the thyroid. Generally, they are diagnosed later in childhood and should undergo surgical resection [20].

14.4 Acquired Goiters

14.4.1 Neonatal Goiters (Maternal/Environmental Factors)

- *Transplacental passage of maternal antibodies/goitrogens*. Women with autoimmune thyroid diseases may produce antibodies that cross the placenta, resulting in fetal and neonatal goiter and thyroid dysfunction. Antithyroid drugs (ATDs) (propylthiouracil, methimazole, or carbimazole) for the treatment of maternal Graves' disease or other iodine-containing drugs (expectorans, amiodarone, nutritional supplements, skin disinfectants) all cross the placenta and can cause fetal hypothroidism and goiter. Transplacental passage of TSH receptor blocking antibodies is rarely accompanied by goiter (though typically the gland is normal size or small). In maternal Graves' disease, transplacental passage of TSH receptor stimulating antibodies (TRAb) that mimic the action of TSH can cause fetal and neonatal thyrotoxicosis and goiter [1]. Although transplacental passage of maternal TRAb does occur early in gestation, the fetal concentration is low until the end of the second trimester when placental permeability increases. Therefore, measurement of maternal TRAb concentration during 24–28 weeks of pregnancy is recommended. If the value is over three times normal, close follow-up for fetal and neonatal thyrotoxicosis is needed [21, 22]. Even women who are euthyroid due to ATD or hypothyroid due to thyroidectomy or radioiodine ablation can have persistent high levels of TRAb which can cause fetal or neonatal thyrotoxicosis. The clinical features of fetal hyperthyroidism are tachycardia (>160 beats/min), intrauterine growth retardation, advanced bone maturation, and goiter. Fetal goiter can also be present in fetal hypothyroidism due to transplacental passage of ATD given to the mother, and this iatrogenic fetal goiter usually regresses on reduction of doses of *ATD* [21]. Today, serial fetal ultrasonographic monitoring carried out by a highly experienced operator can be an important tool [6, 23].

Symptoms of neonatal thyrotoxicosis can be apparent at birth or may be delayed due to the effect of transplacental passage of maternal ATD or effect of coexisting blocking antibodies, but they are apparent by 10–15 days of life [24]. The clinical manifestations of neonatal hyperthyroidism are related to the involvement of central nervous system (irritability, restlessness), cardiovascular system (tachycardia, cardiac failure, systemic and pulmonary hypertension), and eye (periorbital edema, lid retraction, exophthalmos). Signs of hypermetabolism include hyperphagia with poor weight gain, diarrhea, and sweating. Other signs are hepatosplenomegaly, acrocyanosis, and thrombocytopenia. Diffuse goiter, usually small but occasionally large enough to cause tracheal compression, is present in most infants. Neonatal thyrotoxicosis patients require emergency treatment. The goal of the treatment is to normalize thyroid functions as quickly as possible by ATD administration, to avoid iatrogenic hypothyroidism while providing management and supportive therapy for the infant's specific signs. The mortality is 12–20 % due to heart failure [6]. *Neonatal thyrotoxicosis* usually resolves spontaneously between 3 and 12 weeks of life, until the maternal antibodies have disappeared, although it can persist for 6 months or even longer.

- *Severe iodine deficiency* (*ID*). Various studies have shown that during pregnancy not only severe but also moderate ID may cause significant maternal-fetal complications. Normal levels of TH are essential for neuronal migration and myelination of the fetal brain. For the first 12 weeks of gestation, the fetus is completely dependent upon maternal thyroxine. Subsequently, the fetal thyroid becomes able to concentrate iodine and synthesize iodothyronines. However, little hormone synthesis occurs until the 18th–20th week. As ID affects both maternal and fetal thyroid, the risk of goiter development and hypothyroidism is increased in both the mother and fetus. Cretinism represents the most severe form of the broad spectrum of developmental changes caused by maternal ID, with various grades of intellectual impairment depending on ID severity [22, 25].

14.4.2 Goiters in Children and Adolescents

- *Chronic autoimmune* (*Hashimoto's*) *thyroiditis* (*HT*). A firm, bumpy, nontender goiter discovered incidentally during a routine examination is the most common clinical presentation of HT in children [26]. This condition is uncommon in children younger than 4 years of age. The peak age of onset is in early to mid puberty (1–2 % of schoolchildren aged 11–18 years, female-to-male ratio 2:1). It is well known that the risk of HT is higher in individuals with chromosomal abnormalities (Klinefelter, Turner, Down syndromes) and other autoimmune diseases. HT may be generally associated with euthyroid state but also with hypothyroidism (3–13 % of cases) that may be subclinical (up to 35 % of cases) and rarely with hyperthyroidism (Hashitoxicosis). This last condition is caused by autoimmune damage to follicular cells, resulting in the release of preformed TH into circulation. It can present in a fashion similar to Graves' disease, but it

is generally a self-limiting condition and lacks opthalmopathy [27]. In almost all children (85–90 % of cases), high serum concentrations of antithyroid peroxidase (TPO) antibodies and antithyroglobulin (TG) antibodies are detected at first evaluation, while TSH receptor blocking antibodies have been reported in 9.2 % of the cases [28].

In a recent systematic review, Monzani et al. [29] concluded that HT in children may show a more benign evolution than in adults. *Subclinical hypothyroidism* is a remitting process with a low risk of evolution toward overt hypothyroidism (0–28.8 % of cases). The initial presence of goiter and elevated TG antibodies and a progressive increase in TPO antibodies and TSH value seem to predict this evolution [29, 30].

A complete evaluation of goiter includes ultrasonography of the thyroid that would reveal during follow-up scattered hypoechogenicity and eventually nodules. In HT, prevalence of thyroid nodularity has been reported by Corrias et al. [31]. As high as 35 % of cases in a cohort of 365 patients affected by juvenile HT and papillary carcinoma was found in 3 % of patients with thyroid nodes by fine-needle aspiration biopsy. Treatment with L-thyroxine could reduce thyroid size both in children with or without hypothyroidism, as shown by Svensson et al. [32] in a series of 90 children with goiter and HT.

Taking into account the low rate of progression to an overt hypothyroidism, Monzani et al. [29] suggest that treatment of SH should be indicated in the presence of clinical signs or symptoms of impaired thyroid function or goiter or TSH >10 mU/L.

- *Colloid* (*simple*) *goiter*. Colloid goiter is a thyroid diffuse enlargement due to unknown causes, usually occurring in adolescent girls. It is not correlated to thyroidal hormonal dysfunction and may show recurrence in some family pedigrees (autosomal dominant inheritance). In a study on female twins, the cumulative concordance rates for goiter in monozygotic and dizygotic twins were 53 % and 20 %, respectively [33].

  Histological examination reveals flattened epithelium, variable follicular size, and dense colloid. The natural history of this rare condition is generally benign with extremely slow (many years) spontaneous regression that is not influenced by L-thyroxine treatment.
- *Iodine deficiency goiter*. The incidence of endemic goiter due to iodine deficiency has been dramatically reduced in the last 50 years due to iodine supplementation (mainly through the routine utilization of iodized salt) in the western world; however, it has been estimated that about 2 billion people worldwide are at risk for endemic goiter [25]. Urinary iodine excretion less than 50 mcg/L is associated to higher risk of goiter. In children, endemic goiter can be associated with subclinical or mild hypothyroidism, since T3 concentrations may be normal or even high. Association with vitamin A deficiency may increase the risk of thyroid goiter in children with severe iodine deficiency through an increased TSH stimulation, which in turn reduces the risk of hypothyroidism [2].
- *Goitrogens*. The principal goitrogenic substance is iodine. Excessive ingestion of iodine, (i.e., from iodine-containing expectorants) may cause thyroid goiter,

Table 14.2 Frequency of presenting symptoms of Graves disease in pediatric patients followed in our clinic (2000–2012)

Symptoms	Percentage
Goiter	92 %
School performanc and behavioural problems	81 %
Tachycardia	74 %
Weight loss	43 %
Ophthalmopaty	35 %
Increased sweating	28 %
Diarrhea	20 %
Fatigue	14 %
Restless sleep	13 %
Enuresis	13 %
Increased growth	12 %

especially in children with chronic autoimmune thyroiditis. Kelp consumption is another source of possible iodine intoxication. Among other goitrogenic drugs or foods are reported lithium, interferon, cassava, and millet. In association with goiter, children may develop also hypo- or hyperthyroidism. However, when the goitrogenic drug or food is removed, thyroid normal function and size should be restored [34].

- *Graves' disease*. This condition is the most common cause of hyperthyroidism in children and adolescents (overall incidence 1:5,000 children). Adolescent females with a family history of autoimmune thyroid disease are predominantly affected.

  The thyromegaly is present in almost all cases with smooth rubbery texture, but also behavioral manifestations are common findings. The frequency of presenting symptoms is shown in Table 14.2. The clinical onset of Graves' disease can be insidious in most children and the diagnosis delayed up to 6–12 months [27]. The classical hormonal picture at onset is typical, with elevated thyroid hormones and suppressed TSH level. In milder cases, TSH suppression is present without high free T4 levels. Autoantibodies (aTPO and TG) could be detected but are not pathogenetic, as they are TSH receptor antibodies (TRAb). As recently reported by Gastaldi et al. [35], although no clinical variable investigated is constantly associated with a definite outcome, the absence of goiter at the diagnosis may be associated with a better outcome. The most relevant predictor of Graves' disease outcome was serum level of TRAb; TRAb at time of diagnosis less than 2.5 times the upper reference limit, *TRAb* normalization during ATD, and TRAb normalization timing may each predict positive outcomes.

- *Subacute thyroiditis*. When the thyroid goiter is tender and painful, subacute thyroiditis should be suspected also in children, even if uncommon in these young patients. They usually develop thyroid goiter after a viral upper respiratory tract infection. Evaluation of thyroid function reveals a classical hyperthyroid phase (that lasts 2–6 weeks) with subsequent (subclinical) hypothyroidism (2–7 months) and finally restoration of normal thyroid function.
- A*cute suppurative thyroiditis*. Another rare goiter in childhood is represented by acute suppurative thyroiditis. In this condition, goiter onset is abrupt and accompanied by general symptoms of infection as fever, chills, sore throat, and even dysphagia. The goiter is painful, tender, and could be asymmetric for the swelling of a thyroid lobe only (commonly the left one), with generally a pyriform sinus fistula [36].

14.4.3 Nodular Goiters (Thyroid Cysts, Adenomas, Carcinomas)

Thyroid cysts are uncommon in children and are classified as simple cysts or mixed solid and cystic nodules. Among the rare causes of hyperthyroidism, toxic adenomas are reported also in children. Mutations of TSH receptor gene or alpha subunit of G protein have been detected in some children who could show solitary or toxic multinodular goiter [18, 37]. Malignant nodules are solitary or could be found in the context of multinodular goiter. Conditions that are at risk of malignancy are previous irradiation of head and neck, exposure to nuclear fallout, and thyroiditis [31]. The diagnosis of pediatric thyroid nodules should be based on a stepwise evaluation that includes clinical, laboratory, and radiographic modalities. While laboratory assessments establish thyroid function, ultrasonographic imaging identifies clinically unapparent nodules and provides detailed nodule characterization for suspected malignant lesions. Scintiscan in patients with hyperthyroidism and fine-needle aspiration biopsy in patients with euthyroidism represent the next logical step [38].

The fine-needle aspiration biopsy is a safe technique even in childhood and adolescence, offering the best sensitivity, specificity, and accuracy in detecting malignancy compared with conventional approaches.

14.5 Differential Diagnosis of Goiters

The diagnostic evaluation of a child with goiter should take into consideration both the age and the functional thyroid status of the patient [27]. Figure 14.1 shows the algorithm for the initial diagnostic approach to the most common causes of diffuse goiters in the pediatric age.

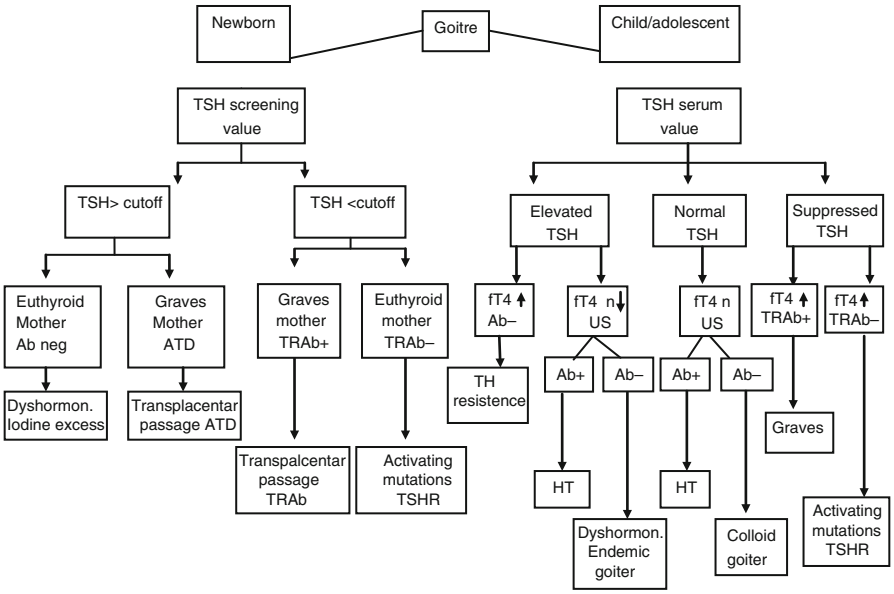

Fig. 14.1 Algorithm for the initial diagnostic approach to the most common causes of diffuse goiters in the pediatric age

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Thyroid Nodules and Carcinoma

15

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Abbreviations

AD	Autosomal Dominant
AR	Autosomal Recessive
ATA	American Thyroid Association
ATC	Anaplastic/undifferentiated thyroid carcinoma
CEA	Carcinoembrionary Antigen
DTC	Differentiated Thyroid Carcinoma
FNAB	Fine-Needle Aspiration Biopsy
FTC	Follicular Thyroid Carcinoma
MEN	Multiple Endocrine Neoplasia
MTC	Medullary Thyroid Carcinoma
PTC	Papillary Thyroid Carcinoma
RAI	Radioactive Iodine
rhTSH	Recombinant Human TSH
THW	Thyroid Hormone Withdrawal

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15.1 Epidemiology and Classification

Thyroid nodules in children are rare [1]: studies estimated a 1.8 % prevalence in children by palpation [2, 3] and 0.2–5 % by ultrasound [4–6]. With respect to adults, infantile thyroid nodules are much less frequent, but conversely, nodule malignancy rate is estimated to be much higher [7]: up to 25 % of pediatric thyroid nodules are reported as malignant [4, 8] *vs* 5–15 % of adult ones [9]. A recent work on nodules ≥1 cm in the two populations statistically confirmed this data reporting a 22 % cancer prevalence in children and 14 % in adults [10]. Thyroid carcinoma is the commonest endocrine tumor in children [11]: in the U.S.A., the incidence across 1975–2006 was of 1 per million for 5–9-year-old children, 5 per million in 10–14-year-olds, and 18 per million in 15–19-year-olds [1, 11].

Most nodules are non-neoplastic and arise as a result of glandular hyperplasia with or without a cystic component, possibly in the context of goiter. Benign tumors account for approximately 10 % of all nodules [3] and are primarily represented by toxic follicular adenomas. Thyroid carcinomas are classified according to the cell of origin: those arising from follicular epithelium and those arising from parafollicular calcitonin-producing C cells. Other less common types of thyroid tumors may occur, either of thyroid or extrathyroid origin [12, 13]. Thyroid follicle-derived tumors, namely, well-differentiated thyroid carcinoma (DTC), are largely prevalent in childhood (90–95 % of cases), including papillary thyroid carcinoma (PTC) and follicular thyroid carcinoma (FTC). PTC and FTC are further subdivided into several histological variants: follicular, tall cell, diffuse sclerosing, and columnar variants for PTC; Hürthle cell, clear cell, and insular variants for FTC [11]. Calcitonin-producing cell-derived tumors and medullary thyroid carcinomas (MTC) are much rarer (5–10 % of thyroid carcinomas). Anaplastic/undifferentiated carcinomas (ATC) are exceptional in childhood and arise from the follicular cells of the thyroid gland but do not retain the biological features of the original cells.

PTC is often multicentric (about 40 % of childhood cases), whereas FTC is mostly sporadic and encapsulated. PTC disseminates primarily through the lymphatic system to pretracheal lymph nodes, but distant metastases, most commonly to lungs, are detected in about 20–30 % children *vs*. 2 % adults [14], while FTC metastasizes through the bloodstream to the lungs and liver and MTC to cervical lymph nodes. Both DTCs have usually a good prognosis with a survival rate of >95 % [15]. The behavior of MTC is usually more aggressive than that of DTC. ATC is considered one of the most aggressive cancers in humans with an overall survival <6 months since diagnosis. Of note, it may arise de novo or from a new mutation (usually in p53) in a preexisting DTC.

Although differentiated thyroid cancer is usually slowly growing, a prompt diagnosis and accurate multistep workup are recommended in children, because greater tumor size, distant spread, and greater atypia are factors associated with a worse prognosis and increased mortality [4]. Of note, because of their rarity, almost all the reports on thyroid nodules and cancer in pediatrics are retrospective, therefore data and guidelines should be interpreted critically and cautiously.

15.2 Causes, Predisposing Factors, and Associated Diseases

Thyroid nodular disease refers to a heterogeneous group of clinical entities encompassing isolated thyroid nodules, syndromic diseases with thyroidal involvement, multinodular goiters, nodules in the context of autoimmune thyroid disease, and thyroid incidentalomas discovered following thyroid or neck ultrasound for familiarity or other conditions [4].

Infrequently, cases of malignant thyroid nodules are found in patients with congenital hypothyroidism due to both dyshormonogenesis or dysgenesis and thyroglossal duct cysts [4]. Recent evidence suggests that genes encoding proteins involved in thyroid hormone synthesis mutated in congenital hypothyroidism may be convincing candidates for contributing to inheritable forms of goiter and be involved in nodule development [16]. Interestingly, the prevalence of thyroid nodules in infants with congenital hypothyroidism has been recently estimated at 4.2 %, with none malignant [17].

As concerns the relationship between thyroid autoimmunity and cancer, which is a matter of some controversy [18–20], few studies evaluated the topic in the pediatric context. In a recent series of children with autoimmune thyroiditis, 9.6 % had nodules ≥1 cm, and thyroid cancer was diagnosed in one out of three of such cases [21]. This estimate, closely paralleling that of patients without thyroiditis, questions whether autoimmunity itself represents a risk factor for cancer. Moreover, this observation implies that nodules ≥1 cm should be investigated notwithstanding an underlying thyroid benign disease.

TSH signaling *via* TSH-cAMP intrathyroidal pathway has a well-known growth-promoting effect on follicular cells and may hypothetically lead to cancer growth; however, direct evidence linking its elevation with thyroid follicular cells' malignant transformation is lacking as yet. On the other hand, since Boelaert et al. [22] found that mild serum TSH elevations can be employed as a predictor of PTC, many studies in adults [23] replicated their results. Also, recent studies in children [24, 25] confirmed this relationship, which appears to be even stronger than that found in adults: most cases with PTC have serum TSH in the upper normal range (i.e., >2.6 mU/l) or mildly elevated (4.4–10 mU/l). Therefore, serum TSH in children with thyroid nodules demonstrated very high sensitivity in detecting PTC. In spite of these observations, to date evidence supporting the utility of TSH measurement in cancer prediction in clinical practice is limited, also taking into account observations on the natural course of idiopathic subclinical hypothyroidism in childhood [26].

Ionizing radiations are a well-known risk factor for thyroid cancer, and children are much more sensitive to the their carcinogenic action than adults. Thyroid cancer risk increases parallel to radiation dosages of up to 20, is steady up to 29 Gy, and then decreases for doses >30 Gy, probably because of cell killing and gland fibrosis [27]. A great deal of our knowledge in this field has been achieved from observations on the high incidence of thyroid cancers following the widespread employment of radiotherapy for benign pathologies of the head, neck, and chest in the 50s and 60s [28] and the exposure to radioactive isotopes in the fallout from Chernobyl [29, 30]: almost exclusively, PTC developed in such cases [31]. Currently, radiation is mostly employed

in the treatment of childhood malignancies: in the last decade, several observations evidenced that patients who underwent such treatments in childhood are prone to develop both cancerous and noncancerous thyroid diseases subsequently [32]: the radiation-induced risk for thyroid cancer persisted elevated for decades after the primary cancer [33–35]. Some authors advise an ultrasound selective screening of such cases aiming at an early diagnosis: contrarily, other authors do not recommend screening procedures as they may lead to unnecessary and invasive treatment and have not been shown to reduce morbidity or mortality in this population [14]. Recently, data supporting a carcinogenetic role for radiations employed in the diagnostic phase of childhood cancer is accumulating [32]. It has been observed that the increasing incidence of PTC observed in the last decades is paralleled by an increasing exposure to medical radiation; however, at present it is not clear both whether PTC incidence is really increasing (or rather reflects improved detection of sublinical cases) and which portion of PTC cases can be consequent to medical radiation exposure [32].

15.3 Genetics and Inheritance of Thyroid Neoplasms

15.3.1 Medullary Thyroid Cancer Syndromes

Inheritance of thyroid neoplasms of medullary origin is well documented and studied. It accounts for 5 % of all thyroid tumors [36] with the familial form representing 20–25 % of cases. The latter belongs either to pure familial MTC syndrome or to multiple endocrine neoplasia (MEN) type 2 syndromes, when associated with the development of pheochromocytoma. In MEN type 2A, hyperparathyroidism and parathyroid gland tumors are additional features. In MEN type 2B, the association includes mucosal neuromas, intestinal ganglioneuromatosis, and marfanoid habitus [37]. Both MEN syndromes are autosomal dominant and caused by specific gain-of-function mutations in the RET (REarranged during Transfection) proto-oncogene (95 % of cases). Familial MTC is commonly associated with mutations at codons 618 and 620 and with noncysteine mutations at codons 768 and 804 [38, 39]. MEN 2A is caused by germline RET mutations in exons 10 and 11 [39]. On the contrary, in MEN 2B, most mutations occur *de novo*. RET activating mutations lead to constitutive intracellular C-cell signaling, hesitating in hyperplasia, calcitonin hypersecretion, and malignant transformation. Aiming at detecting such cases, serum calcitonin dosage is employed as a screening in thyroid nodules and as a disease marker in patients thyroidectomized for MTC.

15.3.2 Genetics of Pediatric Differentiated Thyroid Cancer of Follicular Origin

Molecular investigations are still limited to the research setting but in the near future will likely contribute to refine the predictability of malignancy on cytology specimens. The mitogen-activated protein kinase (MAPK) signaling pathway is

commonly overactivated in PTC: rearrangements in genes implicated in this pathway have a pivotal role in pediatric cases while adult ones predominately harbor point mutations. Pediatric PTC exhibits an increased rate of various RET/PTC (40–70 %, even more in cases following radiation exposure) or AKAP9–BRAF (11 %) rearrangements leading to the creation of fusion genes with increased signaling activity. Mutations in genes involved in the same PTC pathway may occur in the two oncogenes BRAF and RAS. FTC mostly shows PAX8/PPRAR-γ (peroxisome proliferator-activated receptor-gamma) rearrangements or RAS mutations, while alterations in the PI3K/AKT pathway or CTNNB1 and TP53 mutations have been implicated in the development of poorly differentiated thyroid cancers [40, 41]. Recently, mutations in genes of the thyroid morphogenesis pathway (involved in thyroid gland formation, differentiation, function, and hormone synthesis) have been hypothesized as players in modulating thyroid cancer risk [42].

Interestingly, a certain degree of familial aggregation is observed in histotypes of follicular origin [11, 43], although the genetic background of these conditions is still far from being unraveled. Familiar nonmedullary thyroid cancer represents 3–6 % of all thyroid cancer cases and has an autosomal dominant pattern of inheritance with high penetrance. Its susceptibility genes have not been identified so far, therefore an early genetic screening as for MEN2 syndromes is not feasible; interestingly, no difference between sporadic and familial varieties of nonmedullary cancer is detectable in the type or number of mutations [43]. Besides isolated familial nonmedullary thyroid cancer, two syndromic variants exist: one associated with renal cell carcinoma and one with multinodular goiter.

15.3.3 Syndromic Disorders with Thyroid Nodules and Cancer

In the pediatric context, the variety of inherited syndromic disorders of genetic origin associated with thyroid nodules, goiter and cancer, mostly represented by overgrowth, hamartomatous, or cancer predisposition conditions (Table 15.1). The awareness and attentive scrutiny of such conditions is of crucial importance to detect inheritable disorders, perform appropriate genetic studies and family counseling, and early diagnose various kinds of tumors by appropriate cancer screening strategies.

15.4 Diagnostic Workup

Once a nodule is evidenced, either clinically or echographically, the usual diagnostic includes the collection of patient's history, clinical examination, laboratory tests, thyroid ultrasound, and fine-needle aspiration biopsy (FNAB) [58]. Although it has been pointed out that the diagnostic process employed in children should be the same as that in adults, the peculiarities of the pediatric age should prompt in the clinician a higher degree of suspicion; as Niedziela does [7], we think that the simple application of adult guidelines [9] to the pediatric population should be cautious.

Table 15.1 Syndromes and inheritable conditions with thyroid nodules and cancer

Condition	Additional phenotype	Thyroid involvement	Genetics
Cowden OMIM #158350 [44]	(PTEN – hamartoma tumor syndrome spectrum), benign and malignant tumors of uterus, breast, bowel	Thyroid nodules of follicular type within hyperplastic multinodular goiter (50–67 %); thyroid carcinomas in 5–10 % of cases	Germline inactivating mutations of the PTEN tumor suppressor gene
Bannayan-Riley-Ruvalcaba OMIM #153480 [45, 46]	(PTEN – hamartoma tumor syndrome spectrum), macrocrania, lipomatosis, retarder neuropsychomotor development, scoliosis, seizures, myopathy, joint laxity, hyperpigmented spots of the glades	Thyroid adenomas usually are of follicular type ± autoimmune thyroiditis, multinodular goiter and thyroid carcinomas are encountered in >50 % and 5–10 % of cases	Germline inactivating mutations of the PTEN tumor suppressor gene
Carney complex OMIM #160980 [47, 48]	Skin, breast, and cardiac myxomas, lentiginosis and endocrine glands neoplasias	Goitrous multinodular disease, usually of follicular origin and benign nature; malignant evolution in 10–15 % of cases	AD, gain of function mutations of PKA subunits (PRKACB, PRKAR1A)
Familial adenomatous polyposis OMIM #175100 [47]	Multiple intestinal polyps, initially benign but prone to malignant transformation ± mandibular osteomas, fibromas, and sebaceous cysts in Gardner syndrome	Increased risk of thyroid cancer, especially follicular histotype	AD, APC gene mutations
Peutz-Jeghers syndrome OMIM #175200 [49, 50]	Multiple gastrointestinal hamartomatous polyps, melanocytic macules of the lips and oral mucosa, increased cancer risk	Increased risk of thyroid cancer, especially follicular histotype	AD, STK11 and LKB1 gene mutation
MEN IIA OMIM #171400 [51]	Medullary thyroid cancer, pheochromocytoma, and parathyroid tumors	C-cell hyperplasia and medullary thyroid cancer	Proto-oncogene RET mutations
MEN IIB OMIM #162300 [51]	Medullary thyroid cancer, pheochromocytoma, mucosal neuromas, marfanoid habitus	C-cell hyperplasia and medullary thyroid cancer	Proto-oncogene RET mutations
DICER1 OMIM #138800 [52]	Cancer predisposition (pleuropulmonary blastoma, cystic nephroma, cervix embryonal rhabdomyosarcoma, primitive neuroectodermal tumor, ovarian Sertoli-Leydig cell tumors, and Wilms tumor)	Familial multinodular goiter	AD, DICER1 haploinsufficiency

(continued)

Table 15.1 (continued)

Condition	Additional phenotype	Thyroid involvement	Genetics
McCune-Albright OMIM #174800 [53–55]	Polyostotic fibrous dysplasia, cafe-au-lait skin spots, peripheral precocious puberty, hyperfunction of the thyroid, pituitary or adrenal glands	Multinodular/cystic toxic goiter	Mosaic (somatic) GNAS1 gain-of-function mutations
Birt–Hogg–Dubè OMIM #135150 [56]	Genodermatosis with fibrofolliculomas and increased risk of pulmonary air cysts, spontaneous pneumothorax and renal tumours	Euthyroid usually multiple and benign thyroid nodules in 65 % of cases	AD, FLCN tumour-suppressor gene mutations
Werner [57] OMIM #277700	"Adult progeria" more common in Japan, elderly appearance with thin skin, wrinkles, alopecia, and muscle atrophy, osteoporosis, cataracts, diabetes, peripheral vascular disease, melanoma, soft-tissue sarcoma, osteosarcomas	Increased risk for follicular and anaplastic thyroid carcinoma which is the most common among the malignancies (16 % of cases)	AR, WRN gene mutations (DNA repair gene)

Abbreviations: *AD* autosomal dominant, *AR* autosomal recessive

15.4.1 Family and Patient's History

Attention should be focused on family history of thyroid cancer, especially MTC, and on history of exposure to radiation for previous oncohematological diseases. Medical history should be evaluated with peculiar attention to traits and diseases evocative of familial/syndromic forms of thyroid nodules and cancer. Reports suggest that male sex is associated with a higher malignancy likelihood of thyroid nodules.

15.4.2 Clinical Evaluation

The objective examination aims at detecting (a) associated lymph node enlargement, (b) signs and dysmorphic features in syndromic patients, (c) signs or symptoms of local compression (dysphagia, dysphonia, discomfort, or shortness of breath), or (d) signs or symptoms of hyperthyroidism.

Palpation of hard and firm nodules or lymph nodes and compression/invasion symptoms are considered indicative of malignancy. Lymph nodal enlargement is of the utmost importance in children as strikingly more common than in adult patients [8, 59] and presents in 80 % of cases [60, 61], although not implying a worse prognosis [62].

15.4.3 Laboratory Tests

Laboratory tests include the measurement of serum TSH, free T4 (fT4), calcitonin, and free T3 (fT3) in case of suspected hyperthyroidism. Most thyroid nodules occur without symptoms of thyroid hormone excess or defect: >90 % of cases are euthyroid, 5 % hypothyroid (mostly subclinically with normal fT4 and elevated TSH), and 1–5 % hyperthyroid. Calcitonin is usually employed as a screening marker for MTC [7]. If its dosage is mandatory in patients with suspect MTC, MEN2 syndromes, and cytology suggestive of medullary neoplasm, its systematical use in all cases of thyroid nodules is debated [63], mostly because of its cost-effectiveness. There is general agreement that calcitonin levels >100 pg/ml are almost certainly indicative of medullary thyroid cancer [64]. Difficulties arise in mild elevations (the 10–100 pg/ml "gray zone") as calcitonin serum concentration physiologically increases with age and weight, differs according to sex, and may be high also in other conditions (other neuroendocrine cancer, nephropathy, pancreatitis, hypergastrinemia, thyroid autoimmunity, sepsis). In these cases, in order to increase specificity, a confirmatory repeated dosage or a stimulation test (calcitonin dosage 2, 5, and 15 min after pentagastrin 0.5 μg/Kg i.v. bolus) has been suggested [64].

Some ancillary laboratory tests are performed in some specialized/research centers and mostly in adults, as the dosage of thyroglobulin/calcitonin in the washout fluid of neck lymph nodes: these two markers of follicular cancer and medullary cancer, respectively, are sensitive and specific for the early detection of cervical metastases. The test is mostly employed in cases with small thyroid nodules with enlarged lymph nodes [64–66].

15.4.4 Instrumental Evaluations

Thyroid ultrasound has a key role in the diagnostics of nodules, while I131/Tc99 scintiscan is less extensively employed nowadays with respect to some decades ago. On the other hand, novel techniques, like elastography, are progressively introduced in clinical practice. The employment of other imaging techniques like computer tomography and nuclear magnetic resonance is limited to exceptional cases and to define disease extension or characterize masses of unclear origin.

15.4.4.1 Thyroid Ultrasound

Given its advantages, thyroid ultrasound represents the cardinal imaging tool in the diagnostic workup and management of thyroid nodules. The disadvantage of this method is in its being operator dependent. Thyroid ultrasound is fundamental in assessing the number, size, and characteristics of the nodule; in guiding FNAB and in monitoring lymph nodes and remnant thyroid tissue of thyroidectomized patients. In the diagnostic workup, ultrasound allows a first-line screening for selecting nodules with suspicious characteristics and deserving further evaluations. Color Doppler sonography represents a strong asset in providing more detailed characteristics of the nodule and refining the diagnostic decision. Various features are

associated with malignancy: hypoechogenicity, undefined margins, microcalcifications, high intranodular vascular flow at color Doppler [4], and lymph nodal modifications (longitudinal-to-transversal axes ratio <1.5, rounded profile with absence of the ilium, thickened or eccentric cortical, nonhomogeneous pattern, and increased vascular flow [3, 4, 8, 9, 67]), and an increase in nodule size during the follow-up, especially if under levothyroxine therapy [21]. Conversely, cystic pattern, multinodular goiter, regular margins, and peripheral increased vascularization are considered suggestive of benignity.

15.4.4.2 Elastography

Elastography is a novel technology for soft tissue elasticity mapping recently added in clinical practice for the noninvasive prediction of thyroid nodules' malignancy. The analysis of the speed of elastic waves passing through tissues estimates solid nodules' stiffness, which is increased in malignant nodules as they are firmer than the surrounding tissue [68]. In the last years, a number of studies have evaluated its use in this field with encouraging results [69]. It is a promising tool able to increase ultrasound performance in selecting nodules with higher malignancy likelihood and reducing unnecessary FNAB (of up to 60 %) [70–72]. The most relevant drawback of elastography is in its employment in cases with cystic or calcific nodules. Authors agree that further research is needed on its application in the differential diagnosis of indeterminate lesions and in other thyroidal diseases. Specific data on pediatric populations are not available as yet, although in our experience it appears reliable as in adulthood.

15.4.4.3 Scintigraphy

Scintiscan with Tc99 is much less used nowadays with respect to some decades ago. Current indications to perform a scintiscan include almost only benign tumors with overt/subclinical hyperthyroidism, namely, toxic adenoma. Scintiscan is used to confirm the diagnosis: in toxic adenoma, it usually displays a "hot" pattern with silencing of the remnant thyroid tissue. In these cases, FNAB typically does not offer much information [3, 8, 9] and is considered superfluous as surgery is needed in any case. At histological evaluation, PTC can been found in 1–5 % of these nodules [4].

15.4.4.4 Fine-Needle Aspiration Biopsy (FNAB)

FNAB is the most reliable test for nodule diagnosis and is recognized as the cornerstone and gold standard for the evaluation of solitary thyroid nodules. Data on pediatric cases [3, 21, 73, 74, 60, 75] are consistent with those on adults [76] and estimate its diagnostic accuracy as ranging from 75 to 95 %. As a consequence, in the last decades, FNAB has imposed as the gold standard also in pediatric thyroid nodules, demonstrating the highest sensitivity, specificity, and accuracy among other diagnostic investigations [60]. There is general agreement on performing FNAB in euthyroid and hypothyroid patients with palpable nodules and those with nodule diameters ≥1 cm and with sonographic features indicative of malignancy. However, the indications to perform FNAB in children are mostly inferred from adult

guidelines [9]; the increasing data on pediatric thyroid nodules suggest caution as in childhood clinical indications may be different and diagnostic threshold triggering further investigation lower. For nodules <1 cm, FNAB should be considered in selected cases with multiple clues pointing to a malignant lesion [8, 21, 77]: the diagnostic approach should be particularly aggressive in the presence of risk factors like radiation for malignancies of the head, neck, and thorax or family history of thyroid cancer. Besides nodule size, great importance for FNAB indications is represented by the variety of abovementioned anamnestic, clinical, laboratory, and echographic prognostic factors employed in clinical practice to assess malignancy likelihood. It is worth mentioning that multinodular thyroid diseases carry a malignancy risk comparable to that of solitary nodules [3, 63, 78]: clearly, in such cases, all suspect nodules should undergo FNAB.

In spite of high diagnostic accuracy, since a few years ago in up to 20 % of thyroid nodules, FNAB cannot provide diagnostic indications: the large part of results of uncertain interpretation were defined as "follicular lesion of undetermined significance" or commonly referred to as having an "indeterminate cytology" [79]. Major steps toward the standardization of the terminology employed and classification of cytology were reached in 2007 and 2008. In 2007, the British Thyroid Association and the Italian Society of Pathology and Cytology (SIAPEC-IAP) [80, 81] introduced a new classification. In 2008, the Bethesda system for reporting thyroid FNAB specimens [82] recommended that each report begin with one of six general diagnostic categories: I. Nondiagnostic or Unsatisfactory, II. Benign, III. Atypia of Undetermined Significance or Follicular Lesion of Undetermined Significance, IV. Follicular Neoplasm or Suspicious for a Follicular Neoplasm, specifying if Hürthle cell (oncocytic) type, V. Suspicious for Malignancy, VI. Malignant [80, 81]. The result of this novel classification system based on cytoarchitectural patterns was a reduction of superfluous and untimely thyroidectomies.

With the intent to better define malignancy risk of uncertain cytology, several molecular and histochemical markers on cytological smear have been studied in adults [83, 84]. Among them, telomerase [85], galectin-3 [86], CD44v6 [87], and HBME1 [88] alone or variously combined are considered to be more reliable in discriminating malignant cases. Obviously, calcitonin also is a reliable marker of medullary carcinoma. However, the main limitation of this approach is that none of these markers completely fulfill the diagnostic needs, but rather a complete panel of these markers should be employed in a reasoned diagnostic process.

One last critical aspect is in which cases FNAB should be repeated: this aspect should take into consideration that PTC is commonly slow growing with an indolent course even after local and pulmonary metastatization. Studies report that PTC occurs in 1.3 % of patients with a previous benign FNAB repeated yearly [89]. We suggest to monitor clinically and echographically nodules on a 6–12 months basis (based on the initial malignancy likelihood assessment) and repeat FNAB according to change in the clinical and imaging picture. Obviously, in case of multinodular goiter, all suspect nodules should undergo FNAB evaluation.

15.5 Management and Treatment of Benign Thyroid Nodules

Management and treatment guidelines in children with benign nodules are scanty. Surgical intervention, usually hemithyroidectomy, is required to resolve the hyperthyroid state of toxic adenoma [3, 28]. Several options are available in other cytologically benign nodules: in asymptomatic cases, a conservative approach is largely employed, consisting in observation with yearly recheck with or without (sub)suppressive medical treatment with levothyroxine [90, 91] aiming at reducing TSH and inducing nodule shrinkage. When nodules are growing or responsible for symptoms of local compression, (hemi)thyroidectomy and radioiodine thyroid ablation remain the current standard. Recently, several minimally invasive techniques have been introduced to avoid the so far employed surgical/radiotherapy approach: percutaneous ethanol injection therapy is mostly employed in the treatment of prevalently cystic nodules; percutaneous thermal ablation by radiofrequency or laser or microwaves or high-intensity focused ultrasound is employed in highly specialized centers [92, 93]. Further data are needed to assess indications, limitations, and safety of these procedures compared to the standard ones in both adults and children.

15.6 Treatment of Thyroid Carcinoma

15.6.1 Surgery of DTC

Guidelines and randomized trials specific for children have not been designed because of the uncommon occurrence of this disease. Although surgery is the primary therapy for pediatric patients with DTC, there is continuing controversy regarding the optimal surgical option (total thyroidectomy, near-total thyroidectomy, subtotal thyroidectomy, or lobectomy) as well as the role of prophylactic central neck dissection. Currently, total or near-total thyroidectomy in pediatric patients with DTC is considered the best approach by most surgeons and according to the American Thyroid Association (ATA) guidelines [9, 16]. Lobectomy alone may be sufficient treatment for small (<1 cm), low-risk, unifocal, intrathyroidal PTC. The facilitation of radioiodine treatment and imaging and the use of serum thyroglobulin as a tumor marker for recurrent/residual disease [94] are considered other practical advantages of extensive surgery. A primary procedure with less than total thyroidectomy has been demonstrated to significantly increase the need for repeating surgery [95]. Moreover, tumor size should not be considered as a determinant for the type of surgery in children [14]. Although TNM scoring system for differentiated thyroid cancer includes age because of its strong prognostic, it is commonly considered to be imperfect in childhood when the risk of recurrence is high [96].

Since lymph node involvement at the diagnosis is common [8, 59], central neck dissection has been recommended, and modified neck dissection should be performed for clinically apparent and biopsy-proven lateral neck disease. Prophylactic lateral neck dissections are not recommended [94]. On the other hand, complications

of total thyroidectomy and potential harms of the central compartment dissection such as hypoparathyroidism and injury to the recurrent laryngeal nerve should also be considered. Although these risks are minimized when surgery is performed by an experienced endocrine or pediatric surgeon, a high prevalence of hypoparathyroidism and both temporary and permanent recurrent laryngeal nerve palsy has to be taken into account. Recently, age (<16 years), familial history of thyroid cancer, preoperative gross neck lymph node diffusion, tumor diameter, and extrathyroidal invasion were identified as risk factors for disease-free survival in children with PTC. Preoperative gross lymph node metastasis and distant metastasis at diagnosis were identified as relevant factors for cause-specific survival, suggesting that total thyroidectomy alone could not be considered sufficient in all childhood patients [97].

15.6.2 Surgery of MTC

In general, treatment of MTC consists in total thyroidectomy for both sporadic and hereditary forms associated with prophylactic central lymph node dissection, whereas lateral neck dissection is needed for patients with positive preoperative imaging. When distant metastatic disease is detected at diagnosis, less aggressive surgery might be appropriate in order to preserve speech and prevent morbidity. The improved understanding of molecular basis of MEN2 syndromes and isolated MTC allows to define risk groups for cancer development and recommended timing schedule for prophylactic treatment. The latter is the standard of care in pediatrics, since patients with hereditary forms of MTC can develop metastases before the age of 5 [38, 97–99]. Prophylactic thyroidectomy in MEN is recommended within 1 year of age for patients with 883, 918 RET codon mutations, before 5 years for cases with mutations in codons 611, 618, 620, 634, and before 10 years for those with mutations in codons 609, 630, 768, 790, 791, 804, 891.

15.6.3 Radioiodine Therapy

Radioactive iodine (RAI or radioiodine 131I) therapy is a mainstay of postsurgical treatment in DTC. 131I has been demonstrated to destroy thyroid tumor cells several decades ago [100]; moreover, a postsurgery 131I uptake by residual thyroid tissue is usually demonstrated. The frequent multifocal disease extension, nodal involvement, and distant metastases in pediatric patients with DTC together with a sodium iodine symporter expression greater than in adult forms, possibly accounting for a more successful treatment [101], are generally considered as factors making RAI a therapeutic challenge. To date, it is generally suggested that most children should be treated with 131I in order to ablate residual disease, reduce the risk of disease recurrence, and positively affect progression-free survival rate, as recently reviewed [14, 94, 95, 101, 102].

In order to obtain 131I uptake by remnant and residual tissue, TSH elevation greater than 30 mU/l is needed. Levothyroxine administration should be discontinued 2–3 weeks in children and 4 weeks in adults before radioiodine

administration ("thyroid hormone withdrawal," THW); alternatively, patients can be treated with 0.7 mcg/kg triiodothyronine for at least 1 month to be discontinued 2 weeks before 131I administration. TSH rise can also be achieved with recombinant human TSH (rhTSH) to be administered on 2 consecutive days. The use of rhTSH is approved in adults; however, it has to be emphasized that, at present, rhTSH use is not approved for children by drug-regulatory agencies in U.S.A. or E.U. Although it has the potential to reduce whole-body radiation exposure associated with 131I therapy and its clinical use has been reported in children with DTC, data showing comparable efficacy to THW are lacking in pediatrics [9, 14, 94, 103].

Main purposes of the use of RAI treatment include therapy of residual microscopic disease, metastatic or unresectable lesions, together with an accurate patient staging by means of 131I whole-body scanning, usually performed within 4–7 days of RAI therapy, for the detection of distant metastases. In addition, the postsurgery ablation of remaining thyroid tissue in the neck ("thyroid remnant ablation") allows the use of thyroglobulin as a tumor marker during the follow-up. There is no specific recommendation for the timing of 131I after total thyroidectomy; however, it is generally done within 3–6 weeks till 3 months after surgery. 131I administration dosage strategies can be summarized in administering fixed activities (eventually based on the patient's weight); dosing based on the administered activity that is as high as safely administrable, recently defined as the lowest safe limit administered activities up to 5 mCi/kg (185 MBq/kg) for treatment of distant metastases and DTC recurrence in children; and applying specific activities for tumor ablation, dosimetry, which is suggested to be mainly considered for individuals with lung metastases [14, 104]. The use of pretherapy scans is limited because of its low impact on the decision to ablate and because of 131I-induced stunning phenomenon, defined as a reduction in uptake of the RAI therapy dose induced by a pretreatment diagnostic activity. On the other hand, since it can be difficult to distinguish residual disease from thyroid remnant at post-therapy whole-body scan and when the extent of the thyroid remnant cannot be accurately ascertained from the surgical report or neck ultrasonography, 123I (1.5–3 mCi) or low-activity 131I (1–3 mCi) pretherapy scans may provide additional information [9, 14] in order to distinguish residual disease from thyroid remnant and then to plan more adequate therapeutic strategies.

Risks associated with RAI treatment include second primary malignancies, reproductive risks, pulmonary fibrosis, gastritis, and sialoadenitis. Evidence suggests that RAI does not increase the risk of second neoplasms in children nor long-term effect on female fertility. Given the possibility of cumulative gonadal damage in males, sperm banking should be considered before therapy [14].

15.6.4 Levothyroxine Therapy

Levothyroxine therapy is a fundamental part of the treatment of thyroid carcinoma; it is well recognized that TSH suppression can reduce rates of recurrence for DTC, whereas there is no role for it in MTC. The ATA task force recommends in low-risk

adult DTC patients a plasmatic TSH target of 0.1–0.5 mU/l and a more aggressive suppression for high- and intermediate-risk patients, with TSH <0.1 mU/l. Benefits from TSH suppression have been widely reported in adults in terms of decreased progression and recurrence rates and cancer-related mortality. For adults, recommendations state that suppression should be maintained for 5–10 years [9]. On the other hand, specific evidence of benefits from TSH suppression in pediatrics is lacking to date. Moreover, compared with adults, TSH suppression presents peculiar difficulties: actually, in children higher doses of levothyroxine per kg are needed to achieve a complete suppression, and a condition of subclinical iatrogenic hyperthyroidism may impact growth, behavior, and learning ability. Recently, a proposed scheme for children is to initially suppress TSH levels <0.1 mU/l and then allow a TSH rise to 0.5 mU/l once remission is obtained [14, 94].

15.6.5 Other Therapies

External beam radiation does not have a clear role in the treatment of DTC, its use being beneficial as a palliative measure in advanced disease stages. Chemotherapy is not considered in the initial therapy of DTC; newer agents are being evaluated for patients with metastatic or recurrent disease. Treatment of anaplastic thyroid cancer, the most aggressive histotype and one of the most aggressive cancers in humans, has not been standardized as yet and appears largely inefficient; surgery, chemotherapy, radiotherapy alone or in combination are used with almost no impact on survival rate. Most used cytotoxic agents include doxorubicin, cisplatin, and bleomycin.

In advanced MTC, chemotherapy has not shown significant clinical benefit. Radiation may be used in the presence of local invasion or in the setting of bone (together with bisphosphonates to control symptoms) or central nervous system metastasis although there are no clear data indicating an effect on long-term survival. Novel drugs of the family of RET kinase inhibitors may have a relevant clinical impact in the near future: among these compounds, the Food and Drug Administration recently approved vandetanib, which has been shown to lengthen progression-free survival. Prognosis of MTC, however, has been most closely related to the stage of disease at presentation and to the extent of surgery [94, 105].

15.7 Follow-up Recommendations

ATA management guidelines for DTC are considered appropriate to children. Notable exceptions have been considered with regard to timing of repeated ultrasound evaluation in indeterminate FNAB cytology, tumor size as a determinant for the type of surgery, central compartment neck dissection for some lesions, need for RAI administration, TSH suppression, and thyroglobulin measurements as primary tool for assessing treatment effectiveness or recurrence.

15.7.1 Differentiated Thyroid Cancer

Lifelong follow-up of DTC patients is extremely important as tumor recurrences have been demonstrated to occur decades later [14, 94, 101, 102]. Regular assessment of circulating thyroid hormone levels, ultrasonography of the neck, measurement of thyroglobulin, and whole-body 131I scans are employed in the follow-up care. TSH, fT4, and fT3 levels' assessment is indicated every 6 months and 1–2 months after every levothyroxine dosage changes. Thyroglobulin measurement is the mainstay of DTC follow-up in the absence of antithyroglobulin antibodies, which is a confounding factor in its measurement. The disease-free state has been reached when thyroglobulin levels after rhTSH challenge or thyroid hormone withdrawal are undetectable. Levels in the 0.1–10 mcg/l range may indicate residual disease, addressing to perform follow-up neck ultrasonography. In case of thyroglobulin levels >10 mcg/l, neck imaging is indicated: if gross cervical disease is detected, reoperation is needed, whereas 131I treatment with 100–150 mCi (3.7–5.5 GBq) is sufficient. A regular 6 months interval basal thyroglobulin measurement by second-generation assays has been recently shown to correlate with stimulated thyroglobulin levels. On levothyroxine treatment, thyroglobulin values <0.1 mcg/l correlate with a stimulated level <2.0 mcg/l; in case of basal thyroglobulin rise, disease relapse needs to be considered [14, 15, 94, 101–103, 105, 106].

Neck ultrasonography should be performed every 6 months in order to detect residual thyroid tissue and lymph nodes. Attention is needed in order to assess whether lymph nodes represent potential metastatic foci. FNAB of lymph nodes may be indicated for persistent or enlarging lymph nodes, together with thyroglobulin measurement in lymph node aspirates.

Diagnostic whole-body scintigraphy is performed using 123I or 131I 2–5 mCi (0.074–0.185 GBq) generally at 6–12 months after diagnosis. In patients with detectable antithyroglobulin antibodies, scintigraphy is useful in identifying potential residual disease. On the contrary, its employment in patients with no metastases is debated: recent data show that three consecutive negative post-treatment scintiscans are strongly predictive for a low risk of recurrence, while other data suggest that it adds only a modest information to the combination of thyroglobulin assessment and ultrasonography [14, 102, 107, 108].

15.7.2 Medullary Thyroid Cancer

Post surgery, monitoring of calcitonin levels is the backbone of MTC follow-up. After surgery, serum calcitonin levels normalize (undetectable) in 60–90 % of cases in patients with no lymph node involvement but in only 20 % of those with lymph node diffusion. Carcinoembrionary antigen (CEA) levels also have a predictive role after surgery. Calcitonin and CEA doubling times should be used to predict outcome and to help plan long-term follow-up of patients with MTC. The first level should be obtained 6 weeks to 4 months after surgery; persistent marker elevation indicates residual disease. There is no agreement on the imaging techniques to be

employed in the follow-up of MTC, and the choice should be driven by disease location. Distant metastases predominantly occur in patients with a large-sized tumor, extrathyroidal growth, and lymph node involvement [94, 99, 105].

15.8 Prognosis

Usually, even in the presence of metastatic disease, the prognosis of pediatric thyroid cancer is reported to be good: in spite of being usually more aggressive at the time of initial evaluation than adult ones, reports show that pediatric form is ultimately less lethal [109]. In the review by Reiners et al. [110], mortality is reported to be usually low, in the range of 1–2 %, and recurrence rates approximate 30 % (7–58 %). Long-term follow-up data show 30-year survival rates for children of 90–99 % [14, 109–111]: this likely reflects that most pediatric patients have well-differentiated tumor histotypes which mostly respond well to therapy. Data concerning prognosis are still scanty, and collaborative studies are needed to provide more accurate figures.

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Autoimmune Thyroiditis

16

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Abbreviations

DS	Down's syndrome
HT	Hashimoto's thyroiditis
US	Ultrasonographic
FNAC	fine-needle-aspiration cytology
GD	Graves' disease
TS	Turner syndrome
Htx	Hashitoxicosis
TG	Thyroglobulin
TSH	Thyrotropin
TGAb	TG-autoantibodies
TRAb	TSH receptor autoantibodies
L-T4	Levothyroxine
TPOAb	Thyroid peroxidase autoantibodies
SH	Subclinical hypothyroidism

16.1 Definition

Thyroiditis is characterized by inflammation of thyroid gland and can present as acute, subacute, or chronic diseases. Chronic autoimmune lymphocytic thyroiditis or Hashimoto's thyroiditis (HT) is by far the most common inflammatory thyroid

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disorder in childhood, whereas the other forms of thyroiditis are very rare [1]. Furthermore, HT is the most frequent pediatric thyroid disease and the most common cause of goiter and acquired hypothyroidism in children and adolescents from iodine-replete areas in the world [2]. HT is being increasingly detected during the last years because of the higher awareness among pediatricians, the availability of better autoantibody assays and ultrasonographic (US) machines, and the more diffuse access to fine-needle aspiration cytology (FNAC) [3].

HT diagnosis in children is usually suspected in the presence of goiter, even in the absence of thyroid dysfunction signs and symptoms, and may also be established incidentally, during medical checkups, or in the evaluation of children with other autoimmune diseases [4].

HT was described for the first time in 1912 by the Japanese physician Hakaru Hashimoto in four middle-aged women, who presented with chronic thyroid disease and a histopathological picture characterized by lymphocyte infiltration, fibrosis, parenchymal atrophy, and eosinophilic changes of some acinar cells. Although it is known from over a century ago, HT may still sometimes present with surprisingly different clinical entities and frequently astonishes many physicians with one of its many faces [5].

The aim of this survey is to report the most recent views on epidemiology, pathophysiology, presentation, evolution over time, and long-term prognosis of HT in childhood and adolescence.

16.2 Epidemiology and Risk Factors

HT is a relatively common disease, whose prevalence in pediatric age has been reported to range from 0.3 to 3.3 %, achieving its peak during adolescence, whereas it is only infrequently diagnosed during the first 3 years of life [6]. Its prevalence between genders is markedly different, with five- to ten-fold excesses in girls [7]. HT prevalence is also significantly conditioned by environmental iodine status with lower prevalence rates in the pediatric populations with low urinary iodine levels [8].

An epidemiological peculiarity of HT is the significant clustering within families, with 31.6 % of children exhibiting a family history of thyroid autoimmune disorders in first-degree relatives [9]. A further epidemiological peculiarity of HT in childhood is the relatively frequent association with other autoimmune extrathyroidal diseases (17.6 %) [9]. Among the associated autoimmune diseases, the ones that are most frequently encountered in pediatric age are celiac disease, type 1 diabetes, vitiligo, and Addison's disease [9]. In adulthood, the autoimmune extrathyroidal diseases which are most frequently associated with HT are rheumatoid arthritis, pernicious anemia, vitiligo, Addison's disease, and celiac disease [10]. In the light of the above data and the results of other studies, it is largely accepted that HT segregates within families and that both children and adults with HT are more exposed to other autoimmune diseases [10–12].

Table 16.1 Constitutional, environmental, and clinical factors that may condition an increased susceptibility to Hashimoto's thyroiditis in pediatric age

Constitutional and environmental factors	Clinical factors
Female sex	Antecedents of Graves' disease
Adolescent age	Association with extra-thyroidal autoimmune diseases
Familiarity for thyroid diseases	
Iodine status alterations	Association with Turner syndrome
Selenium deficiency	Association with Down's syndrome

Another relevant risk factor for HT in childhood is the association with either Down syndrome (DS) or Turner syndrome (TS), i.e., two chromosomopathies which have been shown to be linked with increased prevalence rates of both Graves' disease (GD) [13, 14] and HT [15, 16]. According to a very recent study on children' HT, 9 % of young patients with HT are affected by either DS or TS [9]. In particular, the prevalence of HT in TS is generally reported to fluctuate from 10 [17] to either 17 [18] or 21 % [19], and also in DS, HT is by far the most common autoimmune disease [20].

The mechanism responsible for the increased risk of autoimmune thyroid disorders and other autoimmune diseases in TS has been recently postulated to be associated with haploinsufficiency for X-chromosome-related genes [21], which may play an important role in the pathogenesis of autoimmune conditions [22]. Whereas some investigators reported that autoimmune thyroid disorders are especially common in TS girls with X-isochromosome karyotype, according to other authors HT is not associated with any specific karyotype [17, 18, 23].

Also in DS children, the mechanisms responsible for the strong association with HT have not been clearly elucidated [20].

The main epidemiological risk factors for HT are summarized in Table 16.1.

16.3 Pathophysiology

The current dogma is that HT develops in genetically predisposed individuals, in conjunction with exposure to environmental triggers [24].

The strongest evidence for a genetic contribution to the etiology of HT lies in twin studies, which demonstrated a higher concordance rate in monozygotic than in dizygotic twins: 30–40 % vs 0–7 % [25]. Thus, the twin data corroborate the presence of a substantial inherited susceptibility to HT [24].

The HT susceptibility genes can be divided into immunomodulating genes and thyroid-specific genes. The first group includes the cytotoxic T lymphocyte antigen-4 (CTLA-4) and the protein tyrosine phosphatase nonreceptor-22 (PTPN22) genes and especially some HLA haplotypes (DQA1, DQ2, and DRB1-1401) [26]. The second group includes thyroglobulin (TG) and thyrotropin (TSH) receptor genes [24, 26]. All these genes seem to participate in the immunological synapse

and/or the signaling pathways activated by the immunological synapse. This provides a potential molecular explanation for interactions between these HT susceptibility genes [24].

Among the environmental triggers, an important role is played by iodine status alterations [27] and selenium deficiency [28]. In particular, iodine deficiency is seen more frequently in the HT cases with hypothyroidism, while iodine excess is observed more frequently in those with hyperthyroidism [29]. However, it is well known that iodine supplementation is associated with an increasing risk of HT in people from iodine-deficient areas [30, 31] and that patients with HT are prone to develop hypothyroidism following iodine administration. The mechanism underlying the proimmunogenic effect of iodine in humans remains to be explained [7], but in mice the incorporation of iodine increases the immunogenicity of TG [32].

Another environmental factor which might be able to affect an increased susceptibility to HT is selenium deficiency, probably due to the effects of this mineral on immune systems [28]. Experimental studies demonstrated a significant reduction in TG-autoantibodies (TGAbs) following selenium supplementation in mice with iodine-induced autoimmune thyroiditis [33]. Nevertheless, clinical studies on the beneficial effects of this treatment in patients with HT are very few [28].

In the individuals who are genetically predisposed to HT and exposed to environmental risk factors, humoral autoimmunity is triggered by the abnormal stimulation of T lymphocytes, with consequent destruction of thyroid cells by chemotaxis, autoantibodies, and inflammatory cascade. The degradation of thyroid cells may be possibly compensated by increased TSH secretion, with consequent hyperplasia of epithelial cells and gland enlargement. However, increased TSH serum levels and goiter are not always detected in patients with HT.

In addition to the usual form of HT, other variants such as the fibrous type are also known. The most recently recognized variant is immunoglobulin G4 HT, which may occur as isolated thyroid limited disease or as a part of a generalized immunoglobulin G4-related sclerosing disease [34–36].

16.4 Interrelations with GD

Although HT and GD have different phenotypes and the mechanisms leading to their dichotomy are unknown, they are generally believed to share a number of common etiological factors. In fact, there have been reports on monozygotic twins in whom one twin had HT and the other had GD [37]. Moreover, both diseases may aggregate in the same families [38] or may even coexist in the same gland [39], and some patients may progress over time from one form to the other.

The metamorphosis of clinical phenotype from GD to HT or vice versa has been, in recent years, the theme of several reports, which raised interesting questions about the mechanisms of these fluctuations and concluded that, in the general population, there exists a continuum between HT and GD within the spectrum of

autoimmune thyroid diseases [40–44]. A mechanism that has been postulated to account for the switching from HT to GD is the alteration in the biological activity of TSH receptor autoantibodies (TRAbs), from predominantly thyroid-blocking antibodies during the HT phase to thyroid-stimulating antibodies when GD manifests itself [40]. According to this hypothesis, the emergence of thyroid-stimulating antibodies after levothyroxine (L-T4) therapy might be sufficient to counteract thyroid-blocking antibody inhibition [45]. However, although the pathophysiological bases of these conversion phenomena have not been clearly elucidated as of yet, it is well assessed, in the clinical practice, that GD presentation may be preceded in 3.7 % of cases by HT antecedents [41] and that this metamorphosis is by far more frequent (25.7 % of cases) in the patients with DS or TS [46]. It has been suggested, on the basis of these findings, that these chromosomal abnormalities might favor metamorphosis from HT to GD and that children with these chromosomopathies and coexisting HT might be at higher risk of progressing to GD [46]. However, the pathophysiological bases of this predisposition need to be elucidated.

16.5 Criteria for Diagnosis

HT diagnosis is based on the combination of clinical features, positivity of thyroid peroxidase autoantibodies (TPOAbs) and TGAbs, and specific US alterations, while thyroid function tests, radioiodine uptake, and FNAC are less relevant for diagnostic purposes.

TPOAbs are generally considered as the most specific serological marker of HT, since they are detected in around 95 % of HT patients, whereas they are rare in healthy individuals. TPOAb titers, moreover, are closely associated with the degree of US hypoechogenicity. TGAbs are positive in only 60–80 % of HT patients, which demonstrates a low degree of sensitivity. Moreover, they are also less specific since they are positive in a greater proportion of healthy controls. Nevertheless, also TGAbs have their own usefulness [47]. In fact, TGAbs and TPOAbs may represent two different aspects of the autoimmune response against thyroid gland, with TGAbs reflecting a more initial type of immune response and TPOAbs reflecting a later adaptive immune response [7].

US criteria for diagnosis of HT are based on the finding of a reduced echogenicity of thyroid gland, which reflects the histological changes occurring in the parenchyma as consequences of the inflammatory destruction of thyroid follicles. These are replaced by small lymphocytes, so that gland echogenicity progressively decreases, becoming similar to that of the surrounding strap muscles [7]. Thyroid echogenicity may be scored, in the clinical practice, according to the standards assessed many years ago by Sostre and Reyes [48]. These US scores maintain over the years a satisfactory clinical reliability and may be still employed, even nowadays, in the clinical practice, since they are able to depict the severity of inflammatory gland injury. In fact, they may also correlate with gland size and/or thyroid

Table 16.2 Echographic scores vs goiter size, thyroid function clinical and biochemical status and antimicrosomal autoantibodies (MCHAbs) in adults with Hashimoto's thyroiditis

Scores	Goiter size	Euthyroidism	Overt hypothyroidism	MCHAbs positive
	(grams)	(%)	(%)	>1:1,600 (%)
G1	27	100	0	0
G2	37	50	0	62.5
G3	33	25	50	83.3
G4	52	9	83	75.0

According to the study of Sostre and Reyes [48]

function status and/or severity of autoimmune process, as found by Sostre and Reyes [48]. According to that study, the gradual decrease of thyroid echogenicity from G1 to G4 patterns is accompanied by a progressive increase in goiter size, hypothyroidism prevalence, and autoantibody positivity, as well as by a concomitant decrease of euthyroidism prevalence (Table 16.2).

16.6 Thyroid Function Tests at Presentation

At the time of diagnosis, children and adolescents with HT may be asymptomatic, and the main reasons for referral are goiter, hypothyroid symptoms, and findings which occur while working on unrelated problems or for high-risk groups [49].

Thyroid function at presentation may significantly vary in the different pediatric reports [3], ranging from euthyroidism to overt hypothyroidism or, occasionally, overt hyperthyroidism [50]. Further complaints of thyroid function reported in children and adolescents at HT presentation include either subclinical hypothyroidism (SH) [3, 51, 52] or more rarely, subclinical hyperthyroidism [53].

In a very recent study, we retrospectively evaluated clinical and laboratory characteristics at HT diagnosis in 608 children and adolescents from three pediatric endocrinology centers in Northern and Southern Italy [9]. Our test results at presentation showed euthyroidism in 52.1 % of patients, overt or SH in 41.4 %, and overt or subclinical hyperthyroidism in 6.5 %. The mean age of patients with thyroid dysfunctions was significantly lower than that found in euthyroid children. Other variables related to thyroid function patterns were prepubertal status and association with either DS or TS, which correlated with increased risk of thyroid dysfunctions [9]. Overall, thyroid function patterns at HT presentation seem to be mainly conditioned by children's age, with an increased risk of severe gland dysfunctions in the cases with early HT presentation [9]. Other factors that may also be involved in the biochemical presentation pattern of HT are the association with either chromosomopathies or other autoimmune diseases [9, 54] and environmental factors [55].

The different presentation modes of HT have been recently summarized and commented in a commentary of our study group [56].

16.7 Clinical Features

The most frequent clinical manifestation of juvenile variant of HT is goiter, but most children may also be asymptomatic at the time of diagnosis.

The prevalence of goiter is generally higher in hypothyroid children [52]. By contrast, other authors have reported an increased prevalence of goiter in euthyroid patients [49]. Finally, according to others, the prevalence of goiter is comparable in euthyroid, hypothyroid, and SH patients [57].

Other less frequent manifestations are those originating from compression of the cervical structures that are anatomically contiguous to thyroid gland and include hoarseness, cough, dysphonia, dysphagia, or, more rarely, dyspnea.

In the cases with more severe impairment of thyroid function, systemic manifestations may also be observed and include signs of hypothyroidism or even hyperthyroidism in the cases presenting with hashitoxicosis (Htx).

The most frequent symptoms of hypothyroidism at HT presentation are constipation, bradycardia, and changes of skin and appendages (dry, cold, yellowish, and thickened skin, coarse hairs, and thin nails). Less frequent presenting manifestations of hypothyroidism involve hematopoietic system (hypochromic and microcytic anemia), skeletal muscles (increased transaminase serum levels), and neuropsychiatric system (memory and attention loss, with inability to concentrate and impaired scholastic performances). Clinical pictures of hypothyroidism may be associated with a strong positivity of TPOAbs and TGAbs [52] and a more severe degree of hypoechogenicity [48].

In a limited number of cases (3.5 %), HT may present with a transient hyperthyroid picture, and this presentation pattern is known as Htx, which is believed to result from unregulated release of stored thyroid hormones during inflammatory-mediated destruction of thyroid gland. It is the second commonest cause of hyperthyroidism in childhood [58], and its presenting clinical picture is not very different from that observed in GD [59]. However, in the majority of cases, the differential diagnosis with GD is straightforward, considering the milder clinical and biochemical phenotype, the absence of TRABs, and the spontaneous resolution of hyperthyroidism that is frequently observed. Nevertheless, in some cases with clinical and biochemical features overlapping between Htx and GD, differential diagnosis between these two disorders may be very complicated [58, 59], and duration of biochemical hyperthyroidism may be abnormally extended (Fig. 16.1). In these few cases, a prolonged treatment with antithyroid drugs (1–2 years) may be also needed, whereas a nonpharmacological treatment is never needed [60].

16.8 HT and Nodular Disease

The literature contains only few specific studies about children with nodular HT, and the available data on the occurrence of thyroid cancer in HT refer almost exclusively to adults.

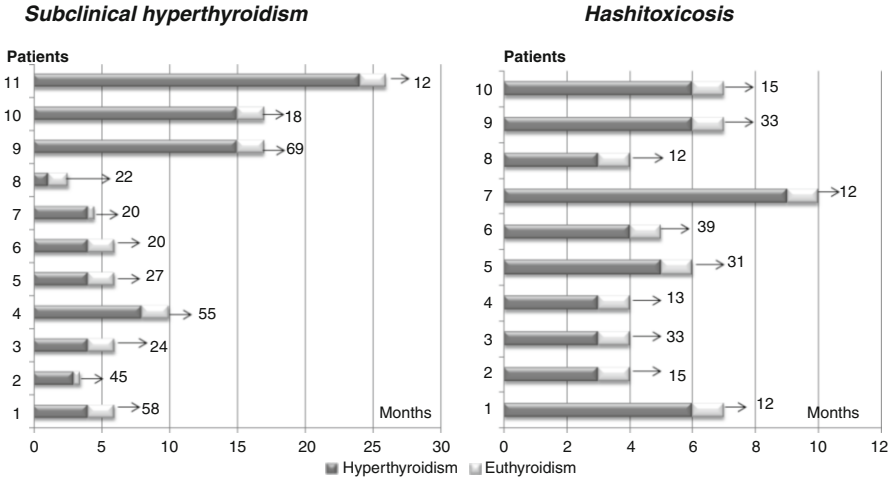

Fig. 16.1 Duration of biochemical hyperthyroidism in two groups of children with either subclinical hyperthyroidism or hashitoxicosis (Refs. [60, 71], respectively). *Grey bars* refer to the periods during which they were hyperthyroid, whilst white bars indicate the development of persistent euthyroidism. The *arrow* and number at the end of each bar refer to the overall duration of follow-up (months) after resolution of hyperthyroidism

The only available study aiming to analyze the relationships among HT, thyroid nodules, and cancer in a large population of pediatric patients has recently demonstrated that nodular disease occurs in 31.5 % of young patients with HT, while cancer occurs in 3 % of cases and in 9.6 % of the subset with nodules, with papillary carcinoma being the most common histological type [55]. This cancer prevalence in HT patients is equal to or higher than that reported in other pediatric studies [61, 62] and much lower than that found in other study populations consisting primarily of adults [63].

Among the children of that series [55], the diagnostic accuracy of FNAC in differentiating benign from malignant lesions was 94.4 %, with a sensitivity of 88.9 and a specificity of 100 %. Other two factors that were significantly associated with cancer risk were the clinical finding of locoregional lymphadenopathy and the US evidence of nodular growth under L-T4 therapy. No other clinical, biochemical, or US factors were significantly predictive of cancer risk (Table 16.3).

Overall, the most recent findings on the links between HT, nodular disease, and thyroid cancer do not support the hypothesis [64] that the lymphocytic infiltration of thyroid gland, which is typical of HT, may play any protective role against proliferation of cancerous cells.

16.9 Natural Evolution Over Time and Long-Term Prognosis

The evolution over time of biochemical pictures is conditioned by presentation patterns and may significantly vary according to them.

Table 16.3 Analysis of the factors with or without predictive value for cancer in children with Hashimoto's thyroiditis and nodular disease

Predictive factors	Non-predictive factors
Male sex	Age
Suspicious cytology	Thyroid function tests
Locoregional lymphadenopathy	Uninodularity vs multinodularity
Echographic evidence of nodular growth under L-T4 therapy	Nodule echogenicity

According to the study by Corrias et al. [55]

Among the HT children presenting with biochemical euthyroidism, 42 % remain persistently euthyroid after a 5-year follow-up, and 52 % develop over time an SH condition, whereas only 6 % become overtly hypothyroid [4]. The presence of goiter and elevated TGAbs at presentation, together with progressive increase in both TPOAb and TSH serum levels, may be predictive factors for a future deterioration of thyroid function [4].

Among the children presenting with HT-related SH, the risk of deterioration over time of thyroid function is even higher, even though the process is very slow and not predictable in the single case [65]. The coexistence of additional risk factors such as celiac disease, elevated baseline TSH, and TPOAb serum levels further increases such a risk 3.4–4.0 fold [65]. Therefore, it can be argued that HT children with SH and additional risk factors should be followed up with periodical TSH measurements [65], since the risk of worsening thyroid function over time is higher in the SH children with an underlying HT than in those with no underlying thyroid disease [9, 66, 67]. This inference is supported by the most recent reviews on SH [68–70].

In the children presenting with HTx, a definitive resolution of hyperthyroidism is generally observed on average 8 months after Htx diagnosis, even though there is a wide variability between subjects [60]. Hyperthyroid phase in children with Htx is always followed by definitive resolution (Fig. 16.1) and evolves to permanent euthyroidism or hypothyroidism, with no relapses [60].

Finally, in the cases presenting with HT-related subclinical hyperthyroidism, this biochemical picture may spontaneously resolve in the majority of cases within the first 24 months after HT diagnosis (Fig. 16.1), and the risk of a progression toward clinically overt hyperthyroidism has to be considered very low, irrespectively of both TSH and FT4 baseline serum levels [71].

Long-term prognosis is variable, with remission, recurrence, and evolution into permanent hypothyroidism all being described [7]. However, according to the historical study by Rallison et al. [72] based on 61 children and adolescents between 11 and 18 years, the long-term evolution of HT after a 20-year follow-up is characterized by a permanent remission in 33 % of cases, while in the remaining 67 % of patients the thyroid injury persists over time. This evolutive trend does not seem to be conditioned by L-T4 therapy [72].

Table 16.4 Main risk factors and etiological factors for subclinical hypothyroidism in children and adolescents

Risk factors	Etiological factors
Familial antecedents of thyroid diseases	Hashimoto's thyroiditis
Obesity	Non-compliance with levothyroixine treatment in primary hypothyroidism
Antecedents of radiation to head and neck	
False positivity at congenital hypothyroidism screening	Chronic treatment with iodine containing medications
TSH receptor gene alterations	Overtreatment with anti-thyroid drugs in
Down's syndrome	Graves' disease
Turner syndrome	Chronic non-thyroidal diseases

16.10 HT and SH

SH is a common clinical problem that is caused by the same thyroid disorders that cause overt thyroid failure and especially HT (Table 16.4). Its average worldwide prevalence has been reported to be in the range 4–10 % in large general population screening surveys [73], 7–26 % in the elderly [74], and <2 % in childhood and adolescence [75].

In the last years, SH has been discussed in a number of editorials, commentaries, controversies, and letters to editors concerning this topic [76]. Discussions are mainly focused on whether SH should be treated or not. In children with the idiopathic form, current views are not in favor of a systematic treatment of SH, considering the low risk of a spontaneous deterioration over time of thyroid function [66]. By contrast, in the children with HT-related SH, the risk of a worsening over time of thyroid function tests seems to be more elevated [66, 77]. Therefore, considering that an underlying HT may negatively affect the evolution over time of children' SH and that L-T4 therapy may have some beneficial effects on the clinical course of HT-related SH and on antibody titers [53], a different treatment policy might be hypothesized for the children with HT vs those with idiopathic SH. Nevertheless, such hypotheses should be verified through comparative investigations based on very large study populations.

16.11 Treatment

Synthetic L-T4 remains the only effective drug available to patients with HT, many decades after the start on its production on a large scale (in 1960). This treatment is mandatory in the cases with overt hypothyroidism or progressively worsening SH, whereas it is controversial in the cases with mild SH or euthyroid goiter. If thyroid enlargement is severe, it may be efficaciously counteracted by L-T4 treatment [57].

Therapy is given daily at doses of 2 mcg/kg body weight, but this initial dose needs to be periodically monitored.

Although this is a symptomatic treatment, which addresses the symptoms rather than the etiology and the pathogenesis of this disorder, nevertheless it is effective in most patients, and economic and therefore pharmaceutical companies have not been stimulated, over the years, to develop new drugs [7].

This treatment is not always lifelong, considering that in a 33 % of cases HT may have a positive long-term prognosis, with complete remission of the autoimmune process and consequent normalization of biochemical and clinical picture [72].

Thyroidectomy is indicated only in the cases with a clinical picture of severe cervical compression or, more frequently, in the children with a thyroid nodule that, at cytology, is suspicious for malignancy [78]. When advisable, avoiding thyroidectomy is particularly relevant in HT patients [7], since the surgical complications are more numerous in this disorder than in other thyroid diseases [79].

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Subclinical Hypothyroidism

17

Alice Monzani, Flavia Prodam, Simonetta Bellone, and Gianni Bona

17.1 Definition

Subclinical hypothyroidism (SH) is defined as a serum *TSH* concentration above the statistically defined upper limit of the reference range when serum free T4 (fT4) concentration is within its reference range [1, 2]. As serum TSH concentration varies over time in healthy subjects, leading to occasional abnormal values, the measurement of serum TSH and fT4 should be repeated within 3–4 months. If elevated serum TSH concentrations are confirmed and fT4 levels are within the normal range, the diagnosis of SH is made. SH is also defined as *isolated hyperthyrotropinemia*, compensated hypothyroidism, preclinical hypothyroidism, mild thyroid failure, or mild hypothyroidism. The diagnosis of SH is mainly based on a biochemical evaluation because it has been observed that most patients exhibit few or no signs or symptoms of thyroid dysfunction. Many studies suggest that some patients do indeed have clinical or functional manifestations of mild thyroid failure that are more frequent than in age-matched controls [2].

17.2 Epidemiology

SH prevalence in the adult population is reported to be 1–10 % in most community-based studies, being higher in the elderly population, in females, and in white subjects [3–5]. The prospective Whickam survey reported a 7.5 % prevalence of SH in women and 2.8 % in men. SH prevalence rises to 11.6 % in women aged >60 years

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Table 17.1 Etiology of SH

Thyroid-related causes	Systemic causes
Hashimoto's thyroiditis	Diabetes mellitus
Iodine deficiency	Celiac disease
Overtreatment of Graves' disease	Chronic renal failure
Transient neonatal hyperthyrotropinemia	Syndromes (Turner, Down …)
Variations in genes of TSH/thyroid hormone pathway	Overweight/obesity
X-ray treatment of the head-neck region	Medications (carbamazepine, valproic acid, domperidone …)

[6]. In the pediatric population, SH prevalence is reported to be slightly lower than 2 %, even if epidemiological studies in childhood and adolescence are scanty [7, 8].

17.3 Etiology

The possible causes of SH are synthesized in Table 17.1. Many thyroidal and non-thyroidal diseases have been shown to be associated with the pathogenesis of SH in pediatric age; often, however, no overt causes are detected, and a condition of *idiopathic SH* is defined [9]. Among the thyroidal causes, the most frequent are *Hashimoto's thyroiditis*, *iodine deficiency*, and the overtreatment of Graves' disease.

Nonthyroidal illnesses include diabetes mellitus, cystic fibrosis, celiac disease, chronic renal failure, and many syndromes, such as Turner, Down, Klinefelter, and Williams syndrome [10].

A condition often associated with SH is *overweight/obesity*, even if a clear cause-effect relationship has not been established yet. Whether an elevated TSH value in childhood obesity is adaptive, increasing energy expenditure rate in order to prevent further weight gain, or indicates subclinical hypothyroidism or thyroid hormone resistance is still controversial [11, 12].

A condition of SH may also be related to the use of some medications, in particular carbamazepine, valproic acid, or domperidone, and to X-ray treatment to the head and neck for malignant diseases [13].

SH may be the outcome of transient neonatal hyperthyrotropinemia [14]. The prevalence of SH in newborns considered false positive for congenital hypothyroidism (i.e., with high TSH at birth and with normal fT4 and normal or slightly elevated TSH at the confirmatory examination) was reported to be about 50 % in infancy and early childhood and decreases with age, being still >30 % in late childhood [15].

Mutations in genes encoding proteins involved in the TSH pathway have been demonstrated to be responsible for SH [16]. TSH exerts its activity by binding to the extracellular domain of TSH receptor (TSH-R), a G protein-coupled seven-transmembrane domain receptor located in the basolateral membrane of thyroid follicular cells. Some loss of function mutations, which may interfere with the normal receptor function leading to SH, have been identified in the TSH-R gene [17–21]. Moreover, polymorphisms or mutations in genes of the thyroid hormone pathway

such as dual oxidase 2, phosphodiesterase 8B, iodothyronine deiodinases, and thyroperoxidase may be responsible of the elevation of TSH level with normal fT4 values [22–25].

17.4 Clinical Manifestations

The clinical presentation varies widely, ranging from no manifestations to clear signs or symptoms of hypothyroidism. The most common clinical sign is *goiter*, reported to be twice as prevalent as observed in the general population [10]. The abnormalities most frequently associated in the pediatric population are *weight gain* [16], increased cholesterol levels, *impaired growth velocity*, anemia, sleepiness, weakness, and *impaired psychomotor and cognitive development* [8, 26].

However, a recent review analyzing the natural history of SH in pediatric subjects reported in the studies included normal height, BMI, and the age of puberty onset and no clinical manifestations of hypothyroidism, regardless of the evolution of thyroid function [27]. And a recent Italian study analyzing growth and intellectual parameters in a cohort of children with persistent idiopathic SH demonstrated no alterations in growth, bone maturation, BMI status, and cognitive functions despite persistently elevated TSH values [28]. Therefore, thyroid hormones involved in growth and neurocognitive development seem to work properly, regardless of the persistence of elevated TSH levels, in the absence of any replacement therapy.

17.5 Diagnosis

The laboratory detection of a TSH value above the normal upper limit in the presence of normal thyroid hormone levels should raise the suspicion of SH. As serum TSH concentration varies over time even in healthy subjects, an abnormally elevated value may be an occasional finding. Therefore, the measurement of serum TSH and fT4 should be repeated within 3–4 months. A careful case history should be collected, including coexisting diseases, family history for thyroid diseases, and result of the neonatal screening for congenital hypothyroidism. Symptoms of hypothyroidism, such as constipation, poor memory, fatigue, slow thinking, anxiety, depression, muscle weakness, and cold intolerance, should be investigated. An accurate physical examination should be performed in search of signs of thyroid failure, such as overweight/obesity, short stature or impaired growth velocity, hoarseness, and thinning hair. In particular, thyroid region should be inspected and palpated in order to detect goiter. If the isolated TSH elevation is confirmed, *antithyroglobulin antibodies* (TG-Abs) and *antithyroperoxidase antibodies* (TPO-Abs) titer should be evaluated and *thyroid ultrasonography* performed.

The diagnostic flowchart for SH is shown in Fig. 17.1.

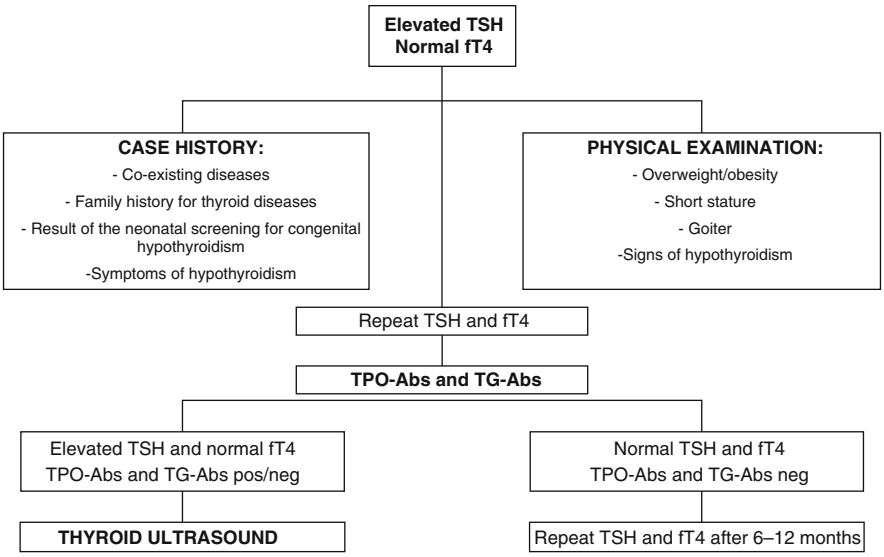

Fig. 17.1 Diagnostic flow-chart for SH

17.6 Natural History

The natural evolution of SH may be the *persistence* of a condition *of SH* (i.e., normal thyroid hormone levels with stably elevated or even raised TSH levels), the *reversion to euthyroidism* (i.e., normalization of TSH values with normal thyroid hormone levels), or the *progression to overt hypothyroidism* (high TSH levels with reduced thyroid hormone levels).

In adult populations, SH was reported to progress toward overt hypothyroidism (reduced circulating thyroid hormones) in proportions ranging from <1 up to 20 % according to different studies [1, 3, 4]. Higher rates of progression to hypothyroidism were reported in patients with higher baseline serum TSH concentration, elevated antithyroid autoantibodies, and a higher degree of hypoechogenicity at thyroid ultrasound [6, 29].

Studies regarding the natural history of SH and its consequences in childhood are scanty [27, 30]. According to the available studies [15, 31–38], SH in children and adolescents seems to be a benign and remitting process with a low risk of evolution toward overt hypothyroidism. Indeed, most of the subjects analyzed in these studies reverted to euthyroidism or remained SH, sometimes with an increase in TSH values. The rate of development of an overt hypothyroidism ranged between 0 and 28.8 %; only one study [36] showed the evolution toward overt hypothyroidism in half of the eight included children.

The initial presence of goiter and elevated TG-Abs, the presence of celiac disease, and a progressive increase in TPO-Abs and TSH value may be predictive of progression toward thyroid failure [38, 39]. Moreover, an initial TSH higher

than 7.5 mIU/l and the female gender are predictive factors for a sustained elevated TSH [37].

Notably, a high persistence of SH in "false-positive" children 'at neonatal screening for congenital hypothyroidism was reported in one study, even if none of the children developed an overt hypothyroidism in the follow-up period [15]. Studying thyroid morphology, the presence of hemiagenesis, hypoplasia of one lobe, or goiter were detected in half of these children and mutations in TPO and TSH-R genes found in two children. Therefore, a mild hyperthyrotropinemia at neonatal screening may be suggestive of congenital anatomic or functional anomalies of the thyroid gland and may be followed by persistent SH later on in childhood.

17.7 Treatment Options

In the decision to treat or not a condition of SH in children, pediatricians should consider on the one hand the risk of progression to overt hypothyroidism and on the other hand the systemic consequences of leaving the hyperthyrotropinemia untreated. In adults, SH has been associated with an increased risk for cardiovascular diseases [40–43] and biochemical abnormalities, including elevated LDL cholesterol, increased serum prolactin concentrations, and a negative influence on the hemostatic profile [44–47]. However, clinicians should likewise consider the risks of an overtreatment, since an overtreated SH could bring on subclinical hyperthyroidism, which has been reported to be responsible for significant bone loss in postmenopausal women [48] and atrial fibrillation in older patients [49]. Moreover, potential benefits of higher TSH levels such as longevity and lower all-cause mortality have been suggested by recent studies [50, 51].

Indeed, the decision regarding the use of *levothyroxine* (L-T4) *replacement therapy* for SH is still a matter of debate. In 2004 and 2005, two expert panels came to different conclusions about the management of SH in adults. One expert panel [1] concluded that patients with normal fT4 and TSH >10 mIU/l may be treated, whereas it advised follow-up of subjects with TSH in the range of 4.5–10.0 mIU/l because of insufficient evidence to support treatment in these latter patients and because of the concern of overtreatment. On the other hand, a consensus statement jointly published by the American Association of Clinical Endocrinologists, the American Thyroid Association, and The Endocrine Society [52] concluded that treatment of SH patients with TSH levels of 4.5–10 mIU/l was appropriate, as the lack of evidence of benefit does not necessarily mean a lack of benefit.

Both panels did not address the issue of SH in the pediatric population. Very few studies have examined the effects of L-T4 replacement therapy in young people with SH [27, 30]. L-T4 therapy in children with SH may be beneficial in the presence of short stature and impaired growth velocity [53, 54] and may be effective in reducing thyroid volume [55]. No effects of L-T4 on neuropsychological functions of children with SH have been reported [26].

Hyperthyroidism due to an *overtreatment* of SH should be considered an infrequent condition in the pediatric age-group.

According to the available evidence, in children with SH L-T4 treatment should be indicated when TSH >10 mIU/l or when TSH 4.5–10.0 mIU/l if in the presence of clinical signs or symptoms of impaired thyroid function, goiter, or coexisting conditions such as syndromes (Down, Turner, etc.) or autoimmune diseases (diabetes mellitus, celiac disease, etc.), possibly predisposing to the progression toward an overt hypothyroidism. In children with SH but with no goiter, negative antithyroid antibodies, and TSH <10 mIU/l, replacement therapy is not justified, both because of the low risk to develop an overt hypothyroidism and because they could simply be euthyroid outliers, representing 2.5 % of normal individuals whose TSH values are above 97.5th percentile of euthyroid distribution. Indeed, a slightly elevated TSH may just be the sign of a physiological adaptation in thyroid-healthy children from iodine-replete areas, whose smaller thyroid volume induced by an improved iodine status does presumably require a higher TSH to maintain an adequate thyroid hormone production [56].

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Hyperthyroidism

18

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Hyperfunction of the thyroid gland could be related to different etiologies. The term *hyperthyroidism* refers only to a situation of hypersecretion of thyroid hormones (THs) by the thyroid gland. However, different conditions may lead to an excess of TH serum levels. This is, for example, the case of a destructive thyroiditis, where there is not a hypersecretion of TH but simply an excessive release of preformed hormones from the thyrocytes damaged by the inflammatory process. Another possibility is the exogenous intake of thyroxine for losing weight, the so-called thyrotoxicosis factitia. However, in the clinical practice ,the term *hyperthyroidism* is generically used for all such conditions. Graves' disease is the most common cause of hyperthyroidism in the pediatric age, followed by Hashimoto's thyroiditis. Other etiologies such us nodular goiter, toxic adenoma, thyroid hormone resistance syndrome, and pituitary adenoma are less frequent.

18.1 Graves' Disease (GD)

GD prevalence in the pediatric age is about 1:5,000 [1], and it is the most common cause of hyperthyroidism in children. GD incidence increases progressively with age peaking at adolescence, being 3:100,000 adolescents [2]. Females are more affected than males (F:M = 5:1).

It is a typically Th2-type autoimmune disease, resulting from a complex interaction between genetic factors, environment, and immune system. Genetic susceptibility to the disease has been linked to HLA antigens DR3, DQ 2, and DQA1*0501,

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to PTPN22 gene on chromosome 1p13, and to cytotoxic T lymphocyte antigen-4 (CTLA-4) gene on chromosome 2q33. It has been hypothesized that T- lymphocyte suppressor cells' function is diminished and their number is reduced, leading to the production of autoantibodies stimulating the thyroid function. These antibodies interact with TSH receptors in a positive functional manner by adenyl cyclase and phospholipase A2 functions, causing thyroid stimulation. Functionally, antibodies mimic TSH action, most of them having a stimulating effect and enhancing the production of thyroid hormones. However, some antibodies bind to the receptor without stimulating it. They thus block the binding of TSH to the receptor and exert an inhibitory effect. These antibodies are known as thyroid stimulation blocking antibodies. The secretion of thyroid hormones depends therefore on the balance between such opposing actions, which may contribute to explain the oscillation of thyroid hormones often seen in GD patients [1–4].

18.1.1 Clinical Aspects

Most of the GD clinical features result from the direct effect of thyroid hormones on the target tissues, but others may be a further expression of the same autoimmune process or of another associated autoimmune disease. Indeed, it is quite common to observe other autoimmune diseases such as type 1 diabetes mellitus, coeliac disease, vitiligo, etc. in GD patients.

Tachycardia is a typical sign of thyroid hormone excess on heart, but other signs such as elevated blood pressure, precordial thrill, and/or an ejection murmur due to functional insufficiency of the mitral valve may be present. There is often a delay in the diagnosis since, before coming to the attention of a pediatric endocrinologist, the child has already been assessed by other specialists, mainly a cardiologist.

Bone is also extremely sensitive to the action of thyroid hormones. Clinically, an increased growth velocity can be observed alongside an enhanced bone maturation resulting in an advanced bone age. However, it takes time for these features to develop, and therefore they can be detected in few cases, due to increased medical knowledge and care. Moreover, thyroid hormones strongly influence bone metabolism, inducing a high bone turnover with an uncoupling between the resorptive and the anabolic phase, which may result in a net bone loss and thus osteoporosis over the years. However, this mostly happens in adults suffering from an unrecognized form of subclinical hyperthyroidism, as in the case of a nodular pathology lasting for many years. In children and adolescents, where the thyrotoxic phase is generally promptly diagnosed, there is only a transitory phase of bone loss which completely recovers after normalization of thyroid hormones [5].

Muscle function may be severely compromised, mostly due to TH-induced protein wasting. A decreased muscle mass, particularly of the proximal muscles, and a reduced force can be observed. It is speculated that the toxic muscle requires more energy to function than normal, presumably because of additional ATP-consuming mechanisms. However, myasthenia gravis, another autoimmune disease. may be associated [6].

Gastrointestinal symptoms are not very common and even when present are mild. They can depend both on TH excess which causes an enhanced intestinal

transit, although not a frank diarrhea, and also on a concomitant coeliac disease. The latter should always be investigated when symptoms do not disappear following normalization of TH.

Eye involvement can be observed in most cases; however, it is not so marked as in adult patients. Signs and symptoms are milder, producing less long-term consequences. The most common sign is lid retraction, which gives a staring expression, and the lag of the lids behind the globes on downward rotation, as well as the failure to wrinkle the forehead on looking upward. All these signs are secondary to thyroid hormone excess which causes the contraction of orbicular muscles and which solves spontaneously following the normalization of TH levels. A real ophthalmopathy with inflammation of extraocular muscles, orbital fat, and connective tissue may be present in some cases, producing a proptosis together with periorbital edema and muscular dysfunction, due to inflammation of medial and lateral rectus muscles. There is now strong evidence that the immune reaction which leads to Graves' ophthalmopathy is directed against TSH receptors expressed in the orbital fibroblasts and adipocytes. The most common symptoms are due to conjunctival or corneal irritation and include burning, photophobia, tearing, pain, and a gritty or sandy sensation. It is very rare to observe a decrease in visual acuity as in adult patients. Eye drops and sunglasses are mostly used to prevent conjunctival dryness, while treatment with corticosteroids may be avoided [7, 8].

Pretibial edema is very uncommon in pediatric age. Its mechanism, similarly to ophthalmopathy, is stimulation of TSH receptors, aberrantly expressed in the skin.

A symmetrical goiter may be present, but in many cases thyroid volume is not particularly increased. A firm goiter at palpation and a thrill can be heard due to increased perfusion of the gland [9, 10].

Biochemical profile: there is usually a favorable lipid profile with low serum total and HDL cholesterol and low total cholesterol/HDL cholesterol ratio with plasma triglycerides in the lower normal range. The carbohydrate profile is characterized by an increased demand of insulin due to increased hepatic glucose production and to reduced insulin sensitivity. On the other hand, type 1 diabetes mellitus may be present. Protein metabolism is globally accelerated. Nitrogen excretion is increased, and nitrogen balance may be normal or negative, depending on whether intake meets the demands of increased catabolism or not [11].

Central and peripheral nervous system are always affected by TH excess. Already from clinical inspection, a tremor is observed, which can be associated to brisk deep tendon reflexes and eventually fasciculation of the tongue. Tremor is best observed asking the child to outstretch both hands. In general, mood swings and behavioral problems can represent a common neuropsychological complaint in hyperthyroid children [12]. Therefore, they can be referred to a child neurologist with a diagnosis of attention hyperactivity disorder, as their attention span is decreased, their sleep pattern is deteriorated, and they are hyperactive in daily life [13].

Involuntary movement disorders, such as chorea, athetosis, ballism, or truncal flexion, eventually associated to ataxia are rarely described in children affected by hyperthyroidism; symptoms remit with treatment of hyperthyroidism [14].

Autoimmune neurological conditions, such as childhood-onset demyelinating disorder [15] or disorders of neuromuscular junction [16], rarely observed as the

only symptom in children affected by GD, can represent a manifestation of the common altered immune state. As other endocrine dysfunctions, hyperthyroidism can be linked to benign intracranial hypertension in children, presenting with chronic headache, papilledema, and normal neuroimaging [17]. Correction of the endocrine alterations is associated with remission of symptoms. Finally, in children presenting with hyperthyroidism and focal neurological deficits, Moyamoya disease should be investigated [18]. Moyamoya disease is a cerebrovascular disorder characterized by bilateral stenosis or occlusion of the terminal portions of the internal carotid arteries. Typical presenting symptoms are cerebrovascular accidents and epilepsy.

Laboratory The diagnosis is very easy and is based on the detection of high levels of free thyroxine (fT4) and free thriiodothyronine (fT3) with a suppressed TSH. Confirmation for GD comes from the presence of TSH receptor antibodies (TSHR-Abs). The best approach is to show the presence of these antibodies with stimulating activity (TSI), which, however, can only be done with a functional assay able to measure the production of cyclic AMP in cultured thyroid follicular cells. This method, however, is still considered a research tool, and in most labs the presence of such antibodies is evaluated by competitive protein binding methods, which shows only the presence of antibodies competing with TSH by binding to its receptor without providing any information about whether it is a stimulating or blocking antibody. In GD, there is actually a mix of antibodies with either stimulating or inhibiting activity and the actual thyroid function results from their balance. Obviously, in a hyperthyroid state is a good suggestion for a prevailing presence of antibodies with stimulating activity. However, a change in the relative ratio might explain the fluctuation of thyroid function often observed in GD patients [11].

18.2 Hashimoto's Thyroiditis (HT)

Hashimoto's thyroiditis is the most common endocrine autoimmune disease in the pediatric age, together with type 1 insulin-dependent diabetes. Furthermore, it is almost the only form of pediatric thyroiditis since the subacute form, the painless form, and Riedel's thyroiditis are seldom seen in pediatric patients. HT is a typical, organ-specific, autoimmune disease caused by an autoimmune-mediated destruction of the thyroid gland involving apoptosis of thyroid epithelial cells. There is a diffuse lymphocytic infiltration of the thyroid, which includes predominantly thyroid-specific B and T cells, and a follicular destruction.

Independently from the actual cause, at one time point a cell-mediated autoimmune attack against the thyroid starts with lymphocytes infiltrating the thyroid. Following destruction of the thyrocytes, new antigens such as thyroid peroxidase (TPO) are released, and the immune system reacts, building new antibodies against TPO (TPOAbs). Thyroid autoantibodies are thus not causative agents but just markers of the damage of the gland. Nevertheless, TPOAbs can activate the complement and thus damage thyroid cells; however, the real role played by this antibody-dependent cell cytotoxicity is still under debate.

TSH receptor antibodies (TSHR-Abs) may also be present in the sera of patients with HT. In contrast to GD where TSHR-Abs are usually stimulatory, in HT they usually inhibit the function of the receptor and thus preferably induce hypothyroidism. However, when TSHR-Abs exert a stimulatory action, they can induce an unusually severe form of hyperthyroidism, the so-called hashitoxicosis.

18.2.1 Hyperthyroid Phase

The hyperthyroid phase is usually caused by inflammation and autonomous release of preformed, stored thyroid hormone; however, if TSHR-Abs with stimulating activity are also present, the hyperthyroid phase may be more severe, lasting several months. This situation called hashitoxicosis must be recognized since the therapeutic approach is very different. In case of simple release of preformed hormones, the treatment is just symptomatic, aimed at alleviating symptoms through the use of beta-adrenergic antagonists if tachycardia or tremulousness are present. Usually, propranolol 1–2 mg/kg divided in three to four doses is employed and titrated according to the clinical picture and the laboratory values. When, however, TSHR stimulating Abs are present, the treatment needs to be different since there is an additional source of thyroid hormones. In this case in addition to beta-adrenergic antagonists, thyreostatic drugs, as in GD, are needed.

18.3 Thyroid Nodules

Autonomous thyroid nodules may be another cause of TH excess. In adults, a multinodular goiter is a very common situation while it is rare in children and adolescents. A situation where a multinodular goiter may be relatively frequent is the McCune–Albright syndrome, caused by a postzygotic activating mutation of the alpha subunit of stimulatory G protein, which allows for a constitutive activation of the TSH receptor in absence of the ligand. Hyperthyroidism is supported by the presence of multiple hyperfunctioning nodules. Very often, there is a concomitant bone fibrous dysplasia, precocious puberty, café au lait pigmentary skin lesions, pituitary adenomas, etc.

Solitary thyroid nodule, the so-called toxic adenoma, is also a relatively rare situation in the pediatric age. Diagnosis is easily made by finding elevated TH and suppressed TSH, a palpable nodule with confirmation at ultrasound. A thyroid scintigram shows a hypercaptation in the nodule with exclusion of the remaining parenchyma. No antibodies are detected [19].

18.4 Cancer Patients

Often, children suffering from malignant diseases receive thyroid irradiation as a complement for disease treatment, following craniospinal or total body irradiation before marrow transplantation. In many cases, an actinic destructive thyroiditis may follow.

18.5 Rare Forms of Hyperthyroidism: The TSHoma and the Thyroid Hormone Resistance (RTH)

18.5.1 TSHoma

TSHoma is a pituitary adenoma, usually benign, which secretes only TSH. More often, microadenomas are observed, though hormone production is often accompanied by an unbalanced hypersecretion of the glycoprotein hormone α-subunit (α-GSU). It is a rare situation in children as compared to adults. The hormonal profile is characterized by high levels of fT3 and fT4 together with a nonsuppressed TSH. This situation cannot be clinically differentiated from the more common RTH syndrome, and thus MRI is needed. Twenty to twenty-five percent of TSHomas are mixed adenomas, characterized by concomitant hypersecretion of growth hormone or prolactin [20, 21].

18.5.2 Resistance to Thyroid Hormones (RTH)

This condition has been recognized for the first time in 1967 while the first mutations in the *THRB* gene, which code for the TSH receptor β were identified in 1989 [22]. This mutation causes a decreased tissue responsiveness to thyroid hormone (TH). It has been always though that the cause were a mutation inactivating the *THRB* gene (*RTH-β*), but lately also mutations in the *THRA* (*RTH-α*) have been detected which result in hypothyroidism. The commonest *THRB* gene mutations are heterozygous and exert a dominant negative effect on wild type receptor. Deletions on the other hand, behave as a recessive dominance since there is no negative effect on the wild type receptor. Homozygous mutations are rare and cause a very severe phenotype. Patients present with elevated fT4 and fT3 and an inappropriately normal TSH. From the clinical point of view, due to the different tissue sensitivity to TH, the patients may be perfectly normal, suffer from hypothyroidism, or be frank hyperthyroid. There may be also a discordance in sensitivity among tissues, and the patients may present with tachycardia and retarded bone age. Hyperthyroid features are predominant in those individuals being deemed to have predominant central or pituitary resistance. The diagnosis is suspected on the basis of the elevated TH together with inappropriately normal TSH; however, a TSHoma must always be excluded by imaging, and a genetic analysis should follow for confirmation. However, in a number of cases, no genetic anomalies can be detected (nonTR-RTH). Treatment is completely independent from TH serum levels and depends on the different sensitivity at tissue levels. If there are clinical signs and symptoms of hyperthyroidism, as in the pituitary form, the aim of the treatment should be lowering TH in serum. The best approach is to lower TSH secretion from the pituitary gland by the use of triiodothyroacetic acid (TRIAC), a TH analogue with low hormonal potency but high affinity to TR. Long-term experiences are promising. Another TH analogue, dextrothyroxine (d-T_4), had been employed but with poor results. A further possible solution is thyroidectomy alongside substitutive therapy. Up to now, there is no evidence that a prolonged period of pituitary stress (the pituitary gland continues to secret a large amount of TSH) may favor the onset of an adenoma [23].

18.6 Imaging

Up to now, thyroid scintigraphy was the most common tool for evaluation of thyroid diseases; however, currently it has been largely replaced by ultrasound evaluation, which has the advantage of identifying in real time a nodule, a lymphocyte infiltration as in the HT and in GD. Furthermore, using color Doppler function, it is possible to assess the degree of vascularization which is typically enhanced in GD (diffuse) or in secreting adenoma (focal) but not in HT. Thyroid ultrasound is so commonly employed in this field that it can be considered an integrated test in outpatient visits of a thyroidologist. Thyroid gland may be also analyzed with MRI under particular circumstances; however, use of MRI is restricted mostly to pituitary gland evaluation [24].

18.7 Fetal and Neonatal Hyperthyroidism

18.7.1 Introduction

Fetal and neonatal hyperthyroidism are rare with a prevalence of neonatal thyrotoxicosis of 1/4,000–1/50,000 pregnancies [25]; it may be a life-threatening event and could lead to death if left untreated.

18.7.2 Pathogenesis

The most frequent cause of fetal and neonatal hyperthyroidism is transplacental transfer of stimulatory TSH receptor antibody (TSHR-Abs) from a mother affected by GD [25]. The prevalence of GD in pregnancy is 0.2 % [26], and 1–12.5 % of the offspring show thyrotoxicosis at birth, and another 3 % show only biochemical signs of hyperthyroidism [25]. The prevalence of hyperthyroidism, however, increases to 22 % in the offspring of women who require treatment for GD in the third trimester of pregnancy [25]. The transplacental passage of stimulatory TSHR-Abs starts early in pregnancy, and the highest level in the fetus is reached during the third trimester, when maternal concentration of TSHR-Abs is the highest. At that time, fetal autoantibodies' levels are similar to those observed in the mother.

For this reason, the American Thyroid Association (ATA) recommends to measure TSHR-Abs during 24–28 weeks of pregnancy and to carefully look for signs of fetal thyrotoxicosis, particularly when TSHR-Abs levels are three times over normal values [27]. It must be reminded that TSHR-Abs can be detected also in women who underwent ablation of the thyroid by surgery or radioiodine as a cure for GD [25, 28, 29].

Nonautoimmune neonatal hyperthyroidism due to activating mutation of the Gsα gene or to an activating mutation of the TSH receptor gene is rare. In these cases, severe permanent fetal and neonatal hyperthyroidism is due to molecular abnormalities of the TSH receptor that lead to its permanent activation. Hyperthyroidism control is generally difficult to achieve, and most patients undergo total thyroidectomy [30]. Rare cases of transient hyperthyroidism related to hydatiform mole and thyrotropin receptor hypersensitive to human chorionic gonadotropin have been reported [31].

18.7.3 Diagnosis of Fetal Hyperthyroidism

At the beginning of pregnancy, determination of TSHR-Abs should be performed in all pregnant women with GD and in euthyroid pregnant women with a history of GD who underwent thyroidectomy. If TSHR-Abs are present, the fetus should be carefully monitored because of the risk to develop thyrotoxicosis [32].

The characteristic clinical features of fetal hyperthyroidism are intrauterine growth retardation [33], nonimmune fetal hydrops, craniosynostosis, tachycardia, and fetal goiter.

Fetal tachycardia related to thyrotoxicosis is very common but not always present [34] and is characterized by a consistent resting fetal heart rate above 160 beats/min measured by Doppler ultrasonography.

The development of goiter in utero, however, might also be related to the overtreatment of the mother with antithyroid drugs; in these cases, goiter usually shrinks following a reduction or discontinuation of the administered drugs.

In selected cases, the confirmation of fetal hyperthyroidism relies on the direct assessment of serum fetal TH by cordocentesis. This procedure, however, may lead to severe complications for the fetus (infection, bradycardia, hemorrhage, death) and should be performed by experienced doctors and only if absolutely necessary [34]. Usually, this approach is needed in doubtful cases, for instance, when a mother with GD is being treated with antithyroid drugs and/or if she underwent ablation of the thyroid [35].

18.7.4 Prognosis of Fetal Hyperthyroidism

Fetal hyperthyroidism could be extremely harmful with an overall mortality of 12–20 % due to heart failure. In untreated women with GD during pregnancy, preterm delivery occurs in 53 % and intrauterine fetal death in 24 % of cases. In treated pregnancies, preterm delivery can occur in 4–11 % of cases and intrauterine death in 5–7 %.

18.7.5 Diagnosis of Neonatal Hyperthyroidism

In neonates, the clinical features of thyrotoxicosis are low birth weight for gestational age, premature birth, microcephaly, frontal bossing and triangular facies, diffuse goiter, warm and moist skin, irritability, hyperactivity, restlessness and poor sleep, stare and occasionally exophthalmos, diarrhea, poor weight gain with good appetite, vomiting, cardiac failure and arrhythmias, systemic and pulmonary hypertension, hepatosplenomegaly, jaundice, hyperviscosity syndrome, thrombocytopenia, and craniosynostosis [35]. Among the other signs, persistent acrocyanosis, hepatosplenomegaly, lymphadenopathy, and thymic enlargement are also reported. Bone age is generally advanced [25].

The diagnosis should be suspected in neonates born to mothers affected by GD who have been treated with antithyroid drugs during the third trimester of pregnancy and excluded in those who underwent ablation of the thyroid for GD [25, 28, 29].

Most cases of neonatal GD are suspected because of the maternal history, but in rare cases they are diagnosed also by neonatal screening [36].

In suspicious cases, serum fT4 and TSH should be measured at delivery or soon thereafter. It must be reminded that the concomitant transplacental passage of antithyroid drugs usually delays the onset of the clinical picture of hyperthyroidism till 10–20 days of life [37].

18.7.6 Prognosis of Neonatal Hyperthyroidism

Neonatal GD is generally a transient disorder, limited by the clearance of maternal antibody from the baby's circulation, and the symptoms of thyrotoxicosis usually disappear after 3–12 weeks from delivery [38]. It could be, however, life threatening, with detrimental effects on heart and central nervous system development. The degree of cardiac involvement determines the short-term prognosis, while the long-term effects of hyperthyroidism involve neurological development [38, 39].

Neonates with thyrotoxicosis improve rapidly with treatment; however, a reduced IQ in some of these patients, even if adequately treated, has been described, supporting the adverse effect of thyrotoxicosis on developing central nervous system. Moreover, growth retardation, craniosynostosis, hyperactivity, and developmental or behavioral problems have been reported as long-term sequelae of neonatal hyperthyroidism, but their correlation with the adequacy of treatment still remains unclear [40].

Finally, the presence of central hypothyroidism has also been reported in neonates born to mothers with GD, attributable to in utero exposure of hypothalamus and pituitary glands to high TH levels. It has been suggested that this is the consequence of an inadequate maternal treatment during pregnancy [41].

18.8 Treatment of Hyperthyroidism

18.8.1 Fetal Hyperthyroidism

Fetal thyrotoxicosis should be treated by administering antithyroid drugs like methimazole (MMI), propylthiouracil (PTU), and carbimazole to the mother [42]. A useful hint for treatment monitoring is the observation that there is a good correlation between maternal and fetal fT4 levels. On the other hand, overtreatment should be avoided, since treating pregnant women with antithyroid drugs in a dosage which lowers the maternal fT4 levels into normal range may lead to fetal hypothyroidism, which should always be suspected when goiter size increases and

bradycardia ensues. Prompt reduction of antithyroid treatment to the mother restores euthyroidism and reduces goiter while rarely intramniotic injections of thyroxin are needed [43].

Altogether, it is commonly suggested to adjust the antithyroid drug treatment in order to keep maternal fT4 in the upper normal to mildly thyrotoxic range [44]. Treatment is usually started with PTU (in the first trimester of pregnancy) at 100–200 mg daily or MMI 10–20 mg daily, and after 1 month the dose should be adjusted to maintain fT4 in the upper one-third of each trimester-specific reference interval [45]. Serum TSH levels of 0.1–2.0 mU/l are appropriate, but TSH <0.1 mU/l is also acceptable if a patient is in good health conditions and fT4 is adequate. Classically, PTU is the drug of choice during the first trimester of pregnancy because the use of MMI has been more often associated to the occurrence of aplasia cutis and other malformations (tracheoesophageal fistula and embryopathy) [46]. In particular, it seems of crucial importance to avoid exposure of the fetus to MMI during gestational weeks 6–10, which is the period of major organogenesis.

Monitoring fT4 levels in the mother, fetal thyroid dimension (monthly thyroid ultrasound to detect goiter onset), fetal growth, and heart rate is mandatory during antithyroid treatment.

18.8.2 Neonatal Hyperthyroidism

Treatment of neonatal thyrotoxicosis is based on antithyroid drugs (MMI, PTU), beta-adrenergic receptor blocking agents (propranolol, atenolol), iodine (lugol solution, SSKI), or iodinated contrast agents and, when needed, glucocorticoids and digoxin. MMI should be administered at a dosage of 0.25–1.0 mg/kg per day every 8 h. PTU is also effective, but it has more frequent and severe side effects, including a risk of hepatotoxicity [47], and for these reasons several scientific associations do not recommend PTU as a first-line treatment for GD in children [48].

Propranolol (2 mg/kg per day every 8 h) should be started to control neuromuscular and cardiovascular hyperactivity while atenolol (1 mg/kg daily, one single daily dose) could be preferred for a more cardio-specific blockage.

Lugol's solution (126 mg iodine/mL) should be administered as one drop (8 mg) orally every 8 h and SSKI (saturated solution of potassium iodide – 1 g/ml) as one to two drops daily, to reduce thyroid hormones release.

Glucocorticoids (prednisolone 2 mg/kg/day) inhibit thyroid hormone secretion and decrease peripheral conversion of T4 to triiodothyronine (T3) and may be helpful in extremely ill infants.

Digoxin could be administered in children with heart failure.

As the clinical and biochemical picture of thyrotoxicosis improves, treatment should be gradually reduced and then discontinued. The treatment may require several adjustments, with the thyroid function being monitored frequently (weekly). Generally, medical treatment would be withdrawn within 12 weeks although it can be continued for 6 months or even longer.

18.8.3 Hyperthyroidism in Childhood and Adolescence

The common treatment options for GD in children include antithyroid drugs (ATDs), subtotal or near-total thyroidectomy, and radioactive iodine (RAI) – I131; however, there is no general consensus on the optimal treatment.

It is common practice to start treatment with ATD; however, this approach may be associated to poor compliance with high relapsing rate or be complicated by toxicity. In case of relapses or drug toxicity, second-line options that are considered radical treatment are advisable and consist in total (or near-total) surgical removal of the thyroid gland or its destruction with radioimmune iodine (I 131).

ß blockers (except in patients with asthma or cardiac failure) could be associated during the first 2 weeks of management to relief tachycardia [49].

18.8.3.1 ATD Therapy

Carbimazole, its active metabolite, methimazole (MMI), and propylthiouracil (PTU) are the antithyroid drugs commonly used in children and adolescents as first-line treatment of GD.

In less than 25 % of cases, patients treated with ATD may show minor side effects (urticaria, rash, gastrointestinal problems, arthralgia). Major side effects such as agranulocytosis, drug-induced hepatitis, and production of cytoplasmic antineutrophil antibodies may be seen, even if with really low incidence. Antibody-positive vasculitis occurs only in exceptional cases.

Side effects of ATD may be dose related and usually occur within the first 6 months in 90 % of patients. MMI has a longer half-life and is effective when given as a single daily dose. MMI dose <10 mg/day is considered safe.

MMI (or carbimazole) starting dose is 0.5–1 mg/kg/day, with a maximum dose of 30 mg per day. After 2–4 weeks, as thyroid function normalizes, the dose should be reduced gradually by 30–50 %. The goal of ATD treatment is to restore normal thyroid function rather than reduce autoimmunity. However, it has been shown that euthyroidism has a beneficial effect on TSHR-Abs production and remission of GD [50]. Currently, there is no evidence of major efficacy of a combination of L-T4 (15) and ATD.

Remission is achieved after 24 months of ATD treatment by <30 % of children [51–54]. More prolonged use of ATD should be planned (2–4 years) in uncured cases.

In patients who do not undergo remission during ATD, the choice of the optimal therapeutical option still shows some uncertainties, as there are no prospective studies on effective long-term efficacy and safety of prolonged use of ATD, and ablative treatments such as surgery or RAI are followed by permanent hypothyroidism [49].

18.8.3.2 Surgical Treatment

Thyroid surgery is generally recommended as radical treatment choice in those hyperthyroid children and adolescents with larger thyroid goiter or with ophthalmopathy.

Nowadays, total (or near-total) thyroidectomy is preferred to subtotal (or partial) thyroidectomy [1]. For 1 week before surgery, five to ten drops of lugol solution

should be administered to reduce thyroid gland vascularity. Thyroidectomy-related risks such as hypoparathyroidism, vocal cord palsy due to recurrent laryngeal nerve injury, and keloid formation have relatively low incidence (15 % of cases) in experienced pediatric surgery centers [55].

18.8.3.3 Radioactive Iodine Treatment (RAI)

In children with hyperthyroidism, RAI is an effective treatment at large doses of I131 (220–275 μCi/g, corresponding to about 250 Gy) [56].

Secondary hypothyroidism that in almost all cases follows RAI treatment should be promptly treated with adequate lifelong levothyroxine therapy. Further prospective long-term studies of potential side effects (like thyroid malignancy, hyperparathyroidism) of this treatment are needed.

18.9 Long-Term Outcome

As mentioned above, less than 30 % of children show remission from GD after 2 years from a first course of ATD (average treatment period of 2 years). About 75 % of patients relapse within 6 months of the end of drug treatment, whereas only 10 % relapse after 18 months.

Due to the lack of prospective studies in children, it is difficult to predict who will relapse and would be better if undergoing early radical treatment. Previous studies take in consideration as predictive markers age, goiter size, severity of hyperthyroidism at the onset, TSHR-Abs levels, and duration of ATD.

A recent study from Leger et al. showed in GD children a positive correlation between the risk of relapses and young age, non-Caucasian origin, and high serum fT4 and TSHR-Abs levels at the onset [57]. Furthermore, the authors showed an inverse correlation between the risk of relapses and duration of ATD, emphasizing that long-term treatment reduces the risk of disease recurrence.

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Generation of Functional Thyroid from Embryonic Stem Cells

19

Francesco Antonica

19.1 Introduction

The thyroid is an elegant butterfly-shaped gland with endocrine function located in front of the trachea. From the histological point of view, thyroid is mainly composed of endothelial cells, C cells (or parafollicular cells) and thyroid follicular cells (TFCs). Endothelial cells make up the vascular network important for the correct TSH-mediated hormonal stimulation of the gland to produce and release thyroid hormones (THs) (T4 and T3) from thyroid follicles. C cells are involved in calcitonin secretion, important for the correct calcium homeostasis and phosphate metabolism. Thyroid hormones are involved in the regulation of many metabolic and biological processes such as skeletal and brain development, and they are considered very crucial already immediately after birth. During the last two decades, several mammalian as well as non-mammalian animal models (i.e. zebrafish [1]) have been intensively used for studying the embryonic development of thyroid gland. Thyroid is an endoderm-derived organ. Its organogenesis begins with the formation of the thyroid anlage at the level of the pharyngeal arches. During this phase (E8.5 in mice and 4th gestational week in humans), so-called specification stage, a specific subpopulation of endodermal cells in the pharyngeal floor becomes committed to thyroid fate and distinguishable due to the co-expression of four specific genes: *Nkx2-1* [2], *Pax8* [3], *Foxe1* [4, 5] *and Hhex* [6, 7]. Afterwards, the entire process of thyroid organogenesis requires, after a first specification of thyroid progenitors, thyroid bud formation and evagination from the endodermal layer, relocalization of the

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thyroid primordium to a position distant from its initial site, then functional differentiation of thyroid follicular cells and formation of functional mature thyroid follicles [4]. During the final morphological and terminal or functional differentiation, thyroid follicular cells organize into polarized monolayer epithelial structures, the follicles, and at the same time, all the genes, such as *Tg*, *Tpo* [2], *Slc5a5* (encoding for NIS) [8] and *Tshr* [2], known to be involved in the complex machinery for thyroid hormone synthesis start to be expressed. Afterwards, the first follicles producing T4 can be detected highlighting the end of thyroid organogenesis [9].

Most of the knowledge on thyroid embryogenesis, the morphogenetic features or the genes controlling such a sophisticated and elegant process is derived from studies performed on mouse embryos. The first studies performed using null mutant mice for the aforementioned transcription factors Nkx2-1 [10–12], Pax8 [10, 13], Hhex [10, 14] and Foxe1 [15] have demonstrated their importance for the correct organogenesis of the gland. Indeed, mice lacking one of these displayed an abnormal embryonic development of the thyroid gland characterized by agenesis. A more detailed analysis of the mutant embryos has demonstrated the function of the aforementioned transcription factors is necessary for the survival of thyroid precursors [10]. Indeed, the specification stage is not affected, but immediately after budding the primordium degenerates, leading to a complete absence of thyroid tissue at birth [10]. Moreover, using *in vivo* and *in vitro* systems, the additional role of Nkx2-1 and Pax8 has been clearly defined in the control of the expression of the functional thyroid-related genes important for thyroid hormone synthesis (such as Tshr, Tg, Tpo, Nis and Duox 1 and 2) [16]. Moreover, other genes have been described to lead to thyroid dysgenesis when knocked out, i.e. *Tshr* [17], *Shh* [18], *Fgf10* [19] and *Fgfr2* [20]. A defective embryonic development of the gland leads to a hypothyroidism state characterized by mice displaying low levels of thyroid hormones associated with most of the typical clinical features such as defective skeletal development. In humans, low plasma levels of thyroid hormones can lead to a physical and mental retardation condition, called also cretinism, if it is not properly diagnosed after birth. Indeed, a rapid diagnosis and a prompt supply of thyroid hormones just after birth can normalize cognitive development [21]. Nevertheless, in some cases IQ deficits are detected in adolescents with congenital hypothyroidism despite a correct substitution therapy [21]. Congenital hypothyroidism (CH) is the most common endocrine disorder affecting 1 in 1,000–2,000 newborns [22]. Two are the major causes of CH: defective embryonic development of the gland or a defective hormone synthesis. A very representative case is the mis-regulation of the hypothalamic-pituitary-thyroid axis due to Trhr loss of function [23, 24]. Thyroid dysgenesis (TD), representing roughly 85 % of CH cases, is a condition that appears with different scenarios: the gland can be absent (agenesis or athyroid), hypoplastic (hypoplasia), located in a different place (ectopy) or with only one lobe absent (hemiagenesis). In the rest of the cases where the synthesis of thyroid hormones is negatively affected, CH has been associated with mutations in genes encoding for TSHR, NIS, TPO, DUOX2, DUOXA2 and DEHAL1 (iodotyrosine dehalogenase 1) [25].

Taking together all the knowledge from studies performed using animal models (such as null mutant mice) and from genetic screening in patients affected by CH,

we can conclude that very little is known about the morphogenetic events occurring during thyroid organogenesis, the genes and epigenetic mechanisms controlling such processes.

Uncovering new genes implicated in pathogenesis represents a big step in improving the pre-natal diagnosis of congenital defect in the structure and function of the gland. In addition, the only known and used therapy to treat CH patients is based on the hormone substitution, so it would be very rational to hypothesize about an alternative approach to cure CH. Nevertheless, even if promptly treated after birth in some cases, the classical hormone substitution does not avoid development of mental retardation or low IQ.

19.2 Embryonic Stem Cells as System Model to Study Embryogenesis

During the last 20 years, embryonic stem cells (ESCs) have emerged as a very promising tool to investigate the molecular and morphological events occurring during the organogenesis of several organs. Embryonic stem cells are pluripotent cells derived from an early embryo that can be used as a cell line in tissue culture. Life begins with the fertilized egg that has the incredible capacity to give rise to the entire body with its whole complexity. From zygote to eight-cell stage (known as morula), an embryo has an extraordinary capacity called totipotency [26] consisting in the potentiality of the cells to become extra-embryonic or embryonic tissue. During this period, the pre-implantation phase, an embryo goes through additional and rapid cell division (cleavage) leading to the formation of the blastocyst, a hollow structure composed of an inner cell mass (ICM) surrounded by an outside cell layer (so-called trophectoderm – TE). The external cells, the trophoblasts (TE), are responsible for the connection to the uterus, formation of large parts of the placenta and also to provide nutrients to the embryo after implantation [27]. Whereas ICM is composed of undifferentiated cells that are no longer totipotent but pluripotent and able to give rise to the embryo proper and additional extra-embryonic tissues. After implantation, the pluripotent cells of the ICM will form the epiblast that afterwards through the gastrulation gives rise to the three different primary germ layers (ectoderm, mesoderm and endoderm) and primordial germ cells (PGCs). Then during the progression of the embryogenesis, each layer represents the starting point for the differentiation of specific lineages, tissues and organs. Indeed, the nervous system, epidermis and neural crest derive from ectoderm; somites (which form muscles), the cartilage of the ribs and vertebrate, the notochord, blood heart, blood vessels and connective tissue derive from mesoderm; gut, lungs, liver pancreas and thyroid are endoderm-derived organs [28].

The concept of the existence of a population of cells that during the normal embryogenesis proliferate and differentiate into many different lineages is clear. How can this capacity be translated *in vitro*? In 1981, two famous groups started to derive and culture pluripotent cells from mouse blastocyst [29, 30]. Even if these cells (called ESCs), derived from ICM, did not show any loss of differentiation

potential, it was necessary to wait a few more years for the final experimental evidence that ESC were really pluripotent. The final demonstration arrived in 1984 when Bradley and colleagues injected ES cells (showing a normal karyotype in contrast to ECCs) into blastocysts and demonstrated their contribution to the development of all cell lineages including germ lines in the generated chimeric mice [31]. There is ample evidence now that ESCs can be differentiated *in vitro* into many cell types, and many approaches and protocols have been proposed to drive the differentiation of ESCs into specific lineages. One of the typical approaches, somehow recapitulating gastrulation *in vitro*, is the formation of aggregates, called embryonic bodies (EBs). It was shown that ESC-derived EBs show molecular and morphological signatures of endodermal, mesodermal and ectodermal lineages. EBs derived by culturing ESCs in suspension can be re-plated and cultured in 3D or adherent conditions for long periods of time [26]. During the *in vitro* differentiation, they can spontaneously differentiate into many of the three germ layer-derived tissues, such as cardiomyocytes, skeletal muscles, smooth muscles, neurons, epithelial cells, pancreatic cells, hepatocytes and glial cells [26]. Many extrinsic factors can both positively and negatively influence the differentiation of EB-forming cells into specific lineages (i.e. cell density, media formulation, amino acids, extracellular matrix proteins, growth factors, morphogens, concentration and quality of the foetal serum). A second common approach for the differentiation of ESC is the adherent monolayer culture. Contrary to EBs method, where a mixture of germ layers is present, it is possible to differentiate a specific cell type derived from a specific germ layer [26]. Many groups in the endocrine field have proposed protocols for the *in vitro* differentiation of endodermal cells, in particular pancreatic beta cells, starting from human or mouse pluripotent stem cells. They generally use a multi-step approach that recapitulates the different embryonic phases that occur during normal development *in vivo*. Briefly, during the first phase, ESCs have to be differentiated into definitive endoderm, and then subsequential exposure of ESC-derived definitive endodermal cells to morphogens (such as FGF10, inhibitors of Notch and Sonic hedgehog pathways, retinoic acid and other growth factors) leads the differentiation into pancreatic hormone-expressing endocrine cells [32, 33]. Recapitulating pancreas development *in vitro* is simply an elegant example of how embryonic stem cells can be used for studying endoderm-derived organ development. Concerning the differentiation of endoderm-derived tissues from pluripotent cells, many studies have focused on the middle and posterior part of the foregut – midgut with pancreatic cells and posterior gut with hepatocytes [34]. Concerning anterior endoderm, Green and colleagues described how to generate anterior foregut endoderm (AFE) starting from human ES and induced pluripotent stem cells (iPS cells) [35]. Concerning the possibility to further differentiate AFE-derived tissues from ESC, two interesting and elegant studies have shown the potential of mouse ESCs to be differentiated into endodermal progenitors positive for the lung and thyroid marker NKX2-1 and additionally expanded and differentiated into thyroid and lung lineage [36, 37].

ESCs can be also efficiently differentiated into mesoderm-derived lineages such as hematopoietic cells, endothelial cells and cardiomyocytes [38], chondrocytes and cartilage-like tissue [39]. ESCs have been also successfully used in the

neurobiology field for differentiating ectoderm-derived lineages such as neural cells for uncovering and characterizing molecular events occurring during neurogenesis [40]. In 2002, Wichterle and colleagues were the first to propose an efficient protocol to differentiate ESCs into a specific neural cell type, spinal motor neurons, using retinoic acid and sonic hedgehog [41]. After this, many independent works have proposed several protocols to differentiate ESCs into different neuronal cell types such as progenitors, retinal photoreceptors, cerebellar neurons and cerebral neurons [38].

Moreover, Sasai and colleagues have recently illustrated the possibility to use both human and mouse ESCs to successfully recapitulate the 3D organization of complex ectoderm-derived tissues such as optic cup [42], retina [43] and adenohypophysis [44]. 3D stem cell culture has been shown to be successfully used to differentiate endoderm-derived tissues such as liver organoids, islet organoids, gut organoids and pulmonary progenitors that in presence of specific scaffolds was shown to reconstitute airway epithelium [45]. Those findings have opened a new avenue in the possibility to use ESCs as *in vitro* model for studying tissue morphogenesis. Moreover, generating 3D organoids resembling a tissue from the morphological and functional point of view represents an additional tool to perform drug screening and physiological studies.

During the last years, many studies have described the possibility to differentiate specific cell lineages acting on the expression of genes known to play a role in the correct embryonic development of several tissues. In 2004, Kyba and colleagues have shown how the ectopic induction of *HoxB4* – known to be involved in haematopoietic stem cell differentiation – in ESC-derived EBs promoted the differentiation into lymphoid-myeloid progenitor cells [46]. Similarly, Ahfeldt and colleagues found that the induction of the expression of *PPARG2* or its co-expression with *CEBPB* and/or *PRDM16* in hEBs-derived mesenchymal progenitor cells committed the cell fate into white and brown adipocytes [47].

During the past, several teams have proposed different protocols of differentiation of pluripotent stem cells into thyroid-like cells. Indeed, all the published works have shown an incomplete analysis of differentiated cells, such as morphological and functional [48, 49]. In 2012, Sabine Costagliola's group published the first protocol of differentiation of pluripotent stem cells into thyroid follicular cells [50].

19.3 Generation of Functional Thyroid from Embryonic Stem Cells

As it has been previously described, two are the major approaches to differentiate embryonic stem cells into specific lineages: (1) defined media approach – differentiation of ESCs into a specific cell type is achieved modulating signalling pathways, known to be important for the development of the specific tissue/organ, by supplementing the medium with the antagonists or agonists of the defined pathway; (2) the second approach is the ectopic expression of defined transcription factors known to be important for the *in vivo* development of the specific tissue.

Concerning thyroid development, the external signalling responsible for the commitment to thyroid fate of a subpopulation of endoderm cells at the level of the pharynx is unknown yet.

The lack of fundamental information made to approach the differentiation of pluripotent stem cells into TFCs by inducing the two genes proposed to be the master genes controlling thyroid embryogenesis, *Nkx2-1* and *Pax8*. In the first instance, embryonic stem cells have been genetically engineered to make possible the induction of both transcription factors [50, 51]. A doxycycline-responsive promoter can make possible to temporally control the co-expression of *Nkx2-1* and *Pax8*. The protocol developed by Costagliola and colleagues can be summarized in three important and key steps: (1) generation of EBs and embedding of EBs into a 3D-Matrigel support; (2) induction of *Nkx2-1* and *Pax8* co-expression by doxycycline-TetOn system; (3) stimulation of folliculogenesis by rhTSH treatment (Fig. 19.1a). During the first step, ESCs are cultured in hanging drops, and this makes possible the initial differentiation of the pluripotent stem cell into the three different germ layers including endoderm. Because thyroid cells have to be organized in a three-dimensional structure to make possible thyroid hormone synthesis, Costagliola and colleagues have taken advantage of growing cells into an extracellular matrix-based environment. This has been aimed at embedding and growing ESC-derived EBs in a gelatinous mixture of proteins called Matrigel (mix of different extracellular matrix proteins) that resembles the extracellular environment that can be found in several tissues including thyroid. The second step that has been described crucial for the *in vitro* thyroid differentiation is the inducible co-expression of both *Nkx2-1* and *Pax8*. As expected from several studies performed using different cell lines, the expression of both transcription factors is sufficient to induce the expression of other thyroid-related genes such as *Tg*, *Tpo*, *Slc5a5* and *Foxe1* [16]. Moreover, other studies have demonstrated the capacity of both transcription factors to regulate their expression in a feedback loop manner [52–55]. Indeed, when *Nkx2-1 and Pax8* are ectopically induced by doxycycline for only 3 days in ESC-derived EBs, the up-regulation of the endogenous forms of both transcription factors as well as *Foxe1*, *Hhex*, *Tg*, *Slc5a5 and Tshr* can be detected [50]. Only when both *Nkx2-1* and *Pax8* are ectopically induced, cells become committed to thyroid fate as suggested from the activation of the endogenous forms of both transcription factors [50]. Those observations have led to the last point of the protocol of differentiation where doxycycline is removed and substituted by rhTSH for the remaining differentiation period (Fig. 19.1a).

At the end of the differentiation, cells differentiated after induction of both transcription factors and subsequent rhTSH treatment show at transcriptional level the up-regulation of the four transcription factors (*Nkx2-1*, *Pax8*, *Hhex* and *Foxe1*), known to play a pivotal role during thyroid development, and of the genes encoding for the protein important for thyroid hormone synthesis such as TG, TPO, TSHR and NIS (Fig. 19.1b). Immunofluorescence analysis of the cells shows clusters of cells co-expressing thyroid markers NKX2-1, PAX8, FOXE1, TG and NIS (Fig. 19.1c–f). Detailed morphological characterization showed that

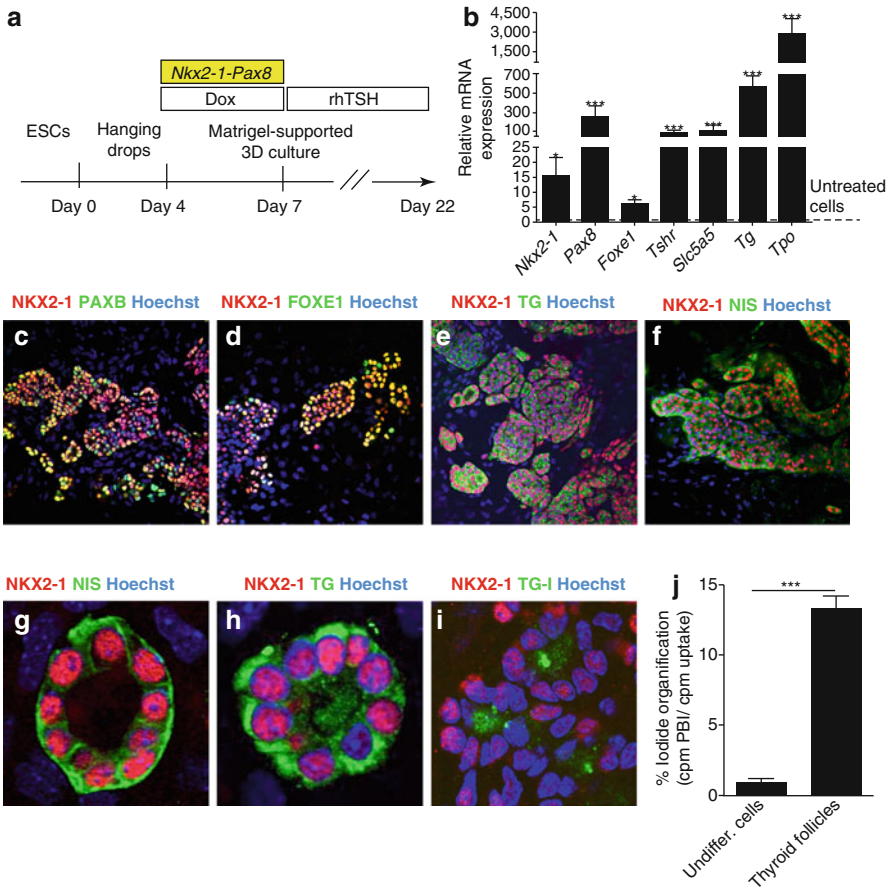

Fig. 19.1 Generation of functional thyroid follicles from pluripotent stem cells. (**a**) Schematic diagram of the thyroid follicle differentiation protocol from ESCs. (**b**) Expression of endogenous *Nkx2-1* and *Pax8*, *Foxe1*, *Tshr*, *Slc5a5*, *Tg* and *Tpo* at day 22 in cells differentiated after Doxycycline-mediated induction of *Nkx2-1-Pax8* and subsequent treatment with rhTSH. Relative expression of each transcript is shown as fold change compared to untreated cells (*baseline*) at day 22 as mean ± s.e.m. ($n=6$). Unpaired *t*-test was used for statistical analysis. *$P<0.05$, **$P<0.01$, ***$P<0.001$. (**c–i**) Immunostaining at the end of the differentiation (day 22) for NKX2-1 and PAX8 (**c**), NKX2-1 and FOXE1 (**d**), NKX2-1 and TG (**e**, **h**), NKX2-1 and NIS (**f**, **g**), NKX2-1 and iodinated TG (TG-I) (**i**). (**j**) Histogram showing the organification percentage of iodine-125 at day 22 in ESC-derived thyroid follicles; undifferentiated cells column refers to cells at day 22 differentiated without Doxycycline and rhTSH (Modified from Antonica et al. [50])

ESC-derived thyroid cells were capable to organize into follicular structures, confirmed by polarization of NIS at the basolateral membrane (Fig. 19.1g) and accumulation of TG in the luminal compartment (Fig. 19.1h), while functional analysis highlighted the capacity of these cells to metabolize iodide (Fig. 19.1i, j) [50]. More interestingly, confirming their functionality, ESC-derived thyroid follicles can be transplanted into hypothyroid mice (Fig. 19.2a). The exogenous tissue,

transplanted under the renal capsule, can perfectly integrate into the host, as shown by the presence of follicular epithelium (Fig. 19.2b, c) histologically positive to the transcription factors (NKX2-1, PAX8 and FOXE1) [50], functional thyroid markers NIS (Fig. 19.2d) and TG (Fig. 19.2e) and finally T4 (Fig. 19.2f) and more interestingly, the formation of angiofollicular units shown as NKX2-1-forming follicles surrounded by endothelial cells forming vessels (Fig. 19.2g). The correct integration, functionality and formation of a vascular network around

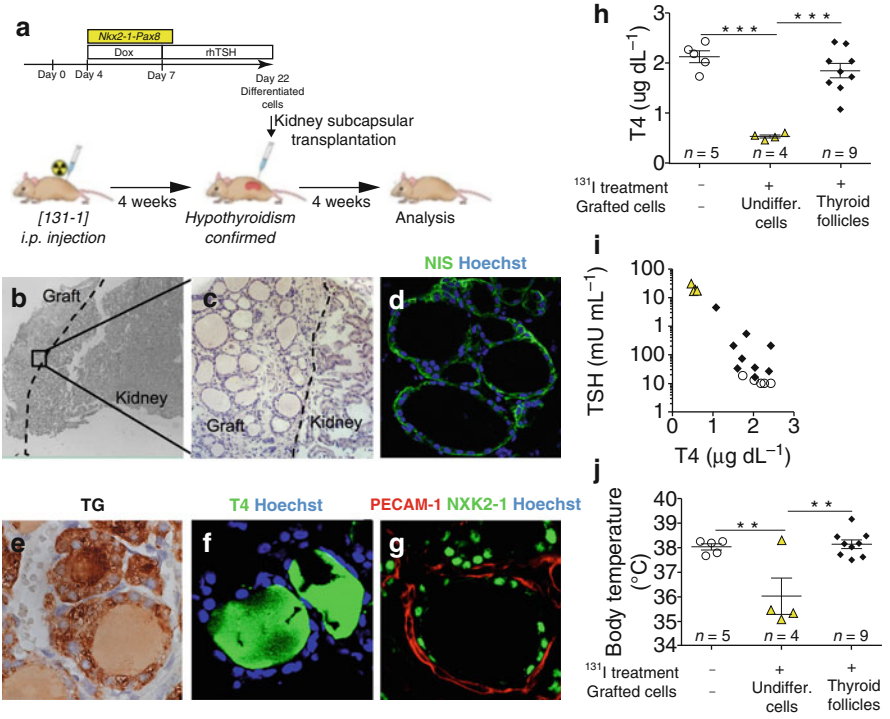

Fig. 19.2 Rescue of hypothyroidism in mice by transplantation of ESC-derived thyroid follicles. (**a**) Schematic diagram of protocol for ESC-derived thyroid follicles transplantation under the renal capsule of mice after radio-iodine ablation of thyroid. (**b–g**) Histological examination of kidney sections 4 weeks after grating. Hematoxylin-eosin staining shows the integration of the transplanted tissue in the cortical area of the host kidney (**b**) characterized by single cuboidal epithelium organization of transplantanted tissue (**c**). Immunofluorescence analysis confirms the expression of Nis (**d**) and Tg (**e**), production of T4 (**f**) and formation of vessels (PECAM-1) surrounding NKX2-1-forming follicles (**g**). (**h**) Total plasma T4 levels 4 weeks after transplantiation of ESC-derived thyroid follicles in iodine-131-treated mice. (**i**) Relationship between plasma TSH and T4 levels 4 weeks after grafting. (**j**) Body temperature measurements 4 weeks after grafting. In (**h–j**), open circles show iodine-131-untreated and ungrafted mice; *yellow triangles* show mice treated with iodine-131 and grafted with undifferentiated cells (cells cultured without Dox and rhTSH) and *black diamonds* show mice treated with iodine-131 and grafted with ESC-derived thyroid follicles. The values are shown as a *dot plot* (**h**, **j**) or scatter plot (**i**) and data are mean ± s.e.m. Tukey's Multiple Comparison Test (**h**, **j**) was used for statistical analysis. $^{**}P<0.01$, $^{***}P<0.001$ (Modified from Antonica et al. [50])

the transplanted ESC-derived thyroid follicles lead to the restoring of the normal serum level of thyroid hormones (Fig. 19.2h) together with the normalization of thyroid homeostasis, shown as a decreased serum level of TSH (Fig. 19.2i) and a symptomatic recovery from the hypothyroidism state shown as a normalization of the body temperature (Fig. 19.2j) [50].

19.4 Embryonic Stem Cell as Model to Study Thyroid Embryogenesis and Modelling Congenital Hypothyroidism

The establishment of the first protocol of differentiation of pluripotent stem cells into functional thyroid cells has provided a novel and complementary system to study thyroid organogenesis. To date, very few studies have been performed *in vivo* in order to obtain a genome-wide expression profile of the thyroid cells at different developmental stages [56]. The *in vitro* model of thyroid organogenesis would be an alternative system to study and uncover new genes playing a role during the embryonic development of the gland. The unlimited source of biological material makes this model very useful to get access to the complete transcriptional profile. Moreover, the model represents also a valid system to study the function of genes (generate ESC mutant lines for specific genes and evaluate the effect during the *in vitro* differentiation) or signalling pathway hypothetically important for the commitment to thyroid fate or for the survival of thyroid cells and even their organization into follicles. Obviously, the system doesn't seem to be suitable to study migration of thyroid cells, another aspect important for the correct development of the gland.

This approach would be interestingly translated from mice to humans in order to uncover new genetic causes of congenital hypothyroidism. During the last years, human embryonic stem cells and more interestingly induced pluripotent stem cells have been emerging as a very interesting tool for the *in vitro* modelling of human diseases.

In 2006, Shinya Yamanaka – Nobel Prize for medicine and physiology 2012 – showed that mouse fibroblasts could be reprogrammed into embryonic stem cell-like cells by the simultaneous ectopic expression of four genes [57]. These cells were called induced pluripotent stem cells (iPSCs). More interestingly, 1 year later they reported that a similar experimental approach was used to reprogram adult human fibroblasts into iPSCs [58]. Briefly, the co-expression of the four key transcription factors *KLF4*, *SOX2*, *c-MYC* and *POU5F1* (also known as *OCT4*) successfully reprograms differentiated somatic cells back to a pluripotent state [58]. During the last 5 years, it has been shown how iPSCs can be differentiated into many different cell types, in similar ways as ESCs.

As it has been discussed, only few mutations in genes already known to be important for thyroid development have been found in CH patients with thyroid dysgenesis [59]. Several studies have been published during the last year demonstrating how iPSCs can be used *in vitro* for modelling genetic diseases [60].

The most reasonable approach would be to take advantage of the iPSC technology to derive pluripotent stem cells from fibroblasts of CH patients with thyroid dysgenesis. Applying the protocol of differentiation to CH patient-derived iPSCs might reveal differences at the transcriptional and morphological levels compared with healthy control-derived iPSC. This approach would lead to identify new genes playing an important role in normal and pathological thyroid development. Nevertheless, the selection of CH cases will be important as well as the good controls essential for the validation of the approach. Due to the fact that the *in vitro* model cannot be used for studying the migration of the gland during the foetal life, CH patients with thyroid ectopy should be excluded. Discordant monozygotic twins represent an interesting category of CH cases, and our model could help us to understand the genetic causes of a different thyroid phenotype in two 'identical' individuals. Our model might represent a good approach to investigate the causes of the differential thyroid organogenesis occurring in discordant monozygotic twins when mutations in coding or regulatory regions (exome sequencing and whole genome sequencing provide an additional tool to discover new mutations) lead to a differential expression of genes important for the development of the gland. Nevertheless, the model might not be a suitable model when epigenetic events occurring during early embryogenesis of the monozygotic twins are the cause of a differential development of the gland. Another interesting study would be the analysis of familial cases of CH. Exome sequencing of an entire family with CHTD (congenital hypothyroidism with thyroid dysgenesis)-affected members in combination with full transcriptional profiling of cells differentiated from healthy and CHTD-derived iPSCs would represent an interesting and valid approach to uncover new genes involved during thyroid embryogenesis. Identifying new genes will have a clinical impact increasing the list of genes that can be screened during pre-natal diagnosis.

Finally, it has been shown that ESC-derived thyroid follicles can be transplanted *in vivo* and restore thyroid homeostasis in mice affected by hypothyroidism. Those findings open new avenues towards conception of stem cell technology for the treatment of hypothyroidism. Nevertheless, I strongly believe we are still far from applying this approach for regenerative medicine. For example, genetic instability, due to culture conditions or the genetic engineering, together with the difficulty to obtain always homogeneous populations of differentiated cells are important pitfalls for regenerative medicine.

19.5 Conclusions

In this chapter, I discussed the first in vitro protocol of differentiation of pluripotent stem cells into functional thyroid follicles. This model enters in the large and steadily growing list of tissues that can be differentiated in 3D stem cell culture. This model would represent an additional and complementary model to study and characterize molecular and morphological events occurring in thyroid organogenesis. More interestingly, this ESC-based model can be used to uncover new genetic causes of thyroid dysgenesis in CH patients. In the far future, we are facing the idea to use the approach of curing severe hypothyroidism using transplantation of autologous ESC-derived thyroid tissue.

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