0021-972X/00/$03.00/0
The Journal of Clinical Endocrinology & Metabolism

Vol. 85, No. 10
Printed in U.S.A.

Two Decades of Screening for Congenital Hypothyroidism in the Netherlands: TPO Gene Mutations in Total Iodide Organification Defects (an Update)

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ABSTRACT

Presented is a cohort study to assess the nature and frequency of thyroid peroxidase (TPO) mutations in 45 patients (35 families) with congenital hypothyroidism due to a total iodide organification defect; incidence is 1:66,000 in The Netherlands. The presentation is consistently similar with a severe form of congenital hypothyroidism and also characterized by a complete and immediate release of accumulated radioiodide from the thyroid after sodium perchlorate administration.

Sixteen different mutations were found, including eight novel mutations; the majority occurs in exons 8, 9, or 10. The GGCC insertion in exon 8 at nucleotide 1277, leading to an early termination signal in exon 9, is the most frequently occurring mutation. These mutations were detected in 29 families in both TPO alleles (13 homozygous and 16 compound heterozygous). In one family, partial maternal isodisomy of 2p was detected, in four families only one mutated TPO allele could be detected, and in one family no inactivating TPO mutation could be found.

Because all patients clearly had the clinicopathologic features of a total iodide organification defect, we conclude that in these five families the mutations in the (other) alleles could be either located in the intronic sequences or in the promoter region. Mutations in the TPO gene result in total iodide organification defects. (*J Clin Endocrinol Metab* **85:** 3708–3712, 2000)

THE INCIDENCE OF permanent congenital hypothyroidism (CH) in The Netherlands is circa 1:3000 newborns (1). Annually, 60–70 cases are detected by the Dutch neonatal CH screening. In approximately 15% of the patients, hereditary disorders in thyroid metabolism (dyshormonogeneses) are found, of which the total iodide organification defect (TIOD) is the most severe condition, with an estimated incidence of 1 in 60,000 newborns (1). The neonatal presentation is consistently similar with low screening T_4 and elevated TSH concentrations in heel puncture blood and mostly even lower plasma FT_4 and T_4 levels (of maternal origin and rapidly declining after birth) and high plasma TSH and thyroglobulin (Tg) concentrations. Also, a rapid and elevated radioiodide ($^{123}I^-$) uptake by the thyroid gland is observed, and the complete and immediate release of the accumulated radioiodine from the thyroid after iv administered sodium perchlorate, indicating that iodide can not be bound to proteins (2). The clinical picture was described 50 yr ago by Stanbury and Hedge (3).

Thyroid peroxidase (TPO) activity in thyroid tissue of patients with TIOD is not detectable (4–7). TPO is a thyroid-specific glycosylated hemoprotein of 110 kDa bound at the apical membrane of the thyrocyte (8). The 933 amino acid-containing protein is encoded by a TPO messenger RNA of 3 kb (9). The TPO gene contains 17 exons, is 150 kb in size, and is located on chromosome 2, locus 2p25 (10–12). Absence of TPO activity implicates the inability to iodinate tyrosine residues in Tg and to couple these residues to form thyroid hormones, mainly T_4 and some T_3 and rT_3 (8, 13). TIOD is inherited in an autosomal recessive way (7). Moreover, mutations in the TPO gene are described to be causative for the diagnosis TIOD (5, 7, 14). In this study, we present the clinicopathologic and molecular biological studies of 45 TIOD patients, the majority of them referred to us over the last 2 decades (since the initiation of the Dutch CH screening).

Patients and Methods

As a nationwide referral and consultation center for CH, 46 TIOD families are known to us, each with at least one patient. After approval by the Academic Medical Center medical ethics committee, patients and (for the younger ones) their parents were informed about the study and asked for participation. Two families in our study are first or second generation Dutch residents from Kurdish and Afghan descent. Thirty-five families gave written permission for DNA studies. A blood sample was drawn from both the patients and their parents at a regularly planned visit to the outpatient clinic. Nine families refused participation, mainly because they experienced DNA diagnostics as threatening; two of them are still considering additional DNA studies.

Patients with the clinicopathologic entity TIOD present with extremely low plasma FT_4 and T_4 levels, high plasma TSH and Tg concentrations, elevated $^{123}I^-$ uptake in the thyroid gland, and an immediate and complete (>90%) release of the accumulated intrathyroidal radioiodine after iv administered sodium perchlorate (2). Physical examination of children with CH in the neonatal period (defined as the first 4 weeks of life), including those with a TIOD, hardly revealed a noticeable goiter, in our experience. Only one patient had congenital goiter that

Received March 15, 2000. Revision received June 26, 2000. Accepted July 6, 2000.

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was noticeable on physical examination and confirmed by ultrasound. Some patients had slightly enlarged thyroid glands detected by ultrasound and/or $^{123}I^-$ scan only.

All patients listed in Table 1 were investigated in the neonatal period, however, during this period not all 45 study patients were investigated; the number (percentage) of examined patients is listed in the last column of Table 1. The patients described were not on T_4 supplementation at the time of the blood test, but began therapy a few hours later. $^{123}I^-$ uptake studies were performed by iv administering 1 MBq $^{123}I^-$, followed by measurements of the uptake above the thyroid every 30 min, and administration of 100 mg $NaClO_4$ iv at 120 min (2). After administration of sodium perchlorate, the release of accumulated iodine was measured at least every 15 min for a maximum of 1 h.

DNA from peripheral blood lymphocytes was isolated. The TPO genes were studied by restriction enzyme digestion and denaturing gradient gel electrophoresis (DGGE) analysis of PCR-amplified genomic DNA (6). Exons with aberrant DGGE patterns were sequenced using ABI Prism BigDye primer cycle sequencing chemistry (PE Applied Biosystems, Foster City, CA) on a ABI 377 DNA sequencer. In the patients without DGGE abnormalities, all exons and intron/exon boundaries of the TPO gene were sequenced.

Results

The baseline clinicopathologic characteristics are listed in Table 1; only the data of patients who are investigated in the neonatal period are presented. The clinical presentation is consistently similar: a severe form of CH with very low plasma (F)T_4 concentrations and highly elevated plasma TSH and Tg levels; all of these measurements were done just before initiation of T_4 supplementation. The level of (F)T_4 greatly depends on the age at the time of the blood test and is clearly declining over time, with a half-life of about 3.5 days (Fig. 1). A high initial $^{123}I^-$ uptake in the thyroid gland is found, the maximum uptake (above 20% of the administered dose) is mostly reached circa 30 min after injection, followed by a gradual, spontaneous decrease in thyroidal $^{123}I^-$ content. An immediate and complete (>90%) release of the accumulated intrathyroidal radioiodine after iv-administered sodium perchlorate is a typical characteristic for the clinicopathologic entity TIOD.

Table 2 shows the different categories of TPO mutations found in the investigated population and sorted by exon number. Mutations in both TPO alleles were found in 29 families, for 13 families in a homozygous fashion and for 16 families in a compound heterozygous fashion. The compound heterozygous mutations had the following combinations (see Table 2): A2/B3 in three families, A2/A5 in two families, and A2/A6 in two families. The next combinations for compound heterozygotes were present in only one family: A1/B6, A1/A2, A2/A3, A2/B3, A2/C1, B1/B3, B3/A5, B3/C2, and B3/C3. In one family, homozygosity was caused by partial maternal isodisomy of 2p (accepted for publication); the mutation involved in this family is listed under A6 (Table 2). In four families a mutation in only one TPO allele was found, and in one family we could not detect any inactivating mutation in the exonic coding sequence, the promoter region, or the exon/intron boundaries of the TPO gene. The mutations involved in these four families are further discussed in the *Discussion.*

Table 2 also shows the four groups of mutations: A, frameshift mutations (six different ones) occur most frequently (39 of the 69 studied TPO alleles, *i.e.* 57%); B, missense mutations (six different ones) found in 15 mutated TPO alleles are second in frequency (22%); followed by C, mutations that putatively affect splicing (three different ones) responsible for five affected alleles (7%); and finally D, one nonsense mutation that affects four TPO alleles (6%). The majority of the detected mutations occur in exons 8, 9, and 10. These exons encode for the active site of the enzyme, the part involved in heme binding. The most frequently occurring single mutation is the GGCC duplication in exon 8, at nucleotide position 1227, detected in 36% of the studied TPO alleles. This mutation is found in 51% of the Dutch TIOD families, either in a homozygous or a compound heterozygous fashion. The duplication results in a Nael restriction site (5′-GCC↓GGC-3′), which allows rapid identification (14, 15).

Based on the data from this study, the incidence of TIOD in The Netherlands was reestablished to be at least 1:66,000 newborns over almost 2 decades of Dutch CH screening (1981—present).

Discussion

By definition, a patient with a TIOD is unable to produce any thyroid hormone. Indeed, the clinicopathologic presen-

TABLE 1. Baseline characteristics of the studied TIOD patients in the neonatal period

Determinant	Reference range	Mean	SD	SEM	Median	95% CI	n (%)
Age at diagnosis (days postpartum)		12	13	1	14	10–14	33 (73%)
Plasma T_4 (nmol/L)	120–220	15	15	3	11	10–21	32 (71%)
Plasma FT_4 (pmol/L)	12–29	2.4	3.0	0.7	1.0	1.0–3.8	21 (47%)
Plasma T_3 (nmol/L)	1.5–3.0	0.7	0.4	0.1	0.75	0.6–0.9	22 (49%)
Plasma TSH (mU/L)	1–10	703	390	74	606	552–855	28 (62%)
Plasma Tg (pmol/L)	20–375	4090	3560	700	2600	2650–5530	26 (58%)
Plasma T_4-binding globulin (nmol/L)	380–750	500	110	20	500	460–550	23 (51%)
Thyroidal $^{123}I^-$ uptake							
Accumulated radioiodide (%)	2–12%[a]	26	10	3	27	20–31	15
Time reached (h)	>>2 h	0.53	0.27	0.07	0.50	0.38–0.68	15
Release after $NaClO_4$ (%)	<10%	98	3	1	100	96–99	15

Not in all patients complete diagnostic determinants were measured during the neonatal period (defined as the first 4 weeks postpartum). The *last column* denotes the number of patients (n) in which these particular determinants were available, and the percentage of the total study population is in *parentheses* (n = 45). Maximum accumulated radioiodide (the maximum uptake) during the first 2 h of the $^{123}I^-$ uptake study is expressed as the percentage of the iv-administered dose of radioiodide.

[a] As measured in healthy adults, 2 h after the radioiodide was given, the percentage depends greatly on the local iodine status (26). Perchlorate-induced release is expressed as the percentage of the radioiodide taken up by the thyroid just before iv $NaClO_4$ administration (see also *Patients and Methods*).

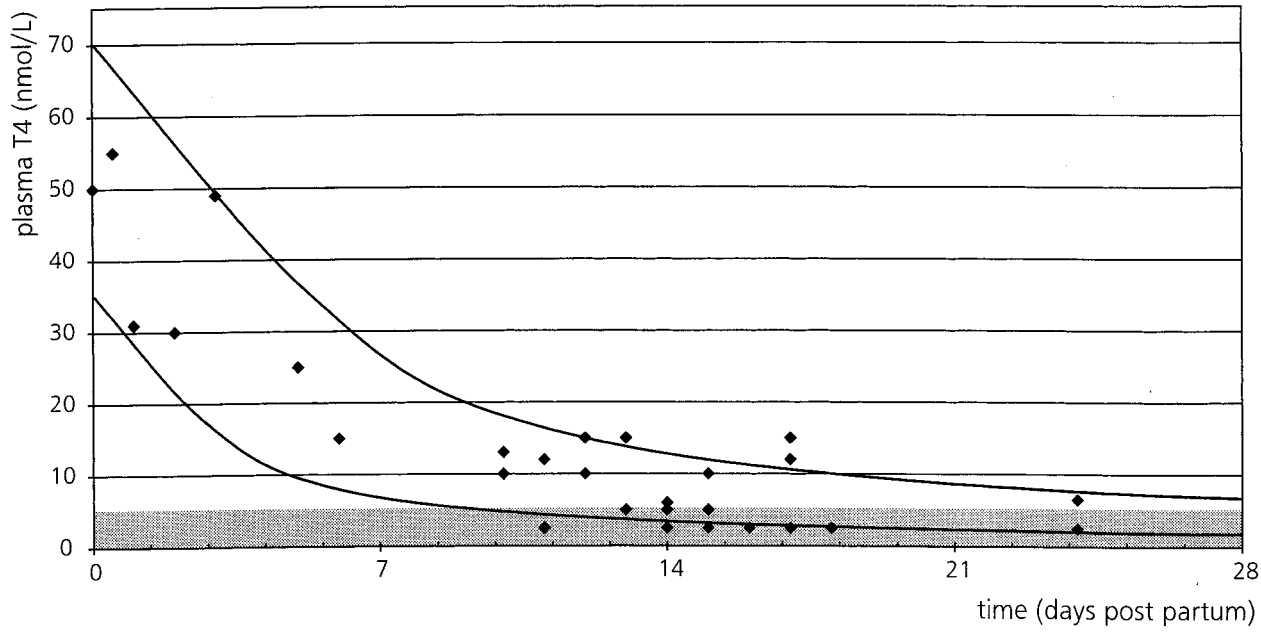

FIG. 1. Plasma T_4 concentrations in 28 TIOD patients after birth. Plasma T_4 values (nmol/L) of individual TIOD patients over the last 2 decades in The Netherlands. The area between the two curved lines represents the 95% CI for the half-life of plasma T_4, as calculated by Vulsma *et al.* (16). This indicates that plasma T_4 in the studied neonates is of maternal origin. The values in the *gray zone* at the bottom of the graph are below the detection limit of the T_4 assay. Note: the period between the day of birth and the day of the initiation of T_4 supplementation has become much shorter since the start of the CH screening. Because initiation of T_4 supplementation is on the same day as the first blood test, the first assessment of T_4, and so forth, has also become earlier after birth over the last years.

TABLE 2. Overview of 16 TPO mutations found in Dutch TIOD patients

Group No.	Exon	Mutation	Position (at nucleotide n)	Effect of the mutation on protein synthesis	n (%)
A					
1	2	ins 20 bp	141	Frameshift, leading to an early termination signal in exon 3[a]	5 (7.2)
2	8	ins GGCC	1277	Frameshift, leading to early termination signal in exon 9	25 (36.2)
3	***8***	***del C***	***1425***	***Frameshift, leading to early termination signal in exon 9***	***1 (1.4)***
4	***12***	***del TT***	***2243/2244***	***Frameshift, directly resulting in a termination signal→Phe 718 stop***	***1 (1.4)***
5	14	ins C	2505–2511[b]	Frameshift, leading to early termination signal in exon 16[c]	4 (5.8)
6	***14***	***del T***	***2512***	***Frameshift, leading to early termination signal in exon 14***	***3 (4.3)***
B					
1	***8***	***G→A***	***1066***	***Ala 326 Thr***	***1 (1.4)***
2	9	A→T	1429	Ile 447 Phe[c]	1 (1.4)
3	9	T→G	1447	Tyr 453 Asp[a,c]	9 (13.0)
4	***9***	***G→T***	***1671***	***Trp 527 Cys***	***1 (1.4)***
5	***12***	***C→T***	***2167***	***Arg 693 Trp***	***2 (2.9)***
6	14	G→A	2485	Glu 799 Lys[c]	1 (1.4)
C					
1	***4***	***G→C***	***439***	***GG/gt→GC/gt (putatively affects splicing)***	***1 (1.4)***
2	10	G→A	1858	AG/gt→AA/gt (putatively affects splicing)	3 (4.3)
3	***10***	***G→A***	***+1 intron 10***	***AG/gt→AG/at (putatively affects splicing)***	***1 (1.4)***
D					
1	10	C→T	1708	Arg 540 stop[a]	4 (5.8)

There are four groups of mutations: A, frameshift mutations; B, missense mutations; C, mutations putatively affecting splicing; D, nonsense mutations. Within each group the different mutations are numbered (*e.g.* A1). Eight mutations are not previously described and are expressed in *bold*/*italic*. n, the number of alleles that carry the particular mutation in the studied population. Thirty-five families are described; in the family where the propositus has partial maternal isodisomy for 2p we counted one TPO allele (*i.e.* 69 studied alleles total). From here, we calculated the frequencies as a percentage (in *parentheses*) of the total number of studied alleles. ins, Insertion; del, deletion; capital letter, exonic sequence; lowercase letter, intronic sequence (27).

[a] TPO inactive in the patient's thyroid tissue (5–7).

[b] Stretch of seven cytosines.

[c] TPO inactive in an *in vitro* expression system (18).

tation of all CH patients in this study is consistently similar, with very low plasma (F)T_4 and T_3 levels and highly elevated plasma TSH and Tg concentrations. However, the (F)T_4 values measured were not undetectable. Because it is known that a limited but substantial maternal-fetal transfer of T_4 exists in these patients and that plasma T_4 disappears with a mean initial half-life of 3.6 days [95% confidence interval (CI), 2.7–5.3], decreasing plasma (F)T_4 and T_3 concentrations

can be expected in the first few weeks of life if not treated with T_4 (16). As clearly depicted in Fig. 1, all measured plasma T_4 levels (at variable age and before treatment was started) illustrate a time course that is consistent with an initial T_4 half-life of 2.7–5.3 days, indicating that the thyroid hormone detected in these patients was of maternal origin. Similar values for the above mentioned diagnostic determinants are also observed in other inborn errors of thyroid hormonogenesis (2). TIOD patients, however, can clearly be distinguished by means of the $^{123}I^-$ uptake study, because sodium perchlorate administration causes an immediate and almost complete release (>90%) of intrathyroidal iodide. Iodine intoxication, being a confounder by temporarily blocking iodide organification is distinguished from a hereditary TIOD by the high urinary iodine excretion, and the medical history.

The combination of the above-described diagnostic determinants is the basis for the selection of patients for DNA diagnostic studies. Because TIOD is inherited in an autosomal recessive manner, the parents of the described TIOD patients are, except for one, carriers of one TPO mutation. It is important to stress that they all have normal thyroid function. In this study, we demonstrate that in almost all patients the clinicopathologic entity TIOD is associated with inactivating mutations in the TPO gene. In four patients only one mutated allele could be detected even after sequencing all exons and the intron/exon boundaries. It is not unlikely that the mutation in the other allele is located in the more upstream part of the TPO gene or within the intronic sequences (*e.g.* branching points), creating an alternative splicing site. Until now, the sequences of these regions are only partly known. The four patients with only one mutated TPO allele had inactivating mutations as mentioned under A1, A2, A4, and B3 (Table 2). In one of the 35 TIOD families, we were not able to detect any inactivating mutation in the TPO gene. It is not unlikely that the TPO gene mutations in this family are undetectable for the same reasons as mentioned above, or that this TIOD might be caused by another enzymatic disorder (*e.g.* related to the thyroid oxidase) (17, 17a).

As a TIOD implies a total inability of the thyroid to iodinate proteins, it is to be expected that the mutated TPO is inactive. For a number of mutations, this has indeed been shown (5, 7, 18). The results in this study are in concordance with those in our previous publications. A recent publication by Kotani *et al.* (19) described a novel missense mutation in exon 7, a G808A transition, replacing Asp with an Asn. Also Pannain *et al.* (20) described a novel mutation, namely a missense mutation due to a G2033A transition in exon 11, replacing Arg with a Gln.

Surprisingly, one patient, homozygous for the GGCC insertion in exon 8 of the TPO gene (see Table 2), was described (14) with a partial release of the radioiodide taken up by the thyroid gland after the administration of perchlorate. The authors measured a very low TPO rest activity, which could explain this partial release. In addition to the normal splicing product of the mutated TPO gene, they also discovered a product of alternative splicing and state the latter could account for the very low residual activity in the goiter of their patient. In our patients with the same mutation in the TPO gene (Table 2), however, a complete release of intrathyroidal iodide after perchlorate was observed. Because the GGCC insertion leads to a frameshift and an early termination signal in exon 9, TPO activity is expected to be absent, corresponding with our findings.

Another study (21) describing three families, in which CH caused by iodide organification defects occurs, showed three mutations in the TPO alleles, of which one of them is novel (in exon 11, see below). In one of these families the patients presented with goiter and mild hypothyroidism at ages 4 and 13 yr. Judging by the presented clinicopathologic data, they definitely seem to have partial organification defects. The patients were compound heterozygote with a C-insertion at nucleotide position 2505–2511 (exon 14) and a missense mutation changing a C to G at nucleotide position 2068 (exon 11), replacing the normal Glu with Gln. The first mutation causes a totally inactive TPO and corresponds with a TIOD, see also Table 2 and the work of Bikker *et al.* (18). The second mutation may inactivate TPO only partially, explaining the partial organification defect and mild hypothyroidism.

A limited number of five patients with a rather mild type of CH due to a partial organification defect (PIOD) (2) was studied for mutations in the TPO alleles; none of the 16 TPO mutations described here (Table 2) could be detected (data not shown). Also, Pendred's syndrome (defined as the combination of a neurosensory hearing loss and a PIOD) is not related to TPO gene mutations (22), but to PDS gene mutations (22–25). TIOD is not associated with neurosensory hearing problems. It seems to us that the clinicopathologic entity PIOD without hearing defects has a different molecular background than TIOD. Hypothetically, mutations in the oxidase or PDS gene or partially inactivating mutations in the TPO gene are possible explanations for the condition PIOD.

This study shows eight TPO mutations that have not been described before yet (see Table 2): three new frameshift mutations, three new missense mutations, and two new mutations that affect splicing. The effect of these (missense) mutations needs to be checked in an *in vitro* expression system. One of the new mutations (a C to T transition at nucleotide position 2167, replacing an Arg with a Trp at amino acid position 693) was discovered only in the family from Afghan descent.

We conclude that there seems to be an all or nothing situation with respect to TPO activity. Where homozygous or compound heterozygous inactivating mutations in the TPO alleles are demonstrated, the peroxidase and hormone-producing activities seem to be zero. In our rather large group of patients, there is not a trace of variable expression or variable penetration. Although inactivating TPO mutations have not been found in all TIOD cases yet, it can be stated that that inactivation of TPO always leads to TIOD and, hence, to a total inability to produce thyroid hormones.

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