Primary congenital hypothyroidism: defects in iodine pathways

Jan J M de Vijlder
Department of Paediatric Endocrinology, Emma Children’s Hospital AMC, Academic Medical Centre, University of Amsterdam, Amsterdam, The Netherlands
(Correspondence should be addressed to Jan J M de Vijlder; j.j.devijlder@amc.uva.nl)

Abstract
The thyroid gland is the only source of thyroid hormone production. Thyroid hormone is essential for growth and development, and is of special importance for the development of the central nervous system. It was for that reason that neonatal screening on congenital hypothyroidism was introduced and is now performed in many countries. Defects in thyroid hormone production are caused by several disorders in hormone synthesis and in the development of the thyroid gland (primary hypothyroidism) or of the pituitary gland and hypothalamus (central hypothyroidism).

This paper describes defects in the synthesis of thyroid hormone caused by disorders in the synthesis or iodination of thyroglobulin, leakage of iodinated proteins by a stimulated thyroid gland and the presence of abnormal iodoproteins, mainly iodinated albumin, in the thyroid gland and blood circulation. Circulating thyroglobulin and abnormal iodoproteins, as well as the breakdown products of these iodoproteins excreted in urine, are used for etiological diagnosis and classification. Moreover, our finding of an enzyme that catalyses the dehalogenation of iodotyrosines, which is important for iodine recycling and required for economical use of iodine, is also referred to.

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Introduction
Thyroid hormone, exclusively produced by the thyroid gland, plays a key role in the growth and differentiation of many organs. It is of special importance for the development of the central nervous system in the pre- and postnatal period (1, 2). For thyroid development and for synthesis, storage and secretion of thyroid hormone a variety of transcription factors and a sequence of precisely tuned events (3) are required. Disorders in one of these factors or in the intermediate reaction steps are the molecular basis of abnormalities in thyroid development and/or in thyroid hormonogenesis that result in congenital hypothyroidism. Severe shortage of thyroid hormone after birth from several weeks on will result in serious mental and motor handicaps. The initial reports on the absence of thyroid tissue leading to sporadic cretinism and myxoedema, and the occurrence of endemic cretinism due to iodine deficiency are from the middle of the 19th century. The first descriptions of inborn errors in thyroid metabolism causing hypothyroidism with goitre are from Pendred (4) and Osler (5) at the end of the 19th century. Nevertheless, even in the middle of the twentieth century, many patients with congenital hypothyroidism were referred to paediatricians at school age (6).

Apart from the children with the classical type of congenital hypothyroidism, there are also special groups of children who are in danger from a shortage of thyroid hormone after birth such as preterm born infants (7) and infants with Down’s syndrome (8).

During pregnancy the mother provides substantial amounts of thyroid hormone to the foetus (9), so the delay in cerebral development caused by congenital hypothyroidism (CH) is mainly the result of postnatal thyroid hormone deficiency. The result of the maternal thyroid hormone provision to the foetus is that hypothyroid newborns do not show clear clinical signs at birth. The risk of mental retardation and the difficulty in recognising the disease were reasons for introducing neonatal mass screening programmes in many countries. The introduction was attended by a considerable increase in the apparent overall incidence of CH, most likely due to the recognition of mild disorders that formerly remained undetected, were detected later and/or were not recognised as congenital problems (10, 11).

Congenital hypothyroidism can be of thyroidal or of central (hypothalamic/pituitary) origin (Table 1), with a widely diverse molecular aetiology. Almost all screening programmes are directed towards the detection of thyroidal congenital hypothyroidism (incidence about 1:3100) (12) by measuring thyrotrophin (TSH) in filter blood spots, and they ignore central congenital hypothyroidism, which has an estimated incidence of about 1:20000 births (12–14). In this
paper, I mainly describe disorders in the mechanism of thyroid hormone synthesis taking place at the apical membrane of the thyroid gland.

**Thyroid metabolism**

Iodination takes place at the apical membrane (see oval in Fig. 1). The active transport of iodide into the follicular cells is mediated by the Na\(^+\)-I\(^-\) symporter (NIS) as the first crucial step in thyroid hormone synthesis (for review see (15)). Iodide taken up by the thyrocytes is transported through the apical membrane by the anion transporter, pendrin (16). Recently, another apical iodide transporter protein (AIT) (17) has been described, which was identified as an NIS homologue.

The oxidation of iodide requires hydrogen peroxide that is synthesised outside the cell at the apical border catalysed by the thyroid oxidase complex. Recently, two proteins of this complex ThOX1 and ThOX2 have been identified (18). The iodination of tyrosine residues, commonly referred to as iodide organification, is catalysed by the membrane-bound thyroperoxidase (TPO). Besides the binding of iodine to tyrosine, TPO also catalyses the coupling of iodotyrosines to iodothyronine residues in thyroglobulin (TG) under oxidative conditions. In order to deliver thyroid hormone to the blood circulation, TG is internalised by endocytosis that can take place by non-selective fluid phase uptake and by receptor-mediated processes. Two receptors playing a role in the endocytosis of TG have been identified: megalin (19) and the thyroid asialoglycoprotein (20) receptor. After endocytosis, thyroxine (T\(_4\)), triiodothyronine (T\(_3\)) and the remaining iodothyrosine residues in TG are released by lysosomal proteolysis. The iodothyronines are secreted into the circulation, non-covalently bound to plasma proteins and transported to target organs. The iodothyrosine molecules are deiodinated and the iodide can be reused as substrate for the iodinating processes in the thyroid.

In all these steps leading to the synthesis and release of thyroid hormone and the recycling of iodine, defects may occur (Table 2).
Table 2  Disorders in thyroid hormone synthesis.

<table>
<thead>
<tr>
<th>Class of defect</th>
<th>Features</th>
<th>Plasma TSH conc.</th>
<th>Plasma T4 conc.</th>
<th>$^{123}$I uptake + wash-out****</th>
<th>Putative mol. defects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iodide transport**</td>
<td>Through basal-lateral membrane. Saliva/blood ratio of iodide near unity Through apical membrane*** deafness</td>
<td>Elevated</td>
<td>Low or elevated</td>
<td>No uptake</td>
<td>Sodium-iodide symporter (NIS) Pendred’s syndrome + apical iodide transporter (AIT) and/or possibly other transporters?</td>
</tr>
<tr>
<td>Iodide organization defect**</td>
<td>Partial** (PIOD) deafness</td>
<td>Normal-elevated</td>
<td>Low-normal</td>
<td>Partial wash-out</td>
<td>Thyroid oxidase (ThOX2), monoallelic mutation</td>
</tr>
<tr>
<td></td>
<td>Partial defect (PIOD)</td>
<td>Normal-elevated</td>
<td>Low-normal</td>
<td>Partial wash-out</td>
<td>ThyOX2 or TPO biallelic mutations</td>
</tr>
<tr>
<td></td>
<td>Total (TIOD)</td>
<td>Elevated</td>
<td>Low or normal</td>
<td>Partial wash-out</td>
<td>ThyOX2 or TPO biallelic mutations</td>
</tr>
<tr>
<td>Iodine recycling defect</td>
<td>Presence of mono- and di-iodotyrosines in urine Variable, dependent on iodine status</td>
<td>Variable, dependent on iodine status</td>
<td>High uptake, with rapid turnover</td>
<td>DEHALI</td>
<td></td>
</tr>
</tbody>
</table>

Conc., concentration; PIOD, partial organization defect; TIOD, total organization defect.

* In thyroid by dyshormonogenesis elevated plasma TSH concentrations, stimulating the thyroid gland, result in elevated plasma TG concentrations, except in the cases of TG synthesis defects and TSH receptor inactivation where elevated TSH concentrations do not give increased plasma TG concentrations.

** Pendred’s syndrome is characterised by a combined thyroid defect and deafness.

*** Pendred’s syndrome is probably caused by an iodide transport defect over the apical membrane, radio-iodine uptake studies reflect a partial organification defect.

**** $^{123}$I taken up that remains as iodide in the thyroid gland will be released rapidly when the NIS transporter is blocked by e.g. perchlorate ions. When the release is between 10% and 90% or >90% in half an hour it is called a partial or total wash-out respectively.
Disorders in thyroid hormone synthesis

Thyroglobulin synthesis

In the process of thyroid hormone synthesis and storage, TG plays a prominent role. TG is encoded by a large gene, mapped to human chromosome 8q24, spanning more than 300 kb (21) containing 48 exons (22). The primary structure of human TG was deduced from the sequence of its 8448-base mRNA (23). The open reading frame for human TG consists of 8307 nucleotides coding for a protein of 2768 amino acid residues which has 66 tyrosine residues and a signal peptide of 19 amino acids (24). The TG sequence contains several repeats – in the N-terminal half eleven type-1 cysteine repeats were described (25). These repeats have been reported in other proteins and may play a role in the self-folding of proteins or may act as a proteinase inhibitor (26).

The structure of TG is optimal for the optimal T4 production. The protein-dimer (molecular mass 660,000) consists of two identical monomers containing 10% carbohydrates. Four to five tyrosine residues (acceptor residues) in the TG monomer, which can be preferentially iodinated (27), are considered to couple with other specific iodo-tyrosine residues (donor residues) to form iodothyronine. The sites in the TG molecule where iodothyronine residues are formed are the so-called hormonogenic sites. An important hormonogenic site is the N-terminal tyrosine residue (tyr 5; acceptor) that most likely couples with iodotyrosine 130 as donor. It can be expected that mutations causing small changes in structure already influence coupling efficiency and, as a consequence, thyroid hormone synthesis and release (28). Even amino acid changes, considered to be polymorphic, may influence the efficiency of thyroid hormone synthesis, changes that may become important under iodine deficient conditions.

Thyroglobulin synthesis defects

The characterization of TG synthesis defects is not easy to establish. The physiological characteristics leading to this classification are shown in Table 2. The concentrations of TSH and thyroid hormones as well as the iodine uptake studies are indicative but one of the parameters important for the diagnosis is the relatively low plasma TG concentration in relation to the plasma TSH level. In practice, the definition 'TG synthesis defect' is a clinical-physiological one. It does not cover defects in the whole process of TG synthesis (29).

Thyroglobulin synthesis defects in animals

Before studies with recombinant DNA and transgenic mice became possible, the availability of animals with inherited congenital hypothyroidism and goitre proved very useful as models for the study of disorders in TG synthesis.

Five animals with TG synthesis defects have been described, and four are elucidated at the molecular level. The Afrikander cattle (30) in which affected animals were borderline euthyroid required special care for normal growth, development and reproduction. The molecular defect appeared to be caused by a nonsense mutation (Arg697Stop) in exon 9 in the gene coding for TG. This mutation gives rise to a thyroglobulin fragment with a molecular mass of 75,000 plus an alternatively spliced TG monomer (molecular mass 250,000), missing exon 9 (31, 32).

The Australian Merino sheep (33) were severely hypothyroid and most lambs were either stillborn or died soon after birth. In the thyroid gland and blood circulation, abnormal iodinated proteins were observed, among which was iodinated albumin. In the thyroid gland no TG (molecular mass: 660,000) could be found. The defect inherited in an autosomal recessive way (34).

In the cog/cog mouse, congenital goitre is linked to the TG locus and is caused by a Leu2366Pro transition in the acetylcholsterase domain of the TG molecule (35). This mutation resulted in an endoplasmic reticulum (ER) storage disease.

Another mutation in the TG molecule that inherited in an autosomal recessive way has been found in severely hypothyroid Rdw rats (plasma T4 concentration: 15 nmol/l; normal: 64 nmol/l, TSH concentration: 9.8 ng/ml; normal: 1.1 ng/l). DNA sequencing revealed a Gly2320Arg mutation in the acetylcholsterase-like domain. The molecular chaperones GRP94, GRP78 and calreticulin were accumulated in the thyroid gland of these animals. TG was not secreted into the follicular lumina, but was retained in the dilated ER (36).

The Dutch goats, a mixed strain of Saanen and dwarf goats, were severely hypothyroid. Almost all affected goats required thyroid hormone treatment or extra iodide administration to survive (37). The defect is inherited in an autosomal recessive way (38). The molecular defect in the TG gene was localised in exon 8 caused by a TAG/Tag/Stop mutation (39). The mutation gives rise to a cys-rich N-terminal TG peptide fragment with a molecular mass of 35 kDa, and about 40 kDa when glycosylated, as in the in vivo situation. These TG fragments are transported to the follicular lumen, conjugated by disulphide bridges to high molecular weight complexes from which the tyrosine residues can be iodinated and coupled to thyroid hormone residues when more than the normal amount of iodine was administered, rendering the animals euthyroid (40, 41). For the neonates, it was very beneficial to give the mothers iodide (37, 42).

Thyroglobulin synthesis defects in man

Five mutations in the TG gene have been described in...
human pathology. A homozygous C886T transition in exon 7 results in the replacement of an Arg residue into a premature stop codon at amino acid position 277 in TG upon translation (43). Clinically, hypothyroidism, observed in three related patients, was not severe indicating that the short TG fragment was still able to produce thyroid hormone, a phenomenon that was also observed in the goat model and in in vitro experiments (44). In these experiments it was shown that the preferential coupling of iodinated tyrosine residues 5 and 130 was still present, but less pronounced than in intact TG.

In a Japanese family a deletion of 68 amino acids from the N-terminal part of thyroglobulin, due to an in-frame mutation of exon 4 was observed (45). The resulting TG molecule lacks the donor tyrosine residue 130 and a cysteine-containing repeated sequence characteristic for the first half of the molecule. Consequently, this patient was severely hypothyroid.

In a Brazilian family, two hypothyroid patients with large goitres were described. Investigation of these goitres revealed that in the TG gene a CGA(Arg)1510 to TGA(stop) transition gave rise to a truncated TG polypeptide of 166.260 Da. Besides this mutation, an alternative splice product was present (46) that is also found in normal thyroid tissue, resulting in a TG lacking 57 amino acids.

Another family has been described in which the TG mRNA lacked 138 nt between position 5590 and 5727. The severely hypothyroid patients, homozygous for this deletion, presented multinodular goitres. Despite high TSH levels the serum TG level was below the detection level (1.0 mU/l) and no increase could be observed after TSH stimulation (47). The disease in the affected patients was associated with massive induction of specific ER molecular chaperones including the hsp90 homologue, GRP94, and the hsp70 homologue, BiP. From thyroid tissues of the four patients, light microscopy demonstrated the presence of intracellular TG despite its absence in thyroid follicle lumina, while electron microscopy indicated abnormal distension of the endoplasmic reticulum. The data suggest that these patients synthesise a mutant thyroglobulin which is defective for folding/assembly, leading to a markedly reduced ability to export the protein from the ER to the follicular lumen (48). Thus, these kindreds suffer from a thyroid ER storage disease, a cell biological defect phenotypically indistinguishable from that found in cog/cog mice (35).

Patients containing a Cys1263Arg mutation (28) are described as having an arrest in the endoplasmic reticulum as well. These patients were mildly hypothyroid to euthyroid, indicating that in the follicular lumen sufficient TG had to be iodinated to deliver adequate amounts of thyroid hormone.

In Fig. 2, the locations of the various mutations in the TG protein molecule in man and animals are shown schematically.

From the localisation of the mutations, we get the impression that patients producing shorter TG fragments are less severely hypothyroid than the patients having mutations in the acetylcholinesterase domain of TG, indicating that shorter TG fragments are secreted more easily into the follicular lumen than TG molecules with more C-terminal mutations.

The patient where exon 4 was spliced out lacks a cysteine-containing repeat and Tyr residue 130. This patient was also severely hypothyroid. Thyroid dyshormonogenesis might be caused because the Tyr130 donor residue was missing. Otherwise the lack of a Cys-containing repeat might cause misfolding of the protein resulting in accumulation of TG in the ER and proteolysis.

‘Abnormal’ iodinated proteins and breakdown products It is known that besides TG other proteins such as albumin can be iodinated. Initial reports suggested that albumin was produced by the thyroid (49). In contrast with this, RT-PCR and labelling experiments showed that albumin in the thyroid gland originates from blood (50) and is iodinated. Some of the iodinated albumin rapidly leaks out, giving a high protein bound iodine value in the blood circulation (51). Some is broken down by lysosomal enzymes in the thyroid, giving rise to the formation of iodinated peptides and iodinated histidine which are excreted in urine, providing a new criterion in the diagnosis of TG synthesis defects, even prenatally (52).

Serum albumin may be taken up by the thyroid gland either by diffusion via the tight junctions or via basolateral endocytosis (53), and is iodinated in the follicular lumen. For the release of iodo-albumin into the circulation intra- or intercellular routes are postulated. The intracellular route follows the internalisation of iodinated albumin by pinocytosis and exocytosis at the basolateral membrane. The intercellular pathway is by leakage via the tight junctions.

The immuno-histological pictures show that the inflow and outflow of protein under these pathological conditions occur mainly intercellularly (Fig. 3) and is strongly TSH dependent. In accordance with these findings we have shown that the width and number of chains of the tight junctions are inversely related to the TSH level, most likely resulting in increased protein leakage at higher TSH levels (54) (Fig. 4A, B).

Iodide organisation

The oxidation and binding of iodine to tyrosine residues in TG are commonly referred to as iodide organisation. Under normal conditions, the iodide uptake is the rate-limiting step in the iodination process. Iodide in the thyroid is derived from two distinct sources: iodide transported into the cell via a transporter system (NIS) (15) and iodide obtained via deiodination of organic iodine compounds within the gland. How far both iodide pools are exchangeable is a subject of
controversy. However, in view of the rapid iodination reaction, catalysed by TPO, the free intracellular iodide concentration is low especially when compared with the total amount of glandular iodine. An impression of the thyroid iodide concentration in the thyroid cells can be obtained by the inhibition of iodide uptake after administration of sodium perchlorate or sodium thiocyanate, visualized by radioiodine ($^{123}$I). Normally no or 10% of the radioiodine taken up will be released (Fig. 5).

Thyroid peroxidase is 110 kDa in size, is encoded by the TPO gene (150 kb) containing 17 exons, and is located on chromosome 2p25. The gene is exclusively expressed in the thyroid gland under the influence of the thyroid transcription factors NKX2.1 (TTF1), FKL15 (TTF2) and PAX8. The protein shows one transmembrane helix, with a large extracellular N-terminal part containing a haem group, essential for enzyme activity.

The generation of hydrogen peroxide is a critical step in thyroid hormonogenesis. The thyroid oxidase 1 (ThOX1) and 2 (ThOX2) genes have recently been identified as coding for components of the hydrogen peroxide generation system of the thyroid (55). The mRNAs coding for ThOX1 and 2 are 5369 and 6126 nucleotides, coding for proteins of 1551 and 1548 amino acids respectively. The structure of these proteins, inserted into the apical membrane, includes seven putative transmembrane domains, an NADPH
and a FAD binding site, two Ca\(^{2+}\) binding sites (EF hands) and haem binding sites. From reconstruction experiments, it is clear that more components have to be included in the thyroid oxidase system (56).

**Iodide organification defects**

Iodination defects can be partial or total. This depends on the degree to which iodide can be organified. Total organification defects (TIODs) are characterised by discharge of more than 90% of the (radio) iodide taken up by the gland within 1 hr after i.v. administration of sodium perchlorate (Fig. 5B). Partial organification defects (PIODs) are characterised by a discharge of more than 10% of the accumulated radioiodine (Fig. 5A).

**Partial iodide organification defects** As iodination needs hydrogen peroxide, iodide and thyroglobulin as substrates at the outside of the apical membrane of the thyrocyte, a shortage in one of these components at the right place will give diminished iodination. A disorder in the function of pendrin may cause a diminished iodide transport over the apical membrane which results in iodide remaining in the thyrocyte. By inhibition of the inflow by perchlorate ions, a partial outflow will occur, resulting in a partial organification defect. In the case of a TG deficiency, insufficient protein may be present for the amount of iodide, occasionally resulting in a partial organification defect.

Furthermore, partial iodide organification defects may be caused by a diminished organification of...
iodide caused by disorders in thyroid peroxidase or thyroid oxidase. Recently, a partial organification defect (perchlorate discharge 76–84%), caused by biallelic mutations in the TPO gene, has been reported. The three siblings, who were mildly hypothyroid, were found to be compound heterozygous for a missense mutation (G1687T) and a deletion in exon 10 (1808–1813del) (57). However, monoallelic mutations in the ThOX2 gene are found to cause partial organification defects, causing transient congenital hypothyroidism after birth (58). Obviously, thyroid hormone production is inadequate to fulfil the amount of thyroxine needed after birth and is most likely caused by too low a production of hydrogen peroxide. The regulation mechanism is not yet known.

**Total iodide organification defects** Patients with severe congenital hypothyroidism, showing a total discharge (>90%) of radio-iodine taken up by the thyroid after administration of sodium perchlorate within 1 hr, are classified under the category of total organification defect. Missense, nonsense, splice defects and frame shift mutations have been described by several groups (59, 60). From a Dutch study in 45 patients from 35 families the molecular diagnosis was made (Table 3). The mutations found are: frameshift mutations (six different ones) which occur most frequently: 39 of the 69 studied TPO alleles i.e. 57%, missense mutations (six different ones) found in 15 mutated alleles (22%), followed by mutations that putatively affect splicing (three different ones) responsible for five affected alleles (7%) and finally one nonsense mutation that affects four TPO alleles (6%). The most frequently occurring single mutation is the GGCC duplication in exon 8 (61), at nucleotide position 1227, detected in 36% of the investigated TPO alleles. This mutation has been found in 51% of the Dutch TIOD families, either in a homozygous or in a compound heterozygous fashion. In one patient showing severe congenital hypothyroidism and a total organification defect, a homozygous deletion (ΔT2512; codon 808) in exon 14 was identified. The homozygosity in the patient was due to partial isodisomy of the short arm of chromosome 2 carrying a defective TPO gene (Table 3). The patient, who was born small for gestational age, was healthy with normal growth and development while being treated with thyroxine. He has a normal phenotype except for a unilateral preauricular skin tag. This shows that partial maternal isodisomy (2pter–2p12) is compatible with no or a minimal influence on normal development (62).

In one patient showing severe congenital hypothyroidism characterised by a total organification defect, no mutations were found in the gene coding for thyroid peroxidase (Table 3). In this case we found a bi-allelic mutation in the ThOX2 gene. These findings prove that the ThOX2 enzymatic activity is essential for thyroidal hydrogen peroxide generation and that transient congenital hypothyroidism can be genetically determined (58). From the results obtained, it is clear that total organification defects show an autosomal recessive inheritance pattern with an incidence of about 1:60 000 births mainly caused by defects in the TPO gene.

**Defects in recycling of iodine** TG, internalised by (micro) pinocytosis from the follicular lumen, has been found to be present in the early and late endosomes. In these organelles, containing proteolytic enzymes, thyroid hormones are freed. The hydrolysate contains amino acids, including mono- and diiodothyrosine (MIT and DIT respectively). Subsequently, MIT and DIT are deiodinated by a specific dehalogenase found not only in the thyroid but also in some peripheral organs (63).

Recently, the identification of a gene called DEHAL1 from a thyroid SAGE library based on expression in thyroid, liver, kidney and mammary gland has been reported by our group (64). The main open reading frame encodes a novel human enzyme with a conserved nitroreductase domain that is FMN dependent and catalyses the dehalogenation of iodothyrosines. It is the first cloned vertebrate dehalogenase, providing the molecular basis for an evolutionary conserved mechanism for iodine recycling.

**Conclusion**

In recent years much progress has been made in the clarification of thyroid defects at the molecular level, especially in the field of thyroid dyshormonogenesis. It might give the impression that most of the inherited defects in thyroid dyshormonogenesis have been elucidated. However, in a large number of patients the thyroid defect cannot be diagnosed at the molecular level. Moreover, results of thyroid SAGE analyses (65) show that about 70% of the expressed transcripts could not be linked to known genes. From this, it is clear that there is still much to be investigated in order to elucidate the mechanism of thyroid hormonogenesis and its associated disorders.

<table>
<thead>
<tr>
<th>Hereditary character</th>
<th>No. of families</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homozygous for a mutation in TPO</td>
<td>13</td>
</tr>
<tr>
<td>Compound heterozygous for mutations in TPO</td>
<td>16</td>
</tr>
<tr>
<td>Isodisomy (chromosome 2p)</td>
<td>1</td>
</tr>
<tr>
<td>Mutation in 1 allele of the TPO gene</td>
<td>4*</td>
</tr>
<tr>
<td>No mutations found in the cDNA coding for TPO</td>
<td>1</td>
</tr>
</tbody>
</table>

* Mutations in promoter regions or intron sequences remain possible.
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