INBORN ERRORS OF SYNTHESIS AND SENSITIVITY TO THYROID HORMONE

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INBORN ERRORS OF SYNTHESIS AND SENSITIVITY TO THYROID HORMONE

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Novel Compound Heterozygous Pathogenic Mutations of *SLC5A5* in a Chinese Patient With Congenital Hypothyroidism

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Background and Objectives: Defects in the human sodium/iodide symporter (*SLC5A5*) gene have been reported to be one of the causes of congenital hypothyroidism (CH). We aimed to identify *SLC5A5* mutations in Chinese patients with CH and to evaluate the function of the mutation.

Methods: Two hundred and seventy-three patients with primary CH were screened for mutations in *SLC5A5* using next-generation sequencing. We investigated the expression and cellular localization of the novel compound heterozygous mutation in *SLC5A5*. The functional activity of the mutants was further examined *in vitro*.

Results: In 273 patients with CH, two previously undescribed pathogenic mutations p.Gly51AlafsTer45 (G51fs) and p.Gly421Arg (G421R) in a compound heterozygous state in *SLC5A5* were identified in a pediatric patient. G51fs was located in the first intercellular loop connecting transmembrane segment I and II, whereas G421R was in the transmembrane segment (TMS) XI. G51fs and G421R resulted in a truncated NIS and reduced protein expression, respectively. *In vitro* experiments further showed that the normal function of iodine transport of sodium-iodide symporter (NIS) mutants was markedly impaired.

Conclusion: The undescribed compound heterozygous mutation of *SLC5A5* was discovered in a Chinese CH patient. The mutation led to significantly reduced NIS expression and impaired iodide transport function accompanied by the impaired location of the NIS on the plasma membrane. Our study thus provides further insights into the roles of *SLC5A5* in CH pathogenesis.

Keywords: iodine transport, mutation, SLC5A5, next-generation sequencing, congenital hypothyroidism

INTRODUCTION

Congenital hypothyroidism (CH), which is defined by inadequate thyroid hormone production in newborn infants, is a common neonatal endocrine disorder with the incidence at about 1:2,000–4,000 worldwide (1). Thyroid hormones are critical for neurodevelopment; severe congenital hypothyroidism can lead to growth retardation and permanent intellectual disability (1, 2).

The human *SLC5A5* (GenBank reference sequence: NM_000453.3) gene, encoding the sodium-iodide symporter (NIS), a 643 amino acid protein, is located on chromosome 19 and consists of 15 exons (3). The NIS protein is composed of 13 transmembrane segments (TMS), an extracellular amino terminus, an intracellular carboxy terminus and three asparagine-linked glycosylation sites. Previous research showed that glycosylation was not critical for NIS targeting to the plasma membrane or normal function (4).

NIS mediates the active transport of iodide from the bloodstream into the tissues, particularly in the thyroid grand, by coupling the inward translocation of Na^+ down its electrochemical gradient to the inward movement of I^- against its electrochemical gradient. The driving force for this process is the electrochemical sodium gradient generated by Na^+/K^+ ATPase (2, 5–9). Sufficient dietary iodine intake is essential for the production of the thyroid hormones. In thyroid tissue, iodide uptake is typically the first step in thyroid hormone synthesis. The *SLC5A5* is responsible for the transport of iodide mainly in the thyroid gland (2, 10). The mutation of *SLC5A5* is a cause of thyroid dyshormonogenesis, which leads to CH in patients. Currently, several iodine transport defect-related mutations of *SLC5A5* have been identified, including G18R, V59E, G93R, R124H, Q267E, V270E, C272X, D331N, Y348D, T354P, G395R, R516X, G543E, and S547R, which have provided valuable structural evidence about the symporter and helped us to understand the pathogenesis of this disease (11–24).

The reported mutations of *SLC5A5* almost all affect its normal functional activity, leading to thyroid dyshormonogenesis (5, 10, 25). Our study, therefore, aimed to identify additional mutations in *SLC5A5* from the Chinese patients with CH and analyzed the function of the mutations *in vitro*, to evaluate the roles of *SLC5A5* in the pathogenesis of CH in the Chinese patients.

Herein, we report a novel compound heterozygous missense mutation G51fs/G421R of *SLC5A5* gene in a pediatric patient diagnosed as congenital hypothyroidism from newborn screening. Our results showed that G51fs and G421R markedly affected the function of iodine intake accompanied by impairment of the location of NIS on the plasma membrane. Our study provided further insights into the roles of *SLC5A5* in CH pathogenesis.

MATERIALS AND METHODS

Clinical Subjects

All subjects were recruited from the Chinese Han population in Jiangsu province, Fujian province, Anhui province, and Shanghai through collaboration with multiple hospitals in China. A total of 273 CH patients (141 females and 132 males) identified through newborn screening were enrolled in this study. The diagnosis of permanent CH was confirmed in infants based on the following criteria: CH patients with elevated TSH (thyrotropin) levels, with or without T4 (thyroxine) or FT4 (free thyroxine) levels less than the normal range, and the restoration of normal thyroid parameters after receiving replacement therapy with L-thyroxine; however, after stopping treatment, a rise in TSH and a drop in FT4 were observed again (26–28). Written consent was obtained from the parents of the CH patients, and the study was approved by the Ethics Committee of Shanghai Ninth People's Hospital affiliated with the Shanghai Jiao Tong University School of Medicine.

Next-Generation Sequencing (NGS) and Bioinformatics Analysis

The sequencing methods and analysis pipelines used were previously reported (28). Genomic DNA was extracted from the whole blood of the probands and family members if available according to standard extraction procedures. All the exons and exon-intron boundaries of *SLC5A5* were amplified by performing multiplex polymerase chain reaction (PCR) using a 48×48Access Array™ microfluidic platform (Fluidigm) according to the manufacturer's protocol. The primers were designed using iPLEX Assay Design software (Sequenom). The HiSeq3000 platform (Illumina, San Diego, CA) was used to perform deep sequencing of these amplicon libraries.

The target sequences were amplified and deep sequenced in duplicate for each sample to avoid base pair variants caused by multiplex PCR. Descriptions of the *SLC5A5* mutations were based on NM_000453.3. We analyzed the raw sequence data in fastq format and obtained the quality scores by following the method indicated by previous studies (28). We filtered out the variants with frequencies >1% in the dbSNP 135 and ESP6500 v2 databases and focused on the functional (protein altering) variants (removal of intergenic and 3'/5'UTR (untranslated region) variants, non-splice-related intronic variants, and synonymous variants) identified in duplicate samples (https://doi.org/10.6084/m9.figshare.13134824.v1). Then, the two remaining variants were selected for validation by Sanger sequencing.

Construction of NIS Plasmids

The ORF (open reading frame, nucleotide 1 to 1932) of human *wild-type* (WT) *SLC5A5* was amplified by PCR from genomic DNA using primers containing the EcoRI and XbaI restriction sites. The amplified PCR fragment was inserted into a p3xFLAG-CMV-10 expression vector and the FLAG tag was inserted upstream to the NIS coding region. Similarly, a WT NIS-EGFP fusion protein was expressed in a pEGFP-N2 expression vector (TransGen Biotech) using EcoRI and BamHI restriction sites, and the GFP was tagged downstream to the NIS coding region. The missense mutations were generated by site-directed mutagenesis using the Fast Mutagenesis System kit (TransGen Biotech). Due to premature termination of NIS, p.Gly51AlafsTer45 (G51fs) containing the EcoRI and BamHI restriction sites was cloned into the pEGFP-

N2 vector to generate the fusion plasmid. All the plasmid constructs were verified by Sanger sequencing. The primers used are listed in **Supplemental Table 3**.

Cell Culture and Transient Transfection

The human embryonic kidney 293T cell line was cultured in Dulbecco's modified Eagle's medium (DMEM)/high-glucose medium (Gibco) supplemented with 10% fetal bovine serum (FBS; Sigma Aldrich) at 37 °C in a humidified atmosphere containing 5% CO_2. Transfections were performed on cells by Lipofectamine 2000 Transfection Reagent (Invitrogen) following the manufacturer's guides. Cells were plated in a 20 mm glass bottom cell culture dish (NEST) and transiently transfected with 1 μg plasmid DNA to detect the cell localization of the WT or mutants of *SLC5A5*-pEGFP-N2 plasmids. Iodide uptake assays were performed on the 293T cells. The cells were cultured in 12-well plates, transfected with 1 μg WT or mutant 3xFLAG-NIS plasmids (cotransfection with 0.5 μg WT and 0.5 μg mutant plasmids to simulate the heterozygous state), and the treated cells were then kept in a humidified incubator at 37 °C with 5% CO_2 for 48 h.

Total RNA Extraction and qPCR (Quantitative PCR)

RNA extraction was performed as described previously (27). RNA was prepared using Trizol reagent (Invitrogen, H10522) and the total RNA (1 μg) was reverse transcribed using the cDNA synthesis kit (Takara). Quantitative PCR (qPCR) was performed using TB Green® Premix Ex Taq™ II (Tli RNaseH Plus), ROX plus (Takara), and the ABI QuantStudio 12K Flex Real-time PCR System (Life Technologies). The primers used are described in **Supplemental Table 3**.

Iodide Transport Functional Studies

The iodide uptake assay was performed as described previously (29). In brief, 293T cells were seeded in 12-well plates, which contained sterilized cover slips (WHB Biotech), incubated at 37°C for 24 h, and transiently transfected alone or co-transfected with WT or mutant pEGFP-N2 plasmids. 48 h after transfection, the 293T cells were washed once in serum-free DMEM medium and incubated for 1 h in 1 ml serum-free medium containing ^{131}I at 5 KBq/ml as the only source of iodide. For the inhibition of NIS-mediated uptake, $NaClO_4$ (final concentration 1 mM) was included in parallel incubations (30). The cells were washed briefly in Hank's Balanced Salt Solution (HBSS) buffer and then incubated with 1 ml HBSS for 5 min. The cells were solubilized by the addition of 1 ml 1 M NaOH, and the radioactivity was measured using a γ counter (GC1200, Anhui, China). DNA was determined by the Qubit Fluorometer using ssDNA Assay Kit (Q10212, Thermofisher). Each value represents the mean ± SD of picomoles ^{131}I per microgram of DNA of three independent experiments done in triplicate.

Western Blot Analysis

Representative Western blot analysis of whole cell lysates from transiently transfected 293T cells was probed with anti-FLAG antibodies (AE063, ABclonal) and anti-GAPDH (AM4300, Thermo Fisher Scientific) antibodies. Proteins were visualized by the Odyssey CLx Infra-Red Imaging system.

Immunofluorescence Analysis of Protein Localization

The 293T cells were seeded in 12-well plates, which contained sterilized cover slips (WHB Biotech), incubated at 37 °C for 24 h, and transiently transfected with WT or mutant pEGFP-N2 plasmids. One day later, the cells were washed twice with PBS (phosphate buffer saline), fixed with 4% PBS-buffered formaldehyde (PFA) for 30 min at room temperature, washed twice with PBS, and then incubated with fluorescent probe Dil (Beyotime Biotech) targeted to the cell membrane for 5 min at 37°C. After washing with PBS, the nuclei were stained with DAPI (Beyotime Biotech) at room temperature for 5 min. Cover slips were mounted, and the plates were examined using a confocal microscope (Nikon A1 Microsystems).

Molecular Modeling and Electrostatic Surface

The sequence of *SLC5A5* was analyzed using the SWISS-MODEL (31). The 3D structure of the Vibrio parahaemolyticus sodium/galactose symporter (vSGLT) was chosen as the modeling template. Theoretical modeling of the protein structure was performed using PyMOL 2.4 (https://pymol.org/2/).

Statistical Analysis

Statistical analysis was performed using Prism 8.0 software (GraphPad Software) from more than three independent experiments. Data were presented as the mean ± SD. Comparisons between two groups were analyzed using unpaired two-tailed Student's t test (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$). Differences were considered statistically significant at values of $p < 0.05$.

RESULTS

Patient

In our study, mutations in *SLC5A5* for congenital hypothyroidism were screened for in the cohort of 273 CH patients. Only one patient was found to carry the mutations in *SLC5A5* with no other mutations in the reported candidate genes. The proband was a full-term girl, born from healthy non-consanguineous Chinese parents. Newborn screening found an abnormally high TSH level (specific value unknown). At 18 days after birth, diagnostic confirmation of congenital hypothyroidism was achieved by measuring the serum TSH at >100 uIU/ml (normal range 0.34–5.6 uIU/ml), free T4 0.45 ng/dl (normal range 0.58-1.64ng/dL), and free T3 0.85 pg/ml (free triiodothyronine normal range 2.5–3.9pg/ml) (**Table 1**). Her height was 54.5 cm, and her weight was 3.3 kg.

Thyroid hormone supplementation was started immediately after diagnosis, with a daily dose of 28 μg levothyroxine. However, 17 days after initial treatment, thyroid function was still abnormal (TSH level >100 uIU/ml; FT4:1.41 ng/dl; FT3:3.83 pg/ml)

TABLE 1 | Thyroid function tests during follow-up before and after thyroid hormone replacement therapy.

	Before therapy	After therapy			Normal Range
		17 d	4 m	1.25 y	
FT3 (pg/ml)	0.85	3.83	4.56	4.32	2.5–3.9
FT4 (ng/dl)	0.45	1.41	1.30	2.74	0.58–1.64
TSH (uIU/ml)	>100	>100	33.43	0.94	0.34–5.6

At 18 days after birth (before therapy), diagnostic confirmation of congenital hypothyroidism was achieved by measuring the serum TSH, FT3, and FT4. The regular thyroid status was monitored at 17 days, 4 months, and 1.25 years after the initial treatment. Abbreviations: d, day; m, month; y, year; FT3, free triiodothyronine; FT4, free thyroxine; and TSH, thyrotropin.

(**Table 1**), and the local pediatrician adjusted the daily dose of levothyroxine to 40 μg. Four months after treatment, her height was 75 cm, and weight was 10 kg. Her thyroid function was slightly improved but still abnormal (TSH:33.43 uIU/ml; FT4:1.3 ng/dL; FT3:4.56 pg/ml) (**Table 1**). At the age of 1 years and 3 months, the proband was recruited to our study. Her height was 81.5 cm, and her weight was 11 kg.

The TSH level returned to the normal range during thyroid hormone replacement therapy (TSH:0.94 uIU/ml; FT4:2.74 ng/dL; FT3:4.32 pg/ml) (**Table 1**). Due to poor compliance, the results of thyroid scintigraphy and the concentration of serum thyroglobulin and autoantibody were unavailable. Normal growth was observed during the first years of life. Of note, the patient's hypothyroidism did not appear to affect her intellectual development. Thyroid ultrasonography showed a normal size and well-located gland. No other members of the family had a known history of hypothyroidism. The proband's parents were euthyroid with no autoantibodies and normal thyroid volume by ultrasound.

Identification of the Novel Compound Heterozygous NIS Mutation

Further molecular analysis of the whole blood of the proband and family members revealed the presence of an undescribed compound heterozygous mutation G51fs/G421R. Based on the current secondary structure model for NIS, G51fs is located in the first intercellular loop connecting transmembrane segment I and II, whereas G421R is in the TMS XI (**Figure 1**). Sequencing of the patient's *SLC5A5* gene revealed a G deletion at nucleotide +152 in exon 1, resulting in a frameshift mutation and, thus, premature termination of NIS and a loss of about 85% of the amino acids of the protein (**Figure 2A**, **Supplemental Table 1**). The patient also had a novel heterozygous G>A transversion at nucleotide +1261 in exon 11, which leads to the substitution of Gly by Arg at residue 421 (**Figure 2A**).

The mutations contribute to a compound heterozygous mutation G51fs/G421R in NIS (**Figure 2B**). On the other hand, analysis of the euthyroid parents of the proband showed that the father was heterozygous for G51fs NIS, and the mother was heterozygous for G421R NIS, suggesting recessive inheritance of the mutation (**Figures 2A, B**). This information is summarized in a pedigree diagram (**Figure 2B**). Multiple amino acid sequence alignment of NIS orthologs from different species revealed that two glycine residues at position 51 and 421 were highly conserved (**Figure 2C**).

We next used the Vibrio parahaemolyticus sodium/galactose symporter (vSGLT) as a template to model the 3D structure of G421R. The substitution of the neutral side chain of Gly by the basic amino acid arginine residue at position 421 changed the net surface positive charges and distorted its surface structure (**Figure 3A**). We observed that the interatomic distance between G421 and T354 was changed from 15.0 Å to 8.7 Å (**Figure 3B**). Substituting gly with a bulkier arginine destabilized

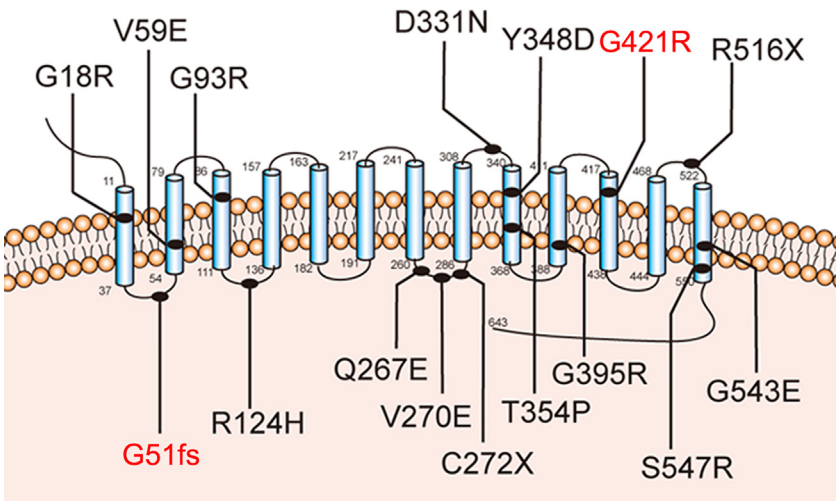

FIGURE 1 | Sodium-iodide symporter (NIS) secondary structure model. Blue cylinders represent the 13 transmembrane segments (TMS). The amino-terminus faces the extracellular space and the carboxy-terminus is in the cytosol. The novel pathogenic variants p.G51fs and p.G421R are highlighted with blue lines.

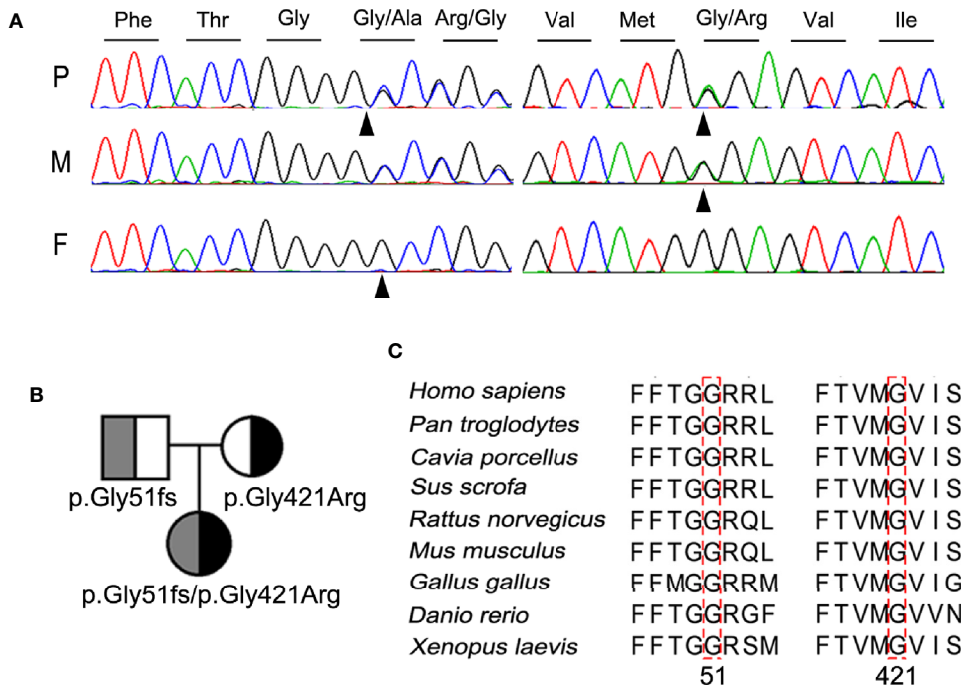

FIGURE 2 | Identification and characterization of the compound heterozygous NIS mutation p.G51fs/p.G421R. **(A)** Partial sequence chromatograms cover the region of the variants in exon 1 (c.152del, p.G51fs) and 11 (c.1261G>A, p.G421R) of *SLC5A5* gene. The nucleotide and corresponding amino acid sequences using the three-letter code are indicated. The proband was compound heterozygous for the mutant alleles p.G51fs and p.G421R NIS. Arrowheads denote the mutated sites. P, proband; F, father; M, mother. **(B)** Pedigree analysis of the members of the proband's family. The mutated alleles for p.G51fs and p.G421R NIS are noted below. **(C)** Amino acid sequences of NIS from other metazoan species were aligned with those of humans by PSI/TM-Coffee (http://tcoffee.crg.cat/apps/tcoffee/index.html). The mutated amino acids in all NIS homologs are indicated by dotted boxes.

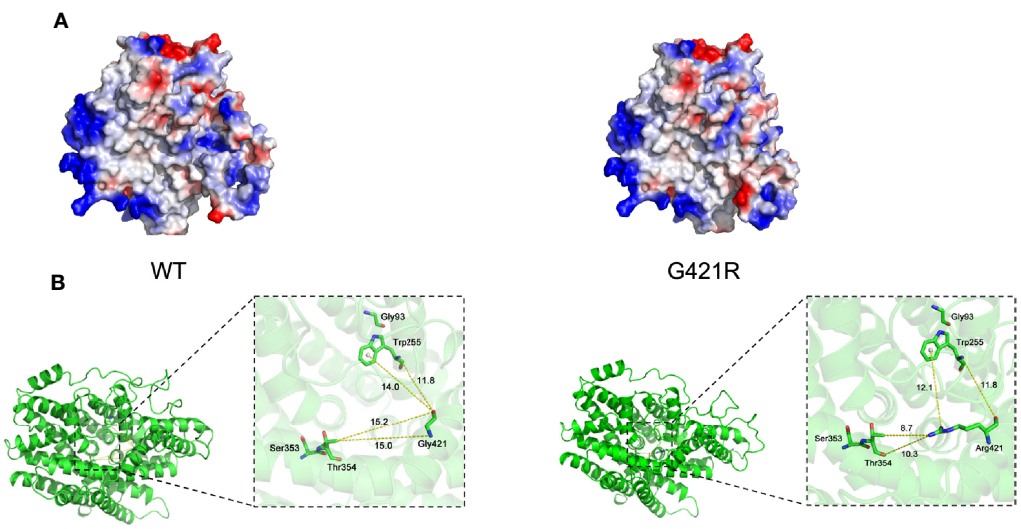

FIGURE 3 | Electrostatic surface and inter-atomic distances of WT NIS and G421R. **(A)** Local solid surface and electrostatic potentials are greatly changed by G421R mutation. Positive and negative potentials are shown in blue and red, respectively. **(B)** The cartoon representation of the crystal structure of NIS based on vSGLT. Those previously reported residues, which are particularly close to the mutation site, are shown in the magnified regions. Dotted lines connect atoms for which inter-atomic distances are given in angstroms. The specific values are shown in the figure. The left part of figure represents WT NIS, and the right G421R.

the helix by causing steric hindrance between the arginine side chain and the nearby amino acid side chains. We further utilized bioinformatic tools to predict the effects of G421R. G421R was categorized by PolyPhen-2 as being "Probably Damaging" with a score of 0.999, and by SIFT as "Damaging" with a score of 0.001 (**Supplemental Table 2**).

Iodide Transport Activity of G421R and G51fs NIS Mutants

To assess the effect of mutations on the activity of iodide transport, we expressed *wild-type* or mutant 3xFLAG-NIS plasmids in 293T cells. A p3xFLAG-CMV-10 empty vector was employed as a negative control, and a reported D331N mutation construct was used as the positive control. Quantitative PCR analysis was performed to ensure effective transcription (**Supplemental Figure 1A**). Western Blot analysis was done to investigate the effect of G51fs and G421R on protein expression. No band was detected in 293T cells transfected with empty vector (**Supplementary Figure 1B**), while truncated NIS with a molecular weight of 13 kDa (monomer) and 26 kDa (dimer) were observed after G51fs transfection (**Figure 4A**). Moreover, protein expression was decreased in 293T cells transfected with the G421R mutant plasmid compared to those transfected with *wild-type* (immature unglycosylated NIS ~ 55kDa, mature glycosylated NIS~ 100 kDa) (**Figure 4A**).

The iodide transport activity assay demonstrated that 293T cells transfected with WT NIS showed a marked accumulation of radio-iodide in the cells, while addition of the competitive inhibitor perchlorate significantly inhibited NIS-mediated iodide transport (**Figure 4B**). Consistent with the previous study, cells carrying the expression vector encoding D331 NIS exhibited partially reduced, although still detectable, ^{131}I uptake, compared with the iodide uptake levels in *wild-type* NIS-expressing cells. G421R and G51fs NIS-expressing cells showed almost no iodide transport activity, with similar radio-iodide levels to cells transfected with a p3xFLAG-CMV-10 empty vector (**Figure 4B**). In addition, substituting of G421 with other amino acids, aspartic acid and phenylalanine), showed the same results (**Supplemental Figure 2**). Further, co-transfection of WT and mutant plasmids to mimic the heterozygous state, no dominant negative effect was observed (**Figure 4B**). Our data showed that G421R and G51fs mutations markedly impaired iodide transport activity of the cells, which was consistent with the impaired membrane patterns.

Cellular Localization of SLC5A5 Mutants

The sodium/iodide symporter has been shown by immunofluorescence analysis to be located at the basolateral side of thyroid follicular cells and to mediate active iodide trapping. To perform its physiological function, this transporter must be properly localized at the plasma membrane. To assess the effect of *SLC5A5* mutations on the membrane localization, we expressed *wild-type* and G421R or G51fs mutants of the *SLC5A5*-pEGFP-N2 plasmid in 293T cells and examined the cellular localization using a confocal fluorescence microscope. A pEGFP-N2 empty vector was employed as a negative control. As we can see from **Figure 5**, NIS was clearly present at the cell membrane in WT cells, while in D331N transfected cells, normal plasma membrane trafficking of NIS was partially affected. In cells transfected with G51fs and G421R mutants, the NIS expressions on the cell membranes were decreased in varying degrees and appeared to localize in the cytoplasm (**Figure 5**), suggesting impaired plasma membrane localization of the mutated NIS proteins.

DISCUSSION

In the present study, the undescribed compound heterozygous mutation of G51fs/G421R in *SLC5A5* was discovered in a

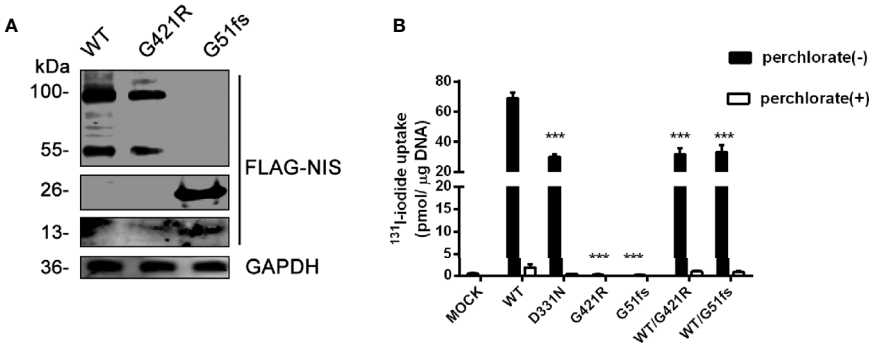

FIGURE 4 | **(A)** Representative Western blot analysis of whole-cell lysates from transiently transfected 293T cells probed with anti-FLAG and GAPDH antibodies. The proteins of WT (wild-type), G421R and G51fs were visualized by Odyssey CLx Infra-Red Imaging system. The molecular weight band was on the right (WT, G421R unglycosylated~ 55 kDa, mature glycosylated~ 100 kDa; G51fs monomer~13 kDa, dimer~ 26 kDa). **(B)** Iodide transport activity for of G421R and G51fs NIS mutants. Mock (p3xFLAG-CMV-10 plasmids), or expressing WT (wild-type) NIS, D331N, G421R and G51fs NIS plasmids were transfected alone or co-transfected into 293T cells. The cells were incubated with with131I at 5 KBq/mL in the absence (black bars) or presence (white bars) of 1 mM perchlorate and the intracellular iodide accumulation was detected using a γ counter. The values are representative of more than three independent experiments and represent the mean ± SD of picomoles ^{131}I per microgram of DNA; each experiment was performed in triplicate. ***$p < 0.001$.

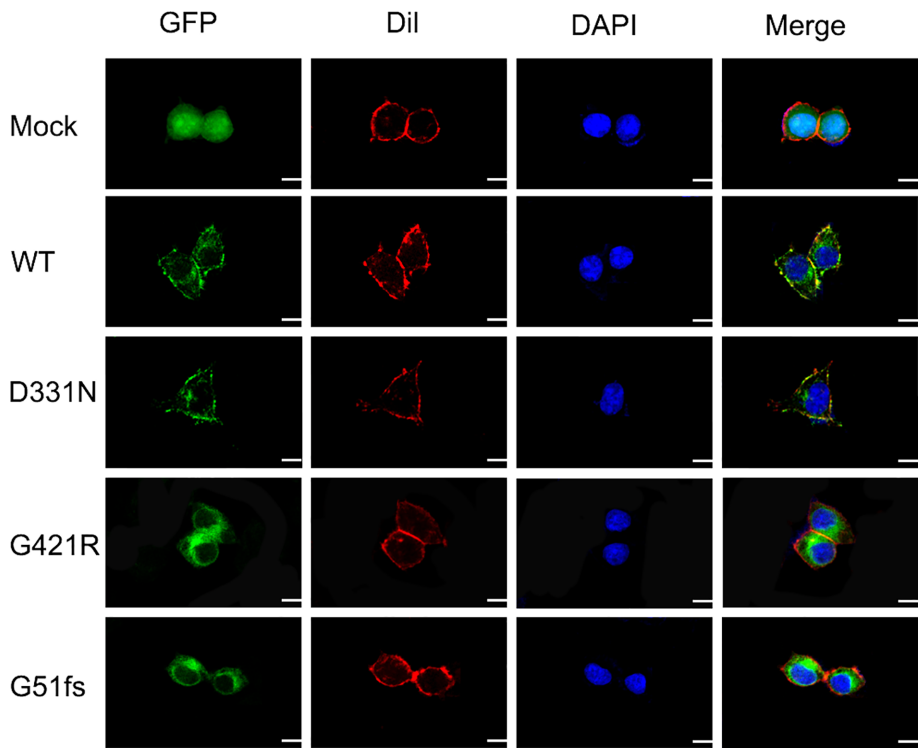

FIGURE 5 | Characterization of cellular localization of G421R and G51fs NIS mutants in 293T cells. pEGFP-N2 empty vector was employed as a negative control (Mock). Human WT NIS, D331N, G421R, and G51fs mutants were cloned into pEGFP-N2 plasmids (green). The cell membrane was labeled with a fluorescent probe DiI (red). Mock, WT NIS, D331N, G421R, and G51fs plasmids were transfected into 293T cells 24 h after seeding. Mutant D331N NIS showed slightly decreased membrane fluorescence, compared with the WT. However, G51fs and G421R mutants displayed severely reduced cell membrane localization in 293T cells. Nuclei were stained with DAPI (blue). Scale: 10 μm.

Chinese pediatric patient with CH. The mutations significantly reduced the NIS expression and impaired iodide transport function accompanied by impairing the location of NIS on the plasma membrane. Our study, thus, provided further insights into the roles of *SLC5A5* in CH pathogenesis.

Clinical heterogeneity in patients with different *SLC5A5* mutations was reported (10). In our patient, hypothyroidism was discovered by neonatal screening. After the diagnosis of CH, the patient was treated with L-thyroxine replacement immediately. However, in many other cases, the onset age of hypothyroidism was variable, ranging from the neonatal period to childhood, and even adulthood (32). Due to delayed treatments, patients presented irreversible intellectual disabilities. Research stated that less than 10% of iodine transport defect patients detected by neonatal screening showed clinical features of congenital hypothyroidism.

In addition, the occurrence of goiters varies between individuals. Because the formation of goiters depends on the level and the duration of TSH overstimulation, as well as on the iodide availability. The patients carrying the same described mutation did not develop goiters. Researchers speculated that early diagnosis and treatment with hormone replacement therapy during the neonatal period might prevent the development of goiters (33). However, even if these patients without goiters at birth are treated and maintain euthyroid, diffuse goiters will still occur after, which indicates that other factors besides TSH stimulation could also cause goiters. Notably, patients carrying the mutation G395R developed goiters has not been reported so far. The majority of these patients were severely hypothyroid, which was detected during the neonatal period (16). The molecular studies of G395R mutation indicated that G395R NIS was synthesized and properly targeted to the plasma membrane but intrinsically inactive (2, 34). It was highly possible that a "milder" pathogenic variant causing a partial loss-of-function might exist and could allow for a lower risk of goiters. Hence, regular long-term follow-up is necessary, and more comprehensive research is required to explore the mechanisms in detail.

In our study, we screened for mutations in *SLC5A5* in a cohort of the 273 CH patients. The frequency of *SLC5A5* mutation in the Chinese patients with CH was about 0.37% (1/273) in our study. However, according to the allele frequency of *SLC5A5* in the Chinese Millionome Database (CMBD), we speculated that the frequency of *SLC5A5* deficiency in CH in Chinese population was about 0.036%, which is significantly lower than what was found in our study. The relatively small

cohort size may be a plausible reason for this discrepancy. The G51fs/G421R compound heterozygous mutation in *SLC5A5* in a pediatric patient diagnosed with congenital hypothyroidism has not been reported before. Analysis of the proband's family showed that the father and mother were heterozygous for G51fs and G421R NIS, respectively (**Figures 2A, B**).

Along with the clinical manifestations of the proband, we proposed that the two novel mutation sites were crucial for normal functional activity of NIS. As expected, cells transfected with the G51fs NIS construct showed no iodide uptake and impaired membrane location due to the loss of most of the functional domains (**Figures 4B**, **5**). Additionally, our studies revealed that the G421R mutation located in the TMS XI was associated with diminished iodide uptake activity (**Figure 4B**). The mutated NIS proteins could not correctly target the plasma membrane (**Figure 5**). *In vitro* experiments in 293T cells transiently co-transfected with WT and mutant plasmids, mimicking the parental heterozygous state, indicated that the mutated allele did not interrupt the activity of WT NIS (**Figure 4B**). This observation was in accordance with the clinical observation that the parents who showed the same mutant allele in the heterozygous state (**Figure 2B**) were euthyroid.

Multiple lines of computational evidence supported a deleterious effect on NIS due to G421R missense mutation (**Supplemental Table 2**). As previously described, the structure of vSGLT, comprised of a central group of seven helices (TMS II-IV, TMS VII-IX, and TMS XI), can form a large cavity participating in the transport of galactose (35). We speculated that the arginine residue may occupy a comparably larger conformational space than glycine in the cavity, thereby, blocking the entry of ions. Previous studies on T354P mutation located in TMS IX revealed that the hydroxyl group at the β-carbon of the residue at position 354 was essential for its function in Na^+ binding and translocation (23, 24). In addition, the architecture of the Na^+ binding site was conserved among other transport proteins, such as bacterial leucine transporter (LeuT) and vSGLT (36, 37). We found that the interatomic distance between G421 and T354 was changed from 15.0 Å to 8.7 Å. Replacement of the hydrophobic neutral glycine residue with the hydrophilic and positively charged amino acid changed the net surface positive charges and distorted the structure of the surface, which may disturb the required rigidity of the protein and affect the trafficking of NIS (**Figure 3**). To perform its physiological function of maintaining an active iodide concentration from the blood using the positive sodium gradient, NIS is required to be properly localized at the plasma membrane (30).

In summary, we identified an undescribed compound heterozygous mutation of G51fs/G421R in *SLC5A5* from 273 Chinese patients with CH. These variants were associated with a significant reduction in NIS protein expression and subsequently impaired the membrane localization, resulting in a dramatic reduction in iodide transport and thyroid hormone biosynthesis. Our data demonstrated that these mutations were the direct cause of the observed CH phenotype. This study advanced our understanding of the possible mechanisms of NIS in CH pathogenesis.

DATA AVAILABILITY STATEMENT

The data presented in the study are deposited in the FigShare repository (https://doi.org/10.6084/m9.figshare.13134824.v1).

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of Shanghai Ninth People's Hospital affiliated to Shanghai Jiao Tong University School of Medicine. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin. Written informed consent was obtained from the individual(s) and minor(s)' legal guardian/next of kin, for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

C-XZ: writing-original draft and data curation. J-XZ: supervision. LY: project administration. C-RZ: methodology. FC: funding acquisition. R-JZ: resources. YF: resources. ZW: software. F-YW: visualization. P-ZL: validation. JL: funding acquisition. RL: funding acquisition, review, and editing. H-DS: funding acquisition, conceptualization, and investigation. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2021.620117/full#supplementary-material

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MINI REVIEW
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Resistance to Thyroid Hormone Beta: A Focused Review

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Resistance to thyroid hormone (RTH) is a clinical syndrome defined by impaired sensitivity to thyroid hormone (TH) and its more common form is caused by mutations in the *thyroid hormone receptor beta (THRB)* gene, termed RTHβ. The characteristic biochemical profile is that of elevated serum TH levels in absence of thyrotropin suppression. Although most individuals are considered clinically euthyroid, there is variability in phenotypic manifestation among individuals harboring different *THRB* mutations and among tissue types in the same individual due in part to differential expression of the mutant TRβ protein. As a result, management is tailored to the specific symptoms of TH excess or deprivation encountered in the affected individual as currently there is no available therapy to fully correct the TRβ defect. This focused review aims to provide a concise update on RTHβ, discuss less well recognized associations with other thyroid disorders, such as thyroid dysgenesis and autoimmune thyroid disease, and summarize existing evidence and controversies regarding the phenotypic variability of the syndrome. Review of management addresses goiter, attention deficit disorder and "foggy brain". Lastly, this work covers emerging areas of interest, such as the relevance of variants of unknown significance and novel data on the epigenetic effect resulting from intrauterine exposure to high TH levels and its transgenerational inheritance.

Keywords: resistance to thyroid hormone, thyroid hormone receptor, variant of unknown significance, autoimmune thyroid disease, thyroid dysgenesis, epigenetic effect

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INTRODUCTION

The term resistance to thyroid hormones (RTH) refers to the clinical syndrome of reduced sensitivity to thyroid hormones (TH) first described in 1967 (1) and until recently it was synonymous with mutations in the *thyroid hormone receptor beta (THRB)* gene. In the past decade, mutations in the *THRA* gene, as well as genetic defects involving TH cell transport and metabolism were added to those of defects of TH action, broadening our understanding of impaired TH sensitivity (2–4).

This mini-review is dedicated to RTH due to mutations in *THRB* gene producing RTHβ, having as a signature elevated serum free iodothyronines levels but non-suppressed thyrotropin (TSH) in the absence of other conditions that may produce some of the characteristic test abnormalities. It focuses on emerging concepts, unusual associations and controversies involving diagnosis and management, while providing a succinct overview of RTHβ covered in most medicine and specialty textbooks (5, 6).

OVERVIEW OF RTHβ

As most neonatal screening programs are based on TSH measured in dry blood spots, the precise incidence of RTHβ is unknown. Surveys of 80,884 and 74,992 newborns using TSH and T_4 measurements identified 2 and 4 infants with *THRB* gene mutations indicating a prevalence of 1 in 40,000 and 1 in 19,000 live births respectively (7, 8). Frequency among sexes is equal, whereas prevalence may vary somewhat among ethnic groups. The inheritance of RTHβ is typically autosomal dominant. This is explained by the formation of dimers between the mutant and normal (wild-type; WT) TH receptor (TR) interfering with the function of the WT TRβ. Since the first description of a *THRB* gene missense mutation causing RTHβ (9), 236 different mutations in 805 families have been identified. They are located in the functional areas of the ligand (T_3)-binding domain and adjacent hinge region (10). In 14% of individuals manifesting the RTHβ phenotype no *THRB* mutations were identified. Rarely familial, they may be caused by mosaicism (11), whereas it has been postulated that mutations in enhancers, repressors or cofactors may be responsible for this subgroup of RTHβ (12).

The distinctive biochemical feature of RTHβ is high serum free iodothyronine levels (principally free T_4) with normal or high TSH concentration. This discrepant correlation has brought the term "inappropriate TSH secretion". Its wide use is deplorable as in fact the degree of TSH secretion is appropriate for the reduced sensitivity of the hypothalamic-pituitary axis to TH. Individuals with RTHβ maintain a nearly euthyroid state compensated by the high TH level in concert with the tissue expression level of the mutant receptor. Thus, features of TH deficiency and excess may co-exist, producing sinus tachycardia in the heart expressing mainly the WT TRα and goiter by TSH stimulation, as the pituitary expresses mainly TRβ including the mutant form. Visual disorders may also be present due to retinal photoreceptor dysfunction (13). Serum TSH determination remains the most sensitive test to determine reduced sensitivity to TH. In contrast, serum markers of TH action on peripheral tissues, such as cholesterol, creatine kinase, alkaline phosphatase, osteocalcin and sex hormone-binding globulin are less reliable, unless they are measured before and after administration of T_3 (14).

After excluding assay interference as a cause of discrepant thyroid function tests (15), the principal other condition to be considered in the differential diagnosis of RTHβ is TSH secreting pituitary adenoma (TSH-oma), particularly in the absence of family history. Thus, testing of first-degree relatives is helpful and cost effective. Characteristics of a TSH-oma include failure to suppress TSH after the administration of supra-physiologic doses of T_3, failure to normally stimulate TSH with TSH releasing hormone (TRH) (although exceptions of TSH-omas with TSH response to TRH have been reported), elevated sex hormone binding globulin levels and increased ratio of pituitary α glycoprotein relative to TSH (16). Co-secretion of growth hormone and prolactin and abnormal pituitary imaging on computerized tomography or magnetic resonance imaging are important diagnostic findings. However, incidental pituitary lesions may be found in up to 24% of patients with RTHβ (15), thus increasing the complexity in differential diagnosis and the value of hormonal investigation and dynamic testing. Conditions that increase the serum iodothyronine levels in the absence of thyrotoxicosis must be considered, including familial dysalbuminemic hyperthyroxinemia (FDH). In a recent study of Khoo et al., the presence of the albumin mutation R218H in FDH interfered with the measurements of free T_4 and T_3 by automated immunometric assays leading to misdiagnosis of FDH as RTHβ or TSH secreting tumor (17). The diagnosis of RTHβ becomes quite challenging in the presence of concomitant thyroid pathology, a subject addressed in greater detail below. Caution should be exercised in the reduction of TH levels with antithyroid medication and ablative therapies (radioactive iodine or surgery) as it leads to difficulty in the subsequent treatment of hypothyroidism.

COMBINED RTHβ AND THYROID DYSGENESIS

The diagnosis of RTHβ is challenging and its management complicated when it co-exists with other disorders, such as congenital hypothyroidism (CH) and thyroid dysgenesis. Children with RTHβ commonly have short stature, goiter and learning difficulties (14) and in association with CH will present high serum TSH and may exhibit hypothyroid symptoms when treated with standard levothyroxine doses. Five reports of RTHβ with CH due to ectopic thyroid tissue have been reported (18–22). Of note, the case reported by Guo et al., had a lingual thyroid with a typical RTHβ phenotype but no detectable mutations in the *THRB* gene (21).

Persistent serum TSH elevation is frequently encountered during the early treatment of CH despite reaching serum T_4 level in the upper limit of normal. This has been attributed to a delayed maturation of the T_4 mediated feedback control of TSH (23). Defining the cause of persistent TSH elevation and addressing it appropriately is of paramount importance, as undertreatment may adversely impact growth and mental development. When non-compliance and suboptimal treatment are excluded by measurement of serum T_4 and T_3, suspicion for co-existence of RTHβ should be raised and, when confirmed, treatment with supraphysiologic doses of levothyroxine aims to bring the serum TSH to near normal while following growth, bone maturation and cognitive development. When RTHβ and ectopic thyroid tissue co-exist, another reason to aim at TSH suppression is to prevent thyroid tissue expansion in anatomic locations, such as the base of the tongue, that may cause dysphonia and hemoptysis.

AUTOIMMUNE THYROID DISEASE AND RTHβ

Autoimmune thyroid disease (AITD) is a common thyroid condition affecting the general population and its coexistence with RTHβ has been considered incidental (24, 25). However, in a study of 330 individuals with RTHβ and 92 unaffected first-degree relatives, subjects with RTHβ had an over 2-fold higher frequency of positive thyroid auto-antibodies (26), suggesting

that this association is not coincidental. A proposed pathophysiologic mechanism by the group of Gavin et al. invoked chronic stimulation of intrathyroidal lymphocytes by elevated TSH in RTHβ leading to pro-inflammatory cytokine production and thyrocyte destruction (27). Yet, in the study of Barkoff et al., the prevalence of AITD by age group was not influenced by the TRβ genotype which argues against high TSH being the cause of AITD (26).

Previous studies have shown that TH activates the immune system by acting on thymic epithelial cells and by direct effect on neutrophils, natural killer cells, macrophages and dendritic cells (28, 29). TH augments dendritic cell maturation and induces pro-inflammatory and cytotoxic responses. Given that dendritic cells are involved in the pathogenesis of AITD (30, 31), this might be a pathway mediating the association between RTHβ and AITD.

VARIABILITY IN RTHβ MANIFESTATION

RTHβ manifestations can be variable in tissue expression and in severity. The terms "generalized", "isolated pituitary" and "peripheral tissue" resistance have been used to describe different clinical manifestations of RTHβ suggesting tissue variability in the resistance to TH. The term generalized resistance to TH (GRTH) was applied to most patients with RTHβ that appear to maintain a euthyroid state whereas pituitary resistance to TH (PRTH) referred to patients with RTHβ that have symptoms of thyroid excess in peripheral tissues or demonstrate changes in peripheral tissue markers compatible to TH action without significant suppression of TSH (32). A single patient with presumed isolated peripheral RTH (PRTH) was reported, in whom administration of high dose of liothyronine (L-T_3) suppressed serum TSH but elicited no clinical signs of TH excess (33). Subsequently shown not to have a *THRB* gene mutation, this case likely represents acquired reduced sensitivity to TH through deiodinase-3 induced hormone inactivation. The clinical spectrum in RTHβ is quite broad and overlapping, even among carriers of the same *THRB* mutation and within the same family, suggesting that the classifications of generalized and pituitary RTHβ are rather semantics to describe a varying range of clinical signs and symptoms resulting from altered sensitivity to TH (34–36).

In some instances, the variability in the severity of the resistance to TH is readily explained on the basis of the character and position of the genetic defect. Homozygous *THRB* mutations are clinically more severe as they lack a WT TRβ and they interfere with the function of the WT TRα through heterodimerization (37, 38). Frame-shift mutations, producing a nonsense extension of the TRβ carboxyl terminus, interfere not only with ligand binding but also with interaction of the cofactors (39). Similarly, mutations with near normal ligand-biding can interfere with function through impaired binding to DNA (R243Q/W) (40, 41) and others (L454V and R383H) have altered binding to coactivators or corepressors (32, 42, 43) leading as in the case of R429Q (44) to more prominent suppression of TSH through predominant effect on genes negatively regulated by TH. Alberobello et al. (45) showed that when a single nucleotide polymorphism located in an intronic enhancer was associated with R338W, it produced pituitary specific over-expression of the mutant TRβ2 receptor illustrating the role of regulatory regions in tissue specific manifestation of RTHβ.

Differences in the level of expression of the mutant *THRB* allele relative to the WT in germline transmitted RTHβ have been shown in fibroblasts (46), but this was not found in another study (47). However, variable tissue expression of a mutant TRβ does occur in *de-novo* mutations resulting in mosaicism (11). The latter can also explain the failure to identify a *THRB* gene mutation in individuals with classical presentation of RTHβ when the only DNA source was circulating leukocytes. Finally, dramatic differences in phenotype observed among members of a family with the same *THRB* gene mutation have remained unexplained despite extensive genetic *in vivo* and *in vitro* functional studies (48).

CURRENT AND FUTURE TREATMENT APPROACHES

No specific therapy to fully correct the TRβ defect is currently available. Based on the mechanism producing the defect, it is clear that developing mutation-specific ligands would abrogate the dominant negative effect of the mutant TRβs, allowing the WT TRβ to elicit T_3 mediated thyroid hormone action. In 2005, the laboratory of the chemist John Kho synthesized TH analogues able to abrogate the dominant negative effect of the TRβ mutants R2320C, R230H and R316H when tested *in vitro* (49). More recently Yao et al. (50) showed that roxadustat, a drug used to treat anemia of renal failure, had 3- to 5-fold higher binding to the TRβ mutants V264D, H435L and R438H than T_3. However, none of these agonists have been tested *in vivo*. Similarly, the development of cell and tissue-specific TH antagonists could reduce the cardiotoxic effects of high serum TH levels acting on the WT TRα predominantly expressed in the heart. Therefore, as of this writing, management of TRβ is tailored to the individuals' symptoms resulting either from tissue TH excess or deprivation. Goiter, hyperactivity and mental "clouding" are clinical features that benefit from judicious treatment with L-T_3 without inducing side effects from TH excess.

Goiter is frequently observed in individuals with RTHβ but is usually of little consequence. However, in the occasion of larger symptomatic goiter, a surgical approach is usually ineffective, as goiter tends to re-occur. Therefore, it is logical to target TSH suppression to inhibit thyroid gland growth (51). An approach of administering supraphysiologic doses of T_3 every other day (250 μg in the case of TRβ R243Q) was successful in drastically reducing goiter size in a young patient without inducing thyrotoxic symptoms, as serum T_3 rapidly declined reaching levels lower than baseline before the ingestion of the next L-T_3 dose (52). The rationale is to deliver a large dose of the short lived L-T_3 to achieve very high peak serum level suppressing the TSH below 0.1 mIU/L to inhibit thyrocyte growth without sustaining elevated TH levels long enough to cause thyrotoxic symptoms (52). Thyroid

nodules are quite prevalent in the general population and thus may occasionally co-exist with RTHβ. Although the majority of thyroid nodules are benign and do not require surgical management, there are few reported cases of papillary thyroid carcinoma in patients with RTHβ. In these cases, thyroidectomy and radioactive iodine ablation to prevent disease recurrence result in lifelong levothyroxine replacement therapy, and in RTHβ persistently high serum TSH. Although the outcomes in the reported cases were fortunately not unfavorable, levothyroxine therapy is challenging and supraphysiologic doses are often needed to maintain serum TSH in lowest tolerable level (53). Alternative options to consider include 3,3,5-triiodothyroacetic acid (Triac), a thyroid hormone analogue with thyromimetic effects on pituitary and liver tissue that may be used to suppress TSH, combination of levothyroxine with beta-blocker to alleviate tachycardia along with calcium and vitamin D supplementation to prevent bone loss acceleration. Lastly, surveillance strategy may be considered for occult, micro-papillary thyroid carcinomas with low potential for aggressive progression.

Attention deficit disorder (ADHD), reported in 48-83% of individuals with RTHβ, is treated using conventional drugs. When such medications are ineffective, treatment with L-T_3 was found beneficial in reducing impulsivity in 5 of 8 and hyperactivity in 4 of 7 individuals with RTHβ and ADHD but not in individuals with ADHD only (54). Every-other-day L-T_3 therapy was also effective to improve the insomnia and hyperactivity in a young child with severe RTHβ phenotype intolerant to daily L-T_4 therapy (55).

The success of treatment with intermittent high dose L-T_3 in improving brain function seems to be linked to the reduction of serum T_4, a hormone more readily available to the brain which expresses predominantly TRα, providing a thyrotoxic local environment. This would be the rationale to consider block-and-replace strategy, proposed by Dr. Alexandra Dumitrescu, and used by the senior author to ameliorate "foggy brain" and anxiety occasionally reported by RTHβ patients, whereas beta blockade may be employed to help with tachycardia.

Lastly, Triac with higher affinity than T_3 for several TRβ mutants may be used to diminish the dominant negative effect of a TRβ mutation. Further, though its short half-life, Triac can effectively reduce TSH with lesser thyromimetic effect on peripheral tissues (56). Triac therapy has been used in few RTHβ cases and was found beneficial in partially alleviating thyrotoxic symptoms including tachycardia, excessive perspiration, attention deficit disorders, as well as goiter. This was the case in patients harboring mutations in the ligand binding domain (residues 310-353 and 429-460), whereas two cases with mutations in the hinge region were refractory to Triac (56, 57). Notably, in a pediatric case of a homozygous *R243Q* mutation with features of thyrotoxicosis and early dilated cardiomyopathy, combination of Triac with methimazole resulted in reduction of thyroid hormones levels and normal TSH accompanied by lower basal metabolic rate and improved growth and cardiac function (58).

A summary of recommendations to guide clinical management of subjects with RTHβ is presented in **Figure 1**.

THE IMPACT OF *TRβ* VARIANTS OF UNKNOWN SIGNIFICANCE

The development of next generation sequencing (NGS) and its increased availability in clinical practice leads to identification of variants of unknown significance (VUS). These include variants of the *THRB* gene not previously reported to be associated with RTHβ. The interpretation of such genetic reports, particularly missense mutations, poses a problem to the practicing physicians; how to explain the findings to the patient and how to proceed with future care. *In vitro* functional analyses of VUS are not commercially available and results cannot be deduced with certainty even when they are.

THRB gene mutations are clustered in three regions of the ligand-binding domain of the TRβ. Yet a major region devoid of mutation ("cold area") contains CG-dinucleotides which are mutagenic hot spots. Artificial mutations created in these CGs produced TRβs weak in dominant negative effect explaining the failure to identify mutations in this region of the receptor (59). This is explained by the fact that the same region is included in the dimerization domain. This region originally encompassed codons 348-437. Later, with the identification of *THRB* gene mutations causing RTHβ, the "cold region" was narrowed down to encompass codons 384-425 (32, 60). Within this region, 12 variants (P384L, G385R, L386V, E390D, R391K, D397G, S398G, N408S, H413D, V414M, K420R, and V425L) were reported in the gnomAD database without information regarding clinical phenotype (61). Although most variants are considered benign based on *in silico* prediction algorithms, conflicting predictions were made for the P384L, D397G and K420R variants and the G385R variant was considered damaging (62). Recently, a 48 year-old patient with AITD, treated with levothyroxine, was found to have high free T_4 with non-suppressed TSH. A mutant TRβ G385E was identified and reported as VUS. Family screening uncovered the same mutation in relatives with normal thyroid function, suggesting that this mutation may not be responsible for the abnormal thyroid pattern (63). Similarly, the G339S variant was identified in a family with AITD after an individual was misdiagnosed with RTHβ, but the same variant was then found in several family members with normal thyroid function, making it unlikely for the G339S variant to be causally related to a RTHβ phenotype (24).

The above paradigms illustrate that *in silico* prediction algorithms may not always be reliable when studying the functional relevance of VUS. Genotype-phenotype co-segregation among family members is useful in characterizing the functional impact of *THRB* mutations. Computational resources that factor in protein specific functional domains may have some predictive functional relevance of VUS but should not be the basis guiding clinical decision making.

EPIGENETIC EFFECT OF RTHβ AND ITS TRANSGENERATIONAL INHERITANCE

The first body of evidence on fertility and pregnancy outcome in RTHβ came from studies in a large Azorean kindred harboring the

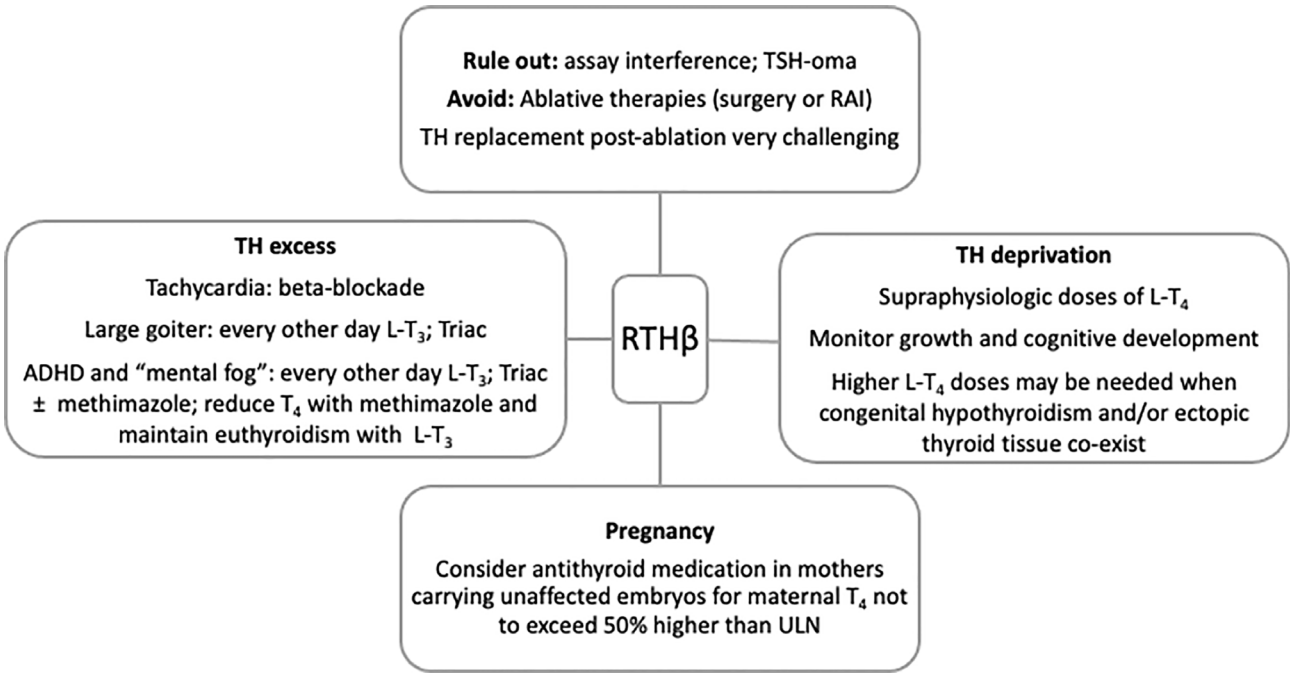

FIGURE 1 | Summary of recommendations to guide clinical management of RTHβ. RAI, radioactive iodine; ADHD, attention deficit and hyperactivity disorder; TH, thyroid hormones; ULN, upper limit of normal.

R243Q mutation. Fertility was not affected and, contrary to women with thyrotoxicosis, RTHβ did not produce an increase in premature labor, stillbirth or pre-eclampsia, jn agreement with the women's euthyroid state despite elevated TH levels (64). However, a significantly higher rate of early miscarriages was observed in women with RTHβ compared to spouses of males with RTHβ or unaffected first-degree relatives independent of maternal age and parity. Furthermore, a tendency was seen for these women to miscarry unaffected fetuses rather than fetuses with RTHβ, suggesting that the miscarriages occurred due to fetal exposure to incongruent high TH levels. In addition, unaffected newborns of mothers with RTHβ had significantly lower birth weight and suppressed TSH at birth compared to offspring of unaffected mothers, arguing that they were exposed in a hypercatabolic intrauterine environment of high TH concentration, whereas infants with RTHβ were protected from the toxic effect of TH excess. Of note, when women with RTHβ carrying unaffected fetuses were given antithyroid medication to avoid free T_4 levels 20% higher than the upper limit of normal, the birth weight and TSH levels at birth of their offspring was similar to infants with RTHβ (65).

In a subsequent study, the long-term effect of intrauterine exposure to high TH levels was examined in WT members of the Azorean kindred. Specifically, the study involved unaffected offspring of mothers with RTHβ and offspring of unaffected mothers, whose fathers had RTHβ, as well as mice mimicking the human phenotype. Unaffected humans and WT mice born to mothers with RTHβ and exposed to high TH levels *in utero* developed reduced central sensitivity to thyroid hormone (RSTH), that persisted during adulthood (66) (**Figure 2**). Increased expression of deiodinase 3, the enzyme that inactivates TH, was found in the pituitaries of the WT mice born to dams with RTHβ (66). This effect was found to be transmitted by male descendants but not in female with likewise RSTH (67). Although the exact mechanism of this transgenerational epigenetic inheritance is not fully characterized, it is thought to involve possible modulation of the imprinted deiodinase 3 gene that regulates local TH availability at a tissue specific level. It remains unclear whether prolonged exposure to high TH levels could have similar implications in adult life. This deserves further investigation as such a finding would have implications in the management of larger populations, such as individuals on long term TSH suppressive levothyroxine therapy for differentiated thyroid cancer.

DISCUSSION–CONCLUSIONS

The diagnosis of RTHβ is challenging and the main condition in the differential diagnosis is TSH-oma. Diagnosis and management of RTHβ are more challenging when other thyroid disorders co-exist, such as CH and ectopic thyroid tissue. More recently, an association has been described between RTHβ and AITD. Although the causal relation remains unclear, proposed pathophysiologic mechanisms include TSH or TH induced stimulation of pro-inflammatory and cytotoxic responses. The observed variability in clinical manifestation of RTHβ can be explained by the type of genetic defect, e.g. homo- *vs* hetero-zygosity, frameshift *vs* insertion/deletion, mutations with predominantly TRβ2 mediated action, mosaicism, and the tissue specific variability in TRβ expression, e.g. heart and brain *vs* pituitary and liver. Management is tailored to

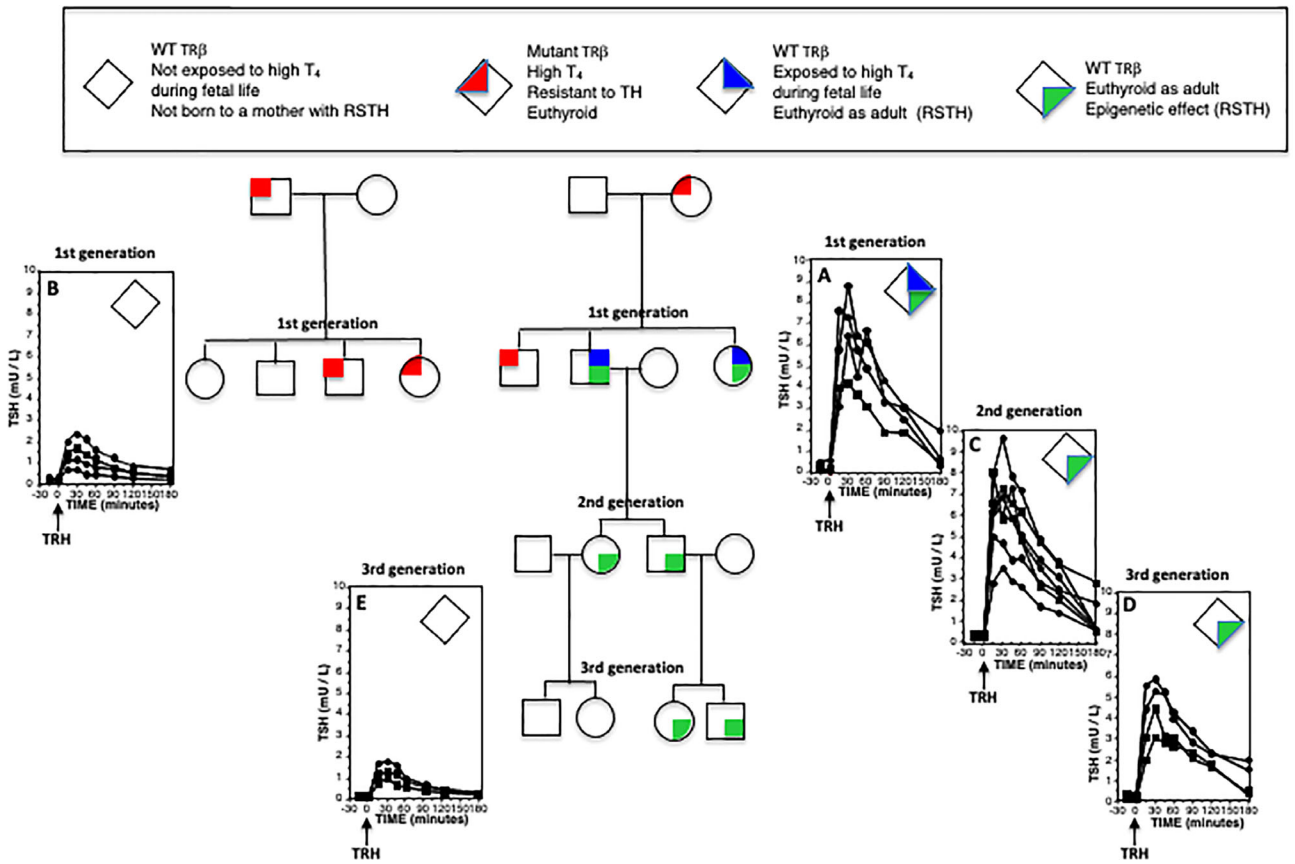

FIGURE 2 | Epigenetic effect of RTHβ and its transgenerational inheritance across the male line. Individuals were given 25 μg L-T_3 twice daily for three days. They were then injected intravenously with 200 μg of thyrotropin releasing hormone (TRH) and samples of blood were obtained at the indicated times for the measurement of serum TSH. Plotted on the graphs are results from female (circles) and males (squares). Increased peak responses in **(A, C, D)**, as compared to those of **(B, E)** indicate reduced sensitivity to thyroid hormone (RSTH). While this epigenetic effect of exposure to high TH levels during fetal life **(A)** is transmitted to both sexes, it is inherited along male line only **(C, D)** but not the female line **(E)**.

control symptoms arising from tissue specific excess or lack of TH. In small case series treatment with every-other-day L-T_3 was beneficial in improvement of goiter and ADHD symptoms. When RTHβ co-exists with CH, supraphysiologic doses of L-T_4 are needed to achieve normal bone and cognitive development. The advances in NGS have led to increasing frequency of VUS identification, where there may be limited data on their functional relevance beyond *in silico* prediction models. Caution should be exercised as to not guide clinical decision making based on computational resources and utilize information from genotype-phenotype co-segregation in family members. Transgenerational studies in humans and mice provide evidence of an epigenetic effect induced by RTHβ, by *in utero* exposure of WT fetuses to high TH concentration. The resulting reduced sensitivity to TH shows transgenerational inheritance across the male but not the female line and is thought to be mediated *via* modulation of deiodinase 3, that regulates local TH availability.

The advances in our knowledge on RTHβ raise novel questions about TH action outside the hypothalamus-pituitary-thyroid axis and the emerging concepts on epigenetic effect of RTHβ need to be explored further, as they may have implications in larger populations, such as patients with thyroid cancer on long term TSH suppression therapy with TH.

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TP and SR designed and wrote this manuscript and both conceptually contributed to this work. All authors contributed to the article and approved the submitted version.

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Screening for Mutations in Isolated Central Hypothyroidism Reveals a Novel Mutation in Insulin Receptor Substrate 4

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Background: Central hypothyroidism (CeH) is a rare condition affecting approximately 1:16 000- 100 000 individuals. Congenital forms can harm normal development if not detected and treated promptly. Clinical and biochemical diagnosis, especially of isolated CeH, can be challenging. Cases are not usually detected in neonatal screening, which, in most countries, is focused on detection of the more prevalent primary hypothyroidism. Until now, five genetic causes for isolated CeH have been identified. Here we aimed to identify the genetic cause in two brothers with impaired growth diagnosed with CeH at the age of 5 years. We further evaluated the candidate gene variants in a large genetic database.

Methods: Clinical and biochemical characterization together with targeted next-generation sequencing (NGS) was used to identify the genetic cause in a family of two brothers presenting with CeH. Screening of *insulin receptor substrate 4* (*IRS4*) variants was carried out in the FinnGen database.

Results: A novel monoallelic frameshift mutation c.1712_1713insT, p.Gly572Trp fs*32 in the X-linked *IRS4* gene was identified by NGS analysis in both affected males and confirmed using Sanger sequencing. Their mother was an unaffected carrier. In addition to the declined growth at presentation, central hypothyroidism and blunted TRH test, no other phenotypic alterations were found. Diagnostic tests included head MRI, thyroid imaging, bone age, and laboratory tests for thyroid autoantibodies, glucose, insulin and glycosylated hemoglobin levels. Examination of the *IRS4* locus in FinnGen (R5) database revealed the strongest associations to a rare Finnish haplotype associated with thyroid disorders (p = 1.3e-7) and hypothyroidism (p = 8.3e-7).

Conclusions: Here, we identified a novel frameshift mutation in an X-linked *IRS4* gene in two brothers with isolated CeH. Furthermore, we demonstrate an association of *IRS4*

gene locus to a general thyroid disease risk in the FinnGen database. Our findings confirm the role of *IRS4* in isolated central hypothyroidism.

Keywords: insulin receptor substrate 4, IRS4, central hypothyroidism, thyroid disorders, genetic screening, FinnGen, congenital hypothyroidism

INTRODUCTION

Central hypothyroidism (CeH) is defined as a reduced thyroid hormone secretion from an otherwise functional thyroid gland due to diminished stimulation of the gland. Reduced stimulation can result either from an impaired secretion of thyroid stimulating hormone (TSH) from the anterior pituitary, defective secretion or action of hypothalamic thyrotropin releasing hormone (TRH), or both (1).

Central hypothyroidism is rarely an isolated defect, but most often appears congenitally as a part of panhypopituitarism affecting also gonadotropin, adrenocorticotrophic hormone (ACTH) or growth hormone secretion. Panhypopituitarism can be potentially life-threatening, primarily because of severe hypoglycemia. The exact prevalence of CeH is unknown, but the presentation of genetic forms shows a peak in childhood, whereas other forms due to pituitary lesions are typical later in adulthood (2). Estimations of CeH prevalence vary between 1:16 000-100 000 individuals (3, 4). Overall, CeH has been reported to be equally distributed in both sexes. However, known X-linked forms suggest a male predominance (2). In addition to the genetic etiology of isolated central hypothyroidism, impairing only TSH secretion, CeH has been described in patients with pituitary tumors, trauma, radiation therapy, diabetes mellitus or it may be idiopathic (5). Genetic defects in pituitary transcription factors can lead to CeH, but are usually associated also with other hormonal defects (1). Isolated TSH deficiency covers approximately 20% of all CeH cases (2). Inherited isolated CeH has been demonstrated to be caused by mutations in *thyroid releasing hormone receptor* (*TRHR*), *thyroid stimulating hormone beta subunit* (*TSHB*), *immunoglobulin superfamily member 1* (*IGSF1*), *transducin (beta)-like 1X-linked* (*TBL1X*) and more recently, in *insulin receptor substrate 4* (*IRS4*) genes (6). Among these genes *TRHR* mutations has been shown to lead to blunted TSH response to TRH, growth retardation and obesity during childhood (7). Furthermore, *TSHB* mutations are characterized by neonatal onset of low TSH, high glycoprotein hormone alpha-subunit levels and pituitary hyperplasia, which is reversible with thyroxine treatment (8). Mutations in both *IGSF1* and *TBL1X* can lead to X-linked isolated CeH, but *IGSF*1 mutations are also associated with low PRL, variable GH deficiency, metabolic syndrome, and postpubertal macroorchidism (9). In addition, the mutations in the *TBL1X* can also lead to impaired hearing (10).

Here we describe a genetic, biochemical and clinical characterization of a family with two brothers diagnosed with CeH at the age of 5 years and shown to carry a novel frameshift mutation in the *IRS4* gene. Furthermore, we evaluate the overall occurrence of *IRS4* variants and their association to other clinical phenotypes in a large national genetic database.

MATERIALS AND METHODS

Study Participants

The study participants were recruited to the study by a pediatric endocrinologist, and they and their parents signed a written consent. The Ethics Committee of the Hospital District of Southwest Finland approved the study (108/180/2010).

The clinical examinations were performed by a pediatrician (SK) and thyroid ultrasound, head MRI and bone age by the pediatric radiologists. Laboratory tests were done at the Turku University Hospital Laboratory, except for the IGF-1 test, which was performed in the Islab-laboratory (Kuopio, Finland). Umbilical serum TSH (uS-TSH), serum TSH, free T4 (fT4), insulin and cortisol concentrations were determined with the Cobas e801 immunoassay analyzer (Roche Diagnostics, Rotkreuz, Switzerland). Serum cholesterol (HDL cholesterol, LDL cholesterol and triglycerides) were determined with the Cobas c702 chemistry analyzer (Roche Diagnostics). Serum glycosylated hemoglobin (HbA1c) was determined with the Cobas c501 immunoturbidimetric assay (Roche Diagnostics). Serum IGF-1 concentrations were determined with the Liaison XL chemiluminescence analyzer (DiaSorin S.p.A, Saluggia, Italy). Growth data were collected from the hospital records based on measurements performed at the visits using stabilized and calibrated scale and wall mounted Harpenden Stadiometer (Holtain Limited, Crosswell, Crymych, Pembs., UK) with ±0.1 cm precision.

TRH Stimulation Test

At the start of the TRH stimulation test, non-fasting serum TSH concentrations were measured. A bolus of 7 μg/kg of TRH (Ferring Pharmaceuticals, Saint-Prex, Switzerland) was given intravenously, and subsequently the serum TSH concentrations were measured at 20 and 60 minutes.

Genetic Analysis

Genetic analyses were performed on DNA extracted from peripheral blood. Amplification of target region was performed with PCR using AmpliTaq Gold 360 (ThermoFisher Scientific, Waltham, MA, USA) according to the manufacturer's protocol in the Veriti 96-Well Thermal Cycler (Applied Biosystems, Foster City, CA, USA). Primers used are listed in the **Supplemental Table 2**. For the index patient an NGS-based targeted panel was performed with Sophia Genetics custom clinical exome solution (Sophia Genetics, Boston, MA, USA) and Illumina sequencing (Illumina, San Diego, CA, USA), including 4400 known disease-causing genes. NGS libraries were prepared using a hybrid capture method according to the manufacturer´s protocol (Sophia Genetics CCE_A_v1). DNA was sequenced with NextSeq sequencer (Illumina, San Diego,

CA, USA) using 2x151bp paired-end technique. The identified variation was visually inspected using the Integrative Genomics Viewer (11). Bioinformatic analysis and annotation was focused on the following candidate gene panel for CeH: *HESX1, IGSF1, IRS4, LEPR, LHX3, LHX4, OTX2, POU1F1, PROP1, SOX3, TBL1X, TRHR* and *TSHB*.

Confirmation of the IRS4 mutation and its segregation was tested with PCR and Sanger sequencing. The primer sequences and PCR conditions are listed in the **Supplementary Materials**. Sequencing reactions were performed by using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). Sequencing was performed with the ABI3500xl Dx (Applied Biosystems) and chromatograms were analyzed using Sequencher v5. (Gene Codes Corporation, MI, USA). The alignment of WT and mutated IRS4 sequences was performed using the Clustal O (1.2.4) multiple sequence alignment tool (12).

IRS4 Variant Analysis in the FinnGen Database

The FinnGen project has been approved by the Ethical Review Board of the Hospital District of Helsinki and Uusimaa with the protocol Nr. HUS/990/2017. The FinnGen data release 5 was used. Detailed information about of the different releases is described on the FinnGen's website[1]. Release 5 comprises of data from 218 792 Finnish participants with disease endpoints[2] constructed from national registries using International Classification of Diseases (ICD), Social Insurance Institute (KELA) drug reimbursement and ATC codes linked with DNA data.

RESULTS

Clinical Characteristics

The two male subjects were referred to the endocrinologist due to mild growth retardation and normal TSH, but low serum fT4 concentrations. They were both diagnosed with CeH between the age of 5 – 6 years. The index case (patient #1) and his brother (patient #2) had normal serum TSH and low fT4 values prior the diagnosis (**Figures 1A, B**). Both cases had blunted TSH response (ΔTSH, <1.6) to TRH stimulation (**Figure 1D**), no interfering antibodies in TSH or TH assays, and the TG or TPO antibody tests were negative (**Figure 1A**, table). Secretion defects or dysfunction of other pituitary hormones were excluded (**Supplemental Table 1**). Both MRI of the head and ultrasound evaluation of the thyroid were normal in both cases at the time of diagnosis. Patient #1 had linear growth until the age of 2 years, after which his height standard deviation scores (SDS) decreased from –1 to –2.4 SD between the age 2 and 5 years (**Figure 1E**). His brother (#2) had similar growth retardation prior to the CeH diagnosis. Both had delayed bone age (2.0 - 2.3 and 0.7 years behind the calendar age) at the time of diagnosis. There were no significant alterations in other biochemical or metabolic variables measured including glucose, glycosylated hemoglobin (HbA1c), insulin, insulin-like growth factor-1, cholesterol or cortisol levels (**Supplemental Table 1**). The thyroxine replacement was started promptly after CeH diagnosis and fT4 levels returned to normal. Furthermore, the serum TSH concentrations, although within the normal range at diagnosis, decreased significantly in both cases after thyroxine supplementation. A small increase in growth velocity was also noted after the initiation of thyroxine treatment (**Figure 1E**).

Both brothers were born at term after a normal pregnancy and uncomplicated birth with >9 APGAR-scores. However, patient #1 received phototherapy for prolonged jaundice as a newborn. The birth weight, height and head circumferences were within average Finnish standards. Patients #1 and #2 had normal umbilical serum TSH levels (patient #1: 11 mU/l and patient #2: 6.6 mU/l) measured at birth as a part of the CH screening program. Both brothers reached all developmental milestones, had normal weight gain and head growth pattern during the first two years (**Figures 1F, G**), normal weight gain and development and no further diagnoses. Both parents were healthy, had thyroid function tests (TSH, fT4, fT3) in normal range, and negative TPO and Tg antibody tests, when measured at the recruitment visit. There was no positive family history for thyroid disease.

Genetic Findings

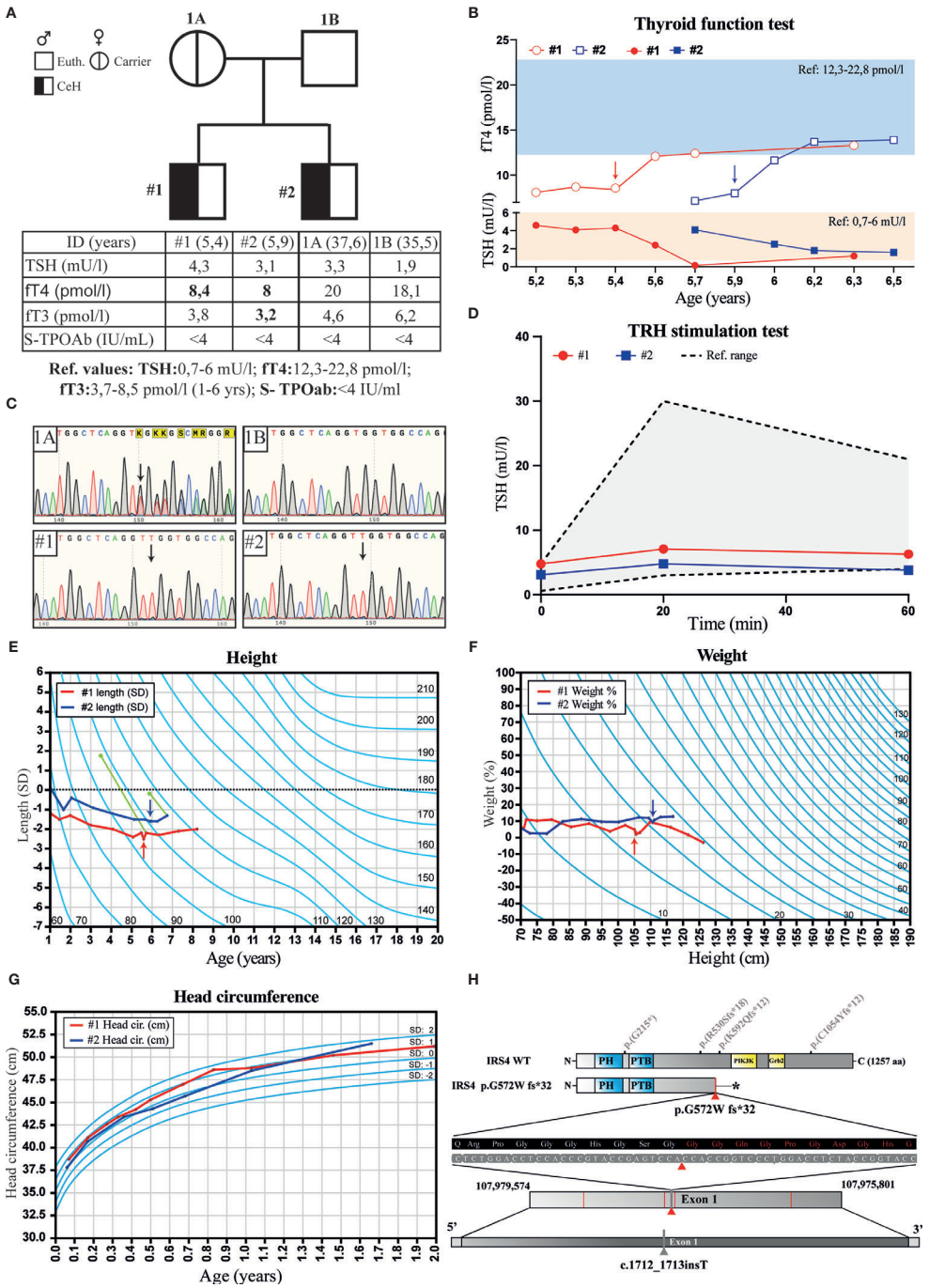
A novel frameshift variant was detected in hemizygous state in exon 1 of the *IRS4* gene located on the X chromosome (X:107977861; NM_003604.2) of the affected male proband (**Figure 1H**). The detected variant had a 1bp insertion (c.1712_1713insT), which leads to a frameshift mutation (p.Gly572Trp fs*32, amino acid sequence listed in **Supplemental Figure 1**), premature stop codon and strongly truncated protein (**Figure 1H**). This variant most likely degrades through nonsense-mediated decay and is not present in the gnomAD and dbSNP databases. Confirmation and co-segregation of the mutant in the family was performed using Sanger sequencing. The same variant was also found in the DNA of the affected brother and the mother, who was a healthy carrier (**Figures 1A, C**). No other pathogenic variants associated with CeH were detected in the clinical exome.

Exploring IRS4 Mutations in the FinnGen Study

To explore the role of IRS4 in both hypothyroidism and across the medical spectrum, we used the large FinnGen population study which in release 5 had integrated genome-wide genotyping and extensive medical history data from 218 792 Finns. We searched for any rare loss-of-function (LoF) or damaging missense mutations (**Table 1**) and found no LoFs. Of note, the only rare predicted damaging missense variant observed (rs766893547, p.Arg8His) was carried by two female congenital hypothyroidism cases (an enrichment odds ratio of 6.7 compared to all females, $p = 0.03$). Furthermore, another variant (rs1801164) had significant association to renal failure (**Supplemental Figure 2**).

We then surveyed the IRS4 locus to see if any medical phenotypes might be associated to this genomic region. This was done by evaluating the DNA variants at the IRS4 locus in

[1] https://finngen.gitbook.io/documentation/
[2] https://www.finngen.fi/en/researchers/clinical-endpoints



ID (years)	#1 (5,4)	#2 (5,9)	1A (37,6)	1B (35,5)
TSH (mU/l)	4,3	3,1	3,3	1,9
fT4 (pmol/l)	**8,4**	**8**	20	18,1
fT3 (pmol/l)	3,8	**3,2**	4,6	6,2
S-TPOAb (IU/mL)	<4	<4	<4	<4

Ref. values: TSH:0,7-6 mU/l; **fT4:**12,3-22,8 pmol/l; **fT3:**3,7-8,5 pmol/l (1-6 yrs); **S- TPOab:**<4 IU/ml

FIGURE 1 | Pedigree, thyroid function tests, growth charts and chromatograms of the family with two brothers diagnosed with isolated central hypothyroidism (CeH). **(A)** Pedigree, serum TSH, fT4, fT3 and TPO antibody (S-TPOAb) concentrations in parents and their offspring. Bolded values indicate concentration below the reference range. **(B)** Follow-up graph of the serum TSH and fT4 concentrations in the affected cases before and after the thyroxin replacement therapy. Blue and orange rectangles show fT4 and TSH reference ranges, respectively. **(C)** Chromatograms of the IRS4 sequence flanking the mutation in mother (1A), father (1B), and the two patients #1 and #2 presenting CeH. Black arrows show the position of thymidine insertion. **(D)** TSH response in TRH stimulation test of the affected cases, gray area indicates a range of normal TSH response. **(E)** Growth (height SD) and **(F)** weight (%) curves of the affected siblings: #1 (red) and #2 (blue). Arrows indicate the time of CeH diagnosis and the start of thyroxine treatment. The green dots and lines show bone age determined at the indicated calendar age using the Tanner-Whitehouse method, and dotted line shows the expected length calculated from the parents´ heights. **(G)** Head circumference in affected siblings carrying IRS4 mutation during the first 2 years. Light blue lines show SD values. **(H)** Location of the IRS4 frameshift mutation detected in this study (marked with red triangle), premature stop codon (indicated with star) previously published (6) *IRS4* mutations (marked with red lines). The location of IRS4 gene shown is based on the GRCh37.p13 primary assembly.

TABLE 1 | IRS4 missense variants in the FinnGen-database.

Location	source	rsid	HGVSp	poly-phen	N(m/f)	fin.AF	nfsee.AF
23:108733710:G:C	I	rs1801164	p.His879Tyr	U	**ND**	0.14554	0.2104144
23:108734099:C:T	C	rs774511400	p.Arg749Lys	U	32/124	0.00049966	0
23:108735030:T:A	I	rs137853896	p.Ser439Cys	B	ND	0.0063782	0.00043206
23:108735113:C:T	I	rs41307415	p.Arg411Gln	B	ND	0.030372	0.05821377
23:108736245:G:A	I	rs1801162	p.Leu34Phe	B	ND	0.030347	0.05845302
23:108736257:C:A	C	rs769861641	p.Val30Leu	B	12/75	0.00025102	0
23:108736322:C:T	C	rs766893547	p.Arg8His	PB	25/110	0.00031291	0

IRS4 Missense variants in the FinnGen-database. Variant source; I (imputed) or C (variant detected from chip); rsid. rs number; HGVSp, the HGVS protein sequence name (genome build 38); Polyphen: U, unknown, B, benign; PB, probably damaging; N, number of males (m) and females (f); ND, not detected; fin.AF, allelic frequency in Finnish; nfsee, non-Finnish-non-Swedish-non-Estonian European.

FinnGen participants and comparing those to disease phenotypes[2] obtained from national registries linked with the DNA data. Across all 2925 phenotypes studied in the FinnGen project, the strongest associations discovered were with thyroid disorders (p = 1.3x10-7) and hypothyroidism (p = 8.3x10-7), which were associated to a rare Finnish-enriched haplotype (tagged by SNP rs1452561670 20 kb downstream of IRS4) (**Supplemental Figure 2**). This haplotype spans a roughly 500 kb interval within which IRS4 is the only documented protein-coding gene.

DISCUSSION

Isolated central hypothyroidism (CeH) in children is rare and challenging to diagnose clinically. It has a multifactorial etiology and a small proportion of cases that are due to gene mutations. Therefore, the etiology for several cases remains unsolved. Currently, mutations in five different genes have been shown to associate with CeH. In this study, we describe a genetic, clinical and biochemical characterization of a family with two brothers presenting CeH at the age of 5 years. Furthermore, an evaluation of candidate gene variants for isolated CeH and their putative association to other phenotypes in a national genetic database was performed. In the present study, both affected cases with a novel frameshift mutation in an X-linked *IRS4* gene were shown to have normal umbilical TSH measured at newborn screening and normal psychomotor development. However, their growth started to slowly decline 2-3 years prior to the diagnosis of CeH, which prompted the investigations. The TSH response was blunted in the TRH-test and TSH levels declined from mid-normal to low- normal after start of the thyroxine replacement. The mother was a healthy carrier with normal TSH and also fT4 concentrations at the upper normal range. The data from this family supports the pathogenic role of IRS4 in isolated central hypothyroidism and the cases showed no obvious additional phenotypes. Furthermore, the candidate gene analysis using the FinnGen database showed an association between the *IRS4* locus and thyroxine purchases.

So far, only one study has reported the association of CeH with pathogenic mutations in *IRS4* (6). Heinen et al. described five families and seven affected male patients with four different mutations in the *IRS4* gene. Most of those patients were detected at birth *via* neonatal screening which can identify both primary and CeH (13). Unfortunately, CeH is not usually detected in congenital hypothyroidism (CH) screening programmes, which are generally targeted to detect the more prevalent primary CH using TSH-based screening (4, 14). However, in some countries with T4-based screening programmes CeH can be detected at birth and potential neurodevelopmental disabilities can be therefore prevented. The importance of such screening was recently demonstrated in a study by Lanting et al. (15), which showed a relatively high (1 in 16 404) occurrence of CeH. Moreover, this finding is also supported by the fact that the clinical recognition of isolated CeH can be challenging without the classic symptoms, such as hypoglycemia, jaundice and micropenis observed typically in panhypothyroidism (2). Thus the debate continues whether the screening should also detect central CH continues (15, 16). In Finland, TSH-based screening is used and the CeH cases have not been routinely detected (17). The two cases described in our study, show that the impaired IRS4 function does not necessary lead to severe CH, as the boys´ development, growth and head growth for the first 2 years was normal. Similarly, in the study from Heinen et al. (6) they report a male case diagnosed with CeH at the age of 12, originally evaluated because of short stature and delayed tooth eruption but otherwise normal development. This suggests that a significant degree of compensation can occur either in the regulation of the growth during the infancy and childhood phase or at the thyroid axis level. In fact, the fT3 levels in our patients were normal or only slightly below the reference range, and normal among the patients described in the previous study (6). In the previously described *IRS4* mutation cases the TSH response to the TRH-test was blunted in six of seven male *IRS4* mutation carriers, similar to that seen in our patients. The value of the TRH-test in the diagnosis of CeH with multiple etiologies has been shown to be controversial (18, 19), and the response can be affected by multiple factors such as initial TSH level, age, weight or nutritional state (20–22). However, the blunted TSH response seen in our CeH cases and the previously described *IRS4* mutation carriers suggest that the IRS4 is needed for the proper signaling of the TRH. The *IRS4* gene codes for a cytoplasmic protein which interacts with tyrosine kinase receptors and mediates their signaling (23). It has been shown to be expressed in the hypothalamus, but also has been found in several other tissues including the pituitary, thyroid and ovaries (10). IRS4 knock-out mice exhibit mild metabolic differences including lower blood glucose levels, impaired glucose tolerance and decreased fertility (24). Additionally, IRS4 female knockout mice have decreased *Tshb* mRNA expression in the pituitary, but no altered serum TSH or thyroid hormone concentrations. In contrast, the lack of IRS4

function in humans impairs TSH pulsatile secretion but the detailed mechanisms remain unclear. Impaired TSH pulsatile secretion may be linked to leptin action (6) that has been shown to participate in TRH response (25) and its action is partially mediated *via* IRS proteins.

A limitation of our study is the lack of functional test of the mutation pathogenicity. However, *IRS4* mutation segregation in the family, the frameshift alteration leading to premature stop codon strongly support the pathogenicity of the mutation. Since the fine regulation of thyroid function *via* hypothalamus and pituitary seems to differ between human and mice (6) further functional studies, for example using induced human pluripotent pituitary cell lines, are warranted to elucidate the detailed mechanisms.

Using FinnGen data we could not identify new LoF mutations. However, one rare predicted damaging missense variant (rs766893547, p.Arg8His) was present in two females with a congenital hypothyroidism diagnosis. Furthermore, an association of the variant rs1452561670 to the thyroid endpoint category was observed. Although these associations include cases with central isolated hypothyroidism (ICD code E23.05), they may also indicate primary thyroid diseases.

To our knowledge, general association of the *IRS4* variants to thyroid diseases has not been previously described in GWAS studies or genetic databases. However, our observation of a Finnish-enriched haplotype spanning IRS4 would not be detectable outside of a large Finnish study. This haplotype spans a roughly 500 kb interval within which IRS4 is the only documented protein-coding gene.

In addition to the association to thyroid endpoint category in FinnGen one IRS4 missense variant (rs1801164) had a significant association to renal failure. However, no renal phenotypes were described in humans with *IRS4* frameshift mutations in the previous publication (6). Furthermore, in our study the creatinine values were normal and there was no sign of kidney dysfunction in the individuals with IRS4 mutation and the family history was negative for any kidney diseases. Therefore, the present clinical findings do not support this database association at least in young individuals.

In summary, our data from patients with a novel frameshift mutation in *IRS4* gene together with the observed association between the rare *IRS4* haplotype and thyroid disease risk supports the pathogenic role of IRS4 in isolated central hypothyroidism.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are publicly available. This data can be found here: https://www.ebi.ac.uk/eva/, PRJEB45041.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by The Ethics Committee of the Hospital District of Southwest Finland (108/180/2010). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2021.658137/full#supplementary-material

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MINI REVIEW
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Thyroid Function in Preterm/Low Birth Weight Infants: Impact on Diagnosis and Management of Thyroid Dysfunction

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Maternal thyroid hormone crosses the placenta to the fetus beginning in the first trimester, likely playing an important role in fetal development. The fetal thyroid gland begins to produce thyroid hormone in the second trimester, with fetal serum T4 levels gradually rising to term. Full maturation of the hypothalamic-pituitary-thyroid (HPT) axis does not occur until term gestation or the early neonatal period. Postnatal thyroid function in preterm babies is qualitatively similar to term infants, but the TSH surge is reduced, with a corresponding decrease in the rise in T4 and T3 levels. Serum T4 levels are reduced in proportion to the degree of prematurity, representing both loss of the maternal contribution and immaturity of the HPT axis. Other factors, such as neonatal drugs, e.g., dopamine, and non-thyroidal illness syndrome (NTIS) related to co-morbidities contribute to the "hypothyroxinemia of prematurity". Iodine, both deficiency and excess, may impact thyroid function in infants born preterm. Overall, the incidence of permanent congenital hypothyroidism in preterm infants appears to be similar to term infants. However, in newborn screening (NBS) that employ a total T4-reflex TSH test approach, a higher proportion of preterm babies will have a T4 below the cutoff, associated with a non-elevated TSH level. In NBS programs with a primary TSH test combined with serial testing, there is a relatively high incidence of "delayed TSH elevation" in preterm neonates. On follow-up, the majority of these cases have transient hypothyroidism. Preterm/LBW infants have many clinical manifestations that might be ascribed to hypothyroidism. The question then arises whether the hypothyroxinemia of prematurity, with thyroid function tests compatible with either non-thyroidal illness syndrome or central hypothyroidism, is a physiologic or pathologic process. In particular, does hypothyroxinemia contribute to the neurodevelopmental impairment common to preterm infants? Results from multiple studies are mixed, with some randomized controlled trials in the most preterm infants born <28 weeks gestation appearing to show benefit. This review will summarize fetal and neonatal thyroid physiology, thyroid disorders specific to preterm/LBW infants and their impact on NBS for congenital hypothyroidism, examine treatment studies, and finish with comments on unresolved questions and areas of controversy.

Keywords: thyroid function, preterm, low birth weight, newborn screening, congenital hypothyroidism, iodine

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INTRODUCTION

Thyroid hormone plays a significant role in development and function of every organ system in the body. The presence of triiodothyronine (T3) occupied nuclear receptors in the fetal brain demonstrated by 10 weeks of gestation provides evidence that thyroid hormone has a critical role in brain development (1). Infants born preterm have lower serum thyroid hormone levels as compared to term infants, a reflection of reduced thyroid stimulating hormone (TSH) surge following birth, immature postnatal pituitary-thyroid function, and loss of the maternal contribution. Preterm/low birth weight (LBW) infants are at increased risk for co-morbidities, leading to changes in serum thyroid hormone levels typical of non-thyroidal illness syndrome (NTIS), but which may be difficult to separate from central hypothyroidism. To properly interpret thyroid function tests, newborn screening programs need to be cognizant of these perturbations in T4 and TSH levels in preterm/LBW infants. Clinicians who are consulted for evaluation of potential thyroid dysfunction need to be aware of these dynamic changes to make appropriate management decisions. This review will describe fetal and neonatal thyroid physiology, thyroid dysfunction specific to preterm/LBW infants and their impact on newborn screening (NBS) for congenital hypothyroidism, examine treatment studies, and finish with comments on unresolved questions and areas of controversy.

MATERNAL-FETAL THYROID RELATIONSHIPS

Evidence supports the notion that thyroid hormone present in the fetus in the first trimester is the result of transplacental transfer of maternal hormone (2). This is primarily T4; fetal T3 levels are low as a result of increased placental deiodinase type 3 activity which converts T4 to reverse T3 (RT3). At term, approximately one-third to one-half of cord blood T4 levels are of maternal origin (3). Increased maternal iodine intake during pregnancy is required to keep both mother and fetus iodine sufficient. The recommended dietary allowance (RDA) increases from 150 mcg daily to 250-300 mcg daily with pregnancy (4). As a subgroup, women of reproductive age in the U.S. are borderline iodine deficient (5). Insufficient maternal iodine intake may be a significant factor contributing to lower serum thyroid hormone levels in preterm infants. Maternal hypothyroidism (and maternal iodine deficiency) also are risk factors for preterm birth (6). In addition, excess iodine exposure during pregnancy may also be associated with neonatal hypothyroidism (the Wolff-Chaikoff effect, resulting in temporary decreased production of thyroid hormone). This is a particular problem for preterm/LBW infants, as they are slow to "escape" from this down regulation and may not recover to normal thyroid hormone production for several weeks. Other drug treatment during pregnancy may also affect neonatal thyroid hormone levels. Treatment with steroids or dopaminergic drugs may decrease TSH secretion, resulting in lower neonatal T4 levels.

Abbreviations: LBW, low birth weight; VLBW, very low birth weight; L-T4, levothyroxine; NBS, newborn screening; NTIS, non-thyroidal illness syndrome; RDA, recommended daily allowance; RT3, reverse T3; TBG, thyroxine binding globulin; T3, triiodothyronine; T4, thyroxine; TSH, thyroid stimulating hormone.

MATURATION OF FETAL THYROID FUNCTION

The thyroid gland starts development at the foramen cecum, migrating to its normal location over the thyroid cartilage. The bilobed thyroid shape is evident by 7 weeks gestation, and thyroid follicles containing colloid are seen histologically by 10 weeks. Thyroglobulin synthesis is detected by 4 weeks, iodine trapping by 8 to 10 weeks, and T4 production by 12 weeks. Significant production of thyroid hormone does not begin until the second trimester (7). Fetal serum T4 concentrations rise from approximately 2 ug/dl (26 nmol/L) at 12 weeks to 10 ug/dL (128 nmol/L) at term (8, 9). Parallel changes are seen in fetal serum free T4 concentrations, rising from approximately 0.1 ng/dL (1.3 pmol/L) at 12 weeks to 2 ng/dL (25.7 pmol/L) at term. As noted above, fetal serum T3 concentrations are low as compared to infant levels, rising from approximately 6 ng/dL (0.09 nmol/L) at 12 weeks to 45 ng/dL (0.68 nmol/L) at term. Thyrotropin releasing hormone (TRH) is present in hypothalamic neurons by 6-8 weeks gestation, and TSH secretion can be detected by 12 weeks. Fetal serum TSH concentrations rise from approximately 4 mIU/L at 12 weeks gestation to 8 mIU/L at term (8, 9). Maturation of the hypothalamic-pituitary-thyroid axis occurs in the second half of gestation, but normal feedback relationships are not mature until term gestation.

POSTNATAL THYROID FUNCTION IN PRETERM/LOW BIRTH WEIGHT INFANTS

The dramatic rise in serum TSH 30 to 60 minutes following delivery is reduced in preterm infants as compared to term infants, generally in proportion to their degree of prematurity (10). The peak approximates 30 to 50 mIU/L in preterm infants vs. 60 to 80 mIU/mL in term infants (**Figure 1**). Cord blood T4 levels are lower in infants born preterm, again generally in proportion to the degree of prematurity (**Table 1**). In response to the dramatic TSH rise, serum T4 and T3 levels are elevated at 24 hours following birth in term infants, gradually falling back to "normal" infant ranges around 5 to 7 days of age. In contrast, in the most preterm infants born at 23 to 27 weeks gestation, serum total T4 levels actually decrease in the first week of life, are "level" in infants born at 28 to 30 weeks gestation, while only those babies born >30 weeks gestation show a rise in total T4 in the first week (**Figure 2**). The differences in total T4 concentrations in term *vs.* preterm babies is explained by a combination of immature thyroid hormone production from the thyroid gland, decreased binding proteins (primarily thyroxine binding globulin [TBG]) by the liver,

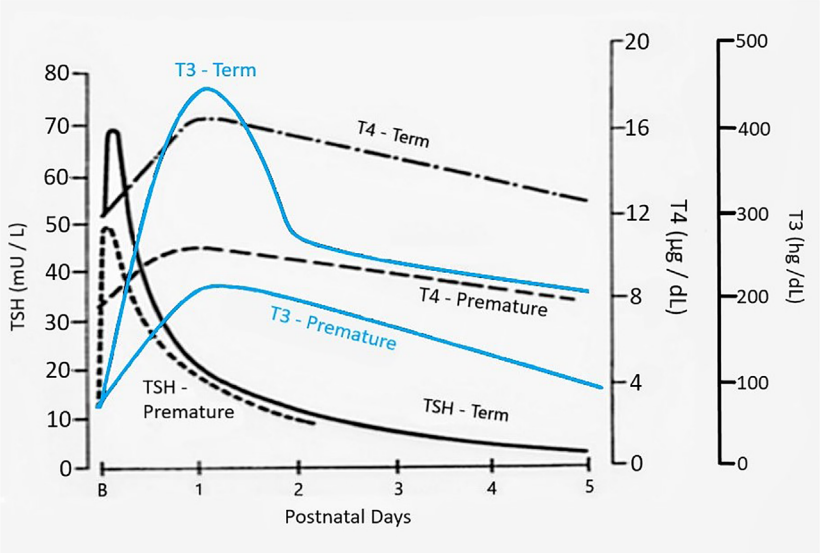

FIGURE 1 | Changes in serum TSH, T4, and T3 following birth in term as compared to preterm newborn infants. Figure developed (with permission) from Fisher DA Thyroid system immaturities in very low birth weight premature infants. Semin Perinatol 2008; 32:387-397.

and the effects of NTIS. Postnatal serum free T4 levels are also lower in the first weeks of life, though proportionally not as low as total T4 levels (**Table 1**, **Figure 3**). Serum total T3 levels follow a pattern similar to total T4, though the levels are proportionally even lower in the most preterm babies, likely a reflection of the impact of NTIS (sometimes referred to as the "low T3 syndrome") (**Table 1**). Administration of certain drugs used to manage co-morbidities in preterm infants may affect thyroid hormone levels; glucocorticoids and dopaminergic agents may inhibit TSH secretion, while excess iodine may inhibit thyroid hormone production.

UNIQUE PATTERNS OF THYROID DYSFUNCTION IN PRETERM/LOW BIRTH WEIGHT INFANTS

Hypothyroxinemia of Prematurity

Serum total T4, and to a lesser extent free T4 concentrations are decreased in proportion to the degree of prematurity, generally without elevation of serum TSH levels, findings described as "hypothyroxinemia of prematurity". In otherwise healthy preterm infants, serum T4 levels gradually rise, such that by 37

TABLE 1 | Concentrations of free T4, T4, T3, and TSH in preterm and term infants in cord blood at birth, and at 7, 14, and 28 days of age (mean +/- 1 SD).

Gestation (weeks)	Age of Infant	Free T4 (ng/dL)	T4 (µg/dL)	T3 (ng/dL)	TSH (mU/L)
23-27 weeks	Cord	1.28 ± 0.4	5.4 ± 2.0	20 ± 15	6.8 ± 2.9
	7d	1.47 ± 0.6	4.0 ± 1.8	33 ± 20	3.5 ± 2.6
	14d	1.45 ± 0.5	4.7 ± 2.6	41 ± 25	3.9 ± 2.7
	28d	1.50 ± 0.4	6.1 ± 2.3	63 ± 27	3.8 ± 4.7
28-30 weeks	Cord	1.45 ± 0.4	6.3 ± 2.0	29 ± 21	7.0 ± 3.7
	7d	1.82 ± 0.7	6.3 ± 2.1	56 ± 24	3.6 ± 2.5
	14d	1.65 ± 0.4	6.6 ± 2.3	72 ± 28	4.9 ± 11.2
	28d	1.71 ± 0.4	7.5 ± 2.3	87 ± 31	3.6 ± 2.5
31-34 weeks	Cord	1.49 ± 0.3	7.6 ± 2.3	35 ± 23	7.9 ± 5.2
	7d	2.14 ± 0.6	9.4 ± 3.4	92 ± 36	3.6 ± 4.8
	14d	1.98 ± 0.4	9.1 ± 3.6	110 ± 41	3.8 ± 9.3
	28d	1.88 ± 0.5	8.9 ± 3.0	120 ± 40	3.5 ± 3.4
≥37 weeks	Cord	1.41 ± 0.3	9.2 ± 1.9	60 ± 35	6.7 ± 4.8
	7d	2.70 ± 0.6	12.7 ± 2.9	148 ± 50	2.6 ± 1.8
	14d	2.03 ± 0.3	10.7 ± 1.4	167 ± 31	2.5 ± 2.0
	28d	1.65 ± 0.3	9.7 ± 2.2	176 ± 32	1.8 ± 0.9

T4, thyroxine; T3, triiodothyronine; TSH, thyroid stimulating hormone.

Adapted with permission from: Williams FL, Simpson J, Delahunty C, et al. Developmental trends in cord and postpartum serum thyroid hormones in preterm infants. J Clin Endocrinol Metab 2004; 89:5314-20 (9).

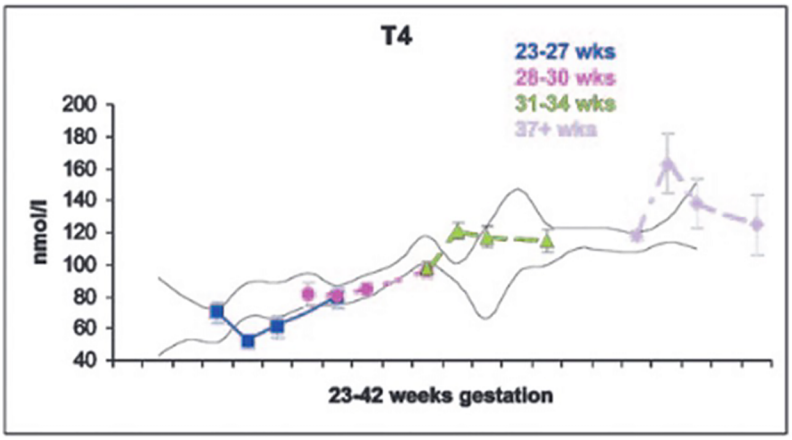

FIGURE 2 | Serum T4 levels in cord blood, and at 7, 14 and 28 days of life in neonates of 23-27, 28-30, 31-34, and 37+ weeks gestation. With permission from Williams FL, Simpson J, Delahunty C, et al. Developmental trends in cord and postpartum serum thyroid hormones in preterm infants. J Clin Endocrinol Metab 2004; 89:5314-5320 (9).

weeks gestation they overlap levels seen in term infants (9). In preterm infants born <28 weeks gestation, serum TSH may rise into the 6 to 15 mIU/L range around 2 to 3 weeks of age (11) (**Figure 4**), a transient elevation speculated to play a role in stimulating the immature thyroid gland to normal T4 production, so compensating for the loss of maternal T4 contribution. Common co-morbidities associated with preterm birth, such as respiratory distress syndrome, sepsis, intraventricular hemorrhage, etc. are associated with thyroid function tests typical of NTIS: normal or low total T4, low total T3, elevated reverse T3, and normal to low serum TSH (12). Serum free T4 concentrations measured by the more common analog assay method may be low in this setting, while free T4 measured by the more accurate equilibrium dialysis method tends to be in the normal range (13).

Delayed TSH Elevation

Some preterm/LBW infants manifest serum TSH concentrations greater than the 15 mIU/L "compensatory" rise described above. The New England NBS program reported an incidence of delayed TSH elevation >20 mUI/mL on the dried blood spot of 1:294 VLBW infants (1000-1500 g) and 1:1878 LBW infants (1501-2500 g), as compared to 1:77,280 in term babies (>2500 g) (14). The delayed TSH elevation occurred at a peak age of 58 days of life (range 11 to 176 days). In even lower birth weight infants, the Wisconsin NBS program reported an incidence of

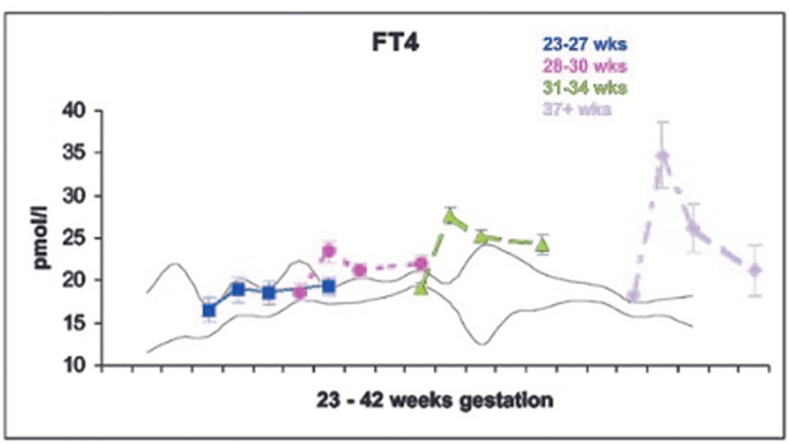

FIGURE 3 | Serum free T4 levels in cord blood, and at 7, 14 and 28 days of life in neonates of 23-27, 28-30, 31-34, and 37+ weeks gestation. With permission from Williams FL, Simpson J, Delahunty C, et al. Developmental trends in cord and postpartum serum thyroid hormones in preterm infants. J Clin Endocrinol Metab 2004; 89:5314-5320 (9).

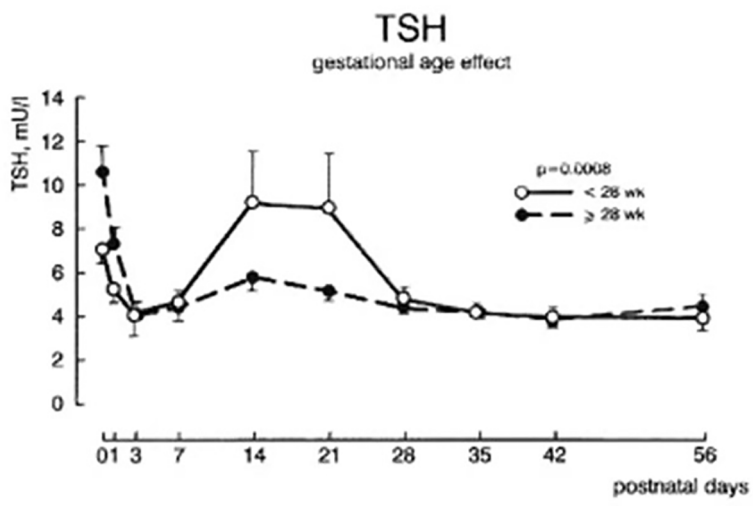

FIGURE 4 | Plasma TSH concentrations ± SEM during the first 8 weeks after birth in infants of 28-30 week gestation and <28 weeks gestation. With permission from van Wassenaer AG, Kok JH, Dekker FW de Vijlder JJ. Thyroid function in very preterm infants: influences of gestational age and disease. Pediatr Res 1997; 42:604-9 (11).

delayed TSH elevation in 1:13 in babies <500 g, 1:28.5 in babies 500 – 1000 g, 1:330 babies 1000-1500 g, and 1:737 babies 1500-2500 g (15). In infants with higher TSH elevation >100 mIU/L, the TSH elevation occurred earlier, ~3 weeks, while those with milder TSH elevation >20 mIU/L occurred later, ~8 weeks of age. Follow-up serum testing has shown that most cases of delayed TSH elevation are transient, particularly if the TSH elevation is mild. In a report from the Rhode Island NBS program, of 19 preterm babies with delayed TSH elevation, three with TSH >50 mIU/L were treated with l-thyroxine, while the other 16 infants monitored without treatment recovered to normal thyroid function between 8 and 58 days of life (16).

Role of Iodine: Too Little, Too Much

Although universal salt iodization has greatly improved the status of iodine sufficiency (17), worldwide iodine deficiency remains one of the most common preventable causes of intellectual disability (18). Preterm infants are particularly vulnerable to iodine deficiency, a result of a combination of loss of the maternal contribution, relatively low levels of iodine in preterm formulas, and near absence of iodine in most parenteral nutritional preparations (19). A study of iodine balance in 27 to 30 week gestational age infants showed that they were in negative iodine balance for the first two weeks of life, and they did not reach the RDA of 30 mcg/kg/day until 120 days of age (20). Lower iodine intake was correlated with lower serum free T4 and T3 and higher TSH levels. If present, iodine deficiency would be a significant factor contributing to slow recovery from hypothyroxinemia of prematurity.

Exposure to excess iodine has also been demonstrated to cause thyroid dysfunction in preterm infants. Excess iodine results in down regulation of thyroid hormone production (the Wolff-Chaikoff effect), thought to be a mechanism to protect against development of hyperthyroidism. Normal thyroid function is maintained by "escape" from this down regulation; however, preterm infants are slow to escape and so may become hypothyroid. Common sources of iodine exposure include iodinated contrast agents and topical iodine antiseptics. Infants with congenital heart disease (CHD) are frequently exposed to such sources; in a study of 183 infants with CHD, one-quarter manifested elevation of serum TSH levels, ranging from 9 to >100 mIU/mL (21). About half were treated with thyroid hormone, while others recovered to euthyroidism after discontinuation of excess iodine exposure. This study involved primarily term infants; likely the effect of excess iodine would apply to preterm/LBW infants.

Impact of Preterm Birth/Low Birth Weight on Newborn Screening for Congenital Hypothyroidism

Several NBS programs from Europe and North America report that the incidence of congenital hypothyroidism is higher in preterm infants. As compared to an incidence of approximately 1:1,500 to 1:2,000 in term infants (22, 23), the incidence in preterm infants approximates 1:900 (24). The incidence is even higher in NBS programs that undertake repeat screening in preterm babies, from 1:300 in LBW to 1:50 in VLBW babies (14, 16).

In NBS programs that employ a primary T4-reflex TSH test strategy, preterm/LBW infants make up a higher percentage of cases that fall below the T4 cutoff (typically the 10th percentile), while the screening TSH is not elevated. These NBS programs must then decide whether to follow-up cases with low T4-non-elevated TSH levels. In some programs, lack of an abnormal TSH

elevation is the end of screening. However, most NBS programs now collect a 2nd dried blood specimen at 2 to 4 weeks of life in infants born <32 weeks gestation or <1500 g birth weight (25). In the very preterm babies <28 weeks gestation, several NBS programs collect a 3rd dried blood specimen at 6 to 8 weeks of life, which is typically around 37 weeks or term gestation. In the majority of preterm/LBW babies, the screening T4 will reach the range seen in term infants by the 2nd or 3rd specimen (5). Collection of the 2nd and 3rd specimens allows detection of preterm babies with delayed TSH elevation, described above.

A few preterm infants will not normalize total T4 by term gestation; such results could be explained by low binding protein levels or NTIS, although central hypothyroidism cannot be ruled out. In these cases, NBS programs recommend serum thyroid function tests, including TSH, total and free T4. In the presence of low binding proteins or NTIS, serum free T4 is most accurately measured by equilibrium dialysis. A normal free T4 and TSH rule out hypothyroidism, while a low free T4 and low or normal range TSH is compatible with central hypothyroidism, leading to evaluation for other pituitary hormone deficiencies. Such cases in preterm infants are rare, likely with an incidence matching term infants:!:25,000 (26).

In NBS programs that employ a primary TSH test approach, otherwise healthy preterm babies will fall below the dried blood TSH cutoff, typically <25 mIU/L (serum units). As described for T4-reflex TSH test programs, most primary TSH test programs collect a 2nd dried blood specimen at 2 to 4 weeks of life in infants born <32 weeks gestation or <1500 g birth weight, and many collect a 3rd dried blood specimen at 6 to 8 weeks of life in preterm babies born <28 weeks gestation. Collection of the 2nd and 3rd specimens allows detection of preterm babies with delayed TSH elevation, whereas stopping screening with a normal TSH on the 1st test risks missing infants with delayed TSH elevation. Re-evaluation after age 2 to 3 years of age shows that many have transient hypothyroidism (27–29).

Unique patterns of Thyroid Dysfunction in Preterm/Low Birth Weight Infants: Physiologic or Pathologic?

Preterm/LBW infants manifest clinical features that might be ascribed to thyroid dysfunction, such as temperature instability, apnea with immature pulmonary function and low surfactant levels, bradycardia, slow to feed with sluggish gut motility, edema, hypotonia, and slow growth and development. Several observational studies demonstrate a correlation between low serum T4 levels and these clinical manifestations (30, 31). As these babies typically have low serum T4-non-elevated TSH levels, clinicians face the dilemma as to whether these features are caused by central hypothyroidism and so might improve with thyroid hormone treatment. However, randomized, placebo-controlled trials using either T4 or a combination of T4 and T3 generally do not show an effect on objective measures, such as O2 requirements, incidence of respiratory distress syndrome, requirement for inotropic agents, progression from parenteral to oral feedings, weight gain, length, or head circumference measurements, or on mortality (32–34). A Cochrane Database Review in 2007 also found no benefit of thyroid hormone treatment in this situation (35).

A more compelling question asks whether deficits in neurodevelopment in preterm infants are a consequence of the hypothyroxinemia. Several studies report an increased odds ratio for endpoints such as disabling cerebral palsy (36), reduced attention span (37), vision disturbances (38), and overall lower IQ (39). However, owing to "confounding variables" common to preterm infants, it is difficult to establish a causal relationship with the hypothyroxinemia alone. In a study of preterm infants <1500 g, a comparison of outcome in infants with and without hypothyroxinemia did not find any significant differences in neurodevelopmental, vision or hearing impairment at 5 years of age (40).

What about treatment trials? A randomized, placebo-controlled trial of T4 treatment in 200 infants less than 30 weeks gestational age with multiple assessments of neurodevelopment over time was carried out by the Dutch. Half were treated with l-T4 8 mcg/kg/d, half with placebo for the first 6 weeks of life. In the first assessment at 24 months of age, there were no differences in the Bayley Infant Scales of Mental and Motor Development (41). However, subgroup analysis showed that the Bayley Mental Developmental Index (MDI) score was 18 points higher in the T4-treated group ≤27 weeks gestation, ($p<.05$), but 10 points lower in the T4-treated group ≥27 weeks gestation (42) (**Table 2**). Re-evaluation at school age (5.7 years) showed that the MDI score had narrowed to 10 points, now not statistically significant (43). Survey of study families at 10 years of age showed that in subjects ≤27 weeks gestation treated with T4, there was a trend toward a lower percentage needing special education, whereas in subjects at 29 weeks gestation, the opposite was true, though only the latter reached statistical significance (**Table 2**). In subjects ≤28 weeks gestation treated with T4, again there was a trend toward better motor outcome as compared to control subjects, but this difference did not reach statistical significance (44). To try and resolve the question of potential benefit in infants born <28 weeks, a more recent trial of thyroid supplementation was undertaken in Amsterdam, Madrid and New York (45). This trial included six treatment arms to investigate different modalities of thyroid hormone administration, combination T4 and T3 treatment, one iodine treatment arm, and one placebo arm. Testing at 36 months of age did not find any differences in neurodevelopmental index scores among the eight groups (**Table 2**). Finally, in a double-blind, randomized, placebo-controlled trial in preterm babies <28 weeks gestation from the United Kingdom, evaluation at 42 months by the Bayley III Mental and Psychomotor Developmental Indices showed statistically better motor, language, and cognitive domains in the group treated with thyroid hormone (46). In summary, the randomized, placebo-controlled trials show mixed results, with potentially some benefit of T4 treatment in infants <28 weeks but potential harm in infants >28 weeks gestation.

TABLE 2 | Summary of studies investigating l-T4 treatment v placebo on neurocognitive outcome in preterm infants.

Study (Ref)	Age at Evaluation	Total Group T4 Rx v Placebo	25/26 weeks T4 Rx v Placebo	27 weeks T4 Rx v Placebo	28 weeks T4 Rx v Placebo	29 weeks T4 Rx v Placebo	>27 weeks T4 Rx v Placebo
van Wassenaer (41, 42)	24 mo	92 v 95	93 v 75	90 v 100	97 v 102	92 v 102	92 v 102
	Bayley Mental	P = 0.62	P= 0.01	P = 0.37	P = 0.49	P = 0.36	P = 0.08
	Bayley Psychomotor	92 v 88	80 v 70	81 v 84	98 v 90	86 v 91	90 v 90
		P = 0.39	P = 0.11	P = 0.35	P = 0.29	P = 0.90	P = 0.90
Briet et al. (43)	5.7 years	93.6 ± 16.2 v 95.7 ± 20.4	94.2v 84.7	90 v 92	98 v 96	90.6 v 105.2	
		P = NS	P = 0.11	P = 0.77	P = 0.58	P = 0.01	
van Wassenaer [Questionaire only] (44)	10.5 years	School outcome, motor function, behavior: T4 Rx v placebo P = NS	Percent in Special Ed T4 Rx =10% placebo=28% P=0.07	Motor Im-pairment T4 Rx=12% placebo=28% P = 0.23		Percent in Special Ed T4 Rx=30% placebo=5% P = 0.05	
van Wassenaer (45)	36 months	**Bayley III Cognitive** T4 Rx v placebo 99.2 11.3 v 105.5 ± 12.3 P= NS		**Bayley III Gross Motor** T4 Rx v placebo 9.3 ± 2.8 v 11.0 ± 2.8 P=NS		**Bayley III Fine Motor** T4 Rx v placebo 11.1 ± 2.2 v 11.8 ± 2.4 P=NS	
Ng et al. (46)	42 months	**Bayley III Cognitive** T4 Rx v placebo 91 ± 10 v 85 ± 13 P = .045		**Bayley III Motor** T4 Rx v placebo 84 ± 12 v 77 ± 13 P = .034		**Bayley III Language** T4 Rx v placebo 92 ± 13 v 83 ± 20 P = .041	

T4 Rx, l-thyroxine treated group.
NS, non-significant.

CONCLUSION: UNRESOLVED QUESTIONS AND AREAS OF CONTROVERSY

Much has been learned over the last few decades about fetal and neonatal thyroid physiology; thyroid function is different in babies born preterm/LBW as compared to term infants. As such, there remain unresolved questions and areas of controversy regarding management of thyroid issues in preterm infants. The following section highlights a few of these areas.

At What Gestational Age Can the Postnatal HPT Axis Compensate for Loss of Maternal T4 Quickly Enough to Avert Untoward Effects of Thyroid Hormone Deficiency?

A reasonable goal for serum T4 or free T4 levels after birth in preterm babies might be to either match *in utero* concentrations present at a similar gestational age or levels in term infants by 1-2 weeks after birth. Evidence suggests that infants born <28 weeks gestation have serum T4 (and to a lesser extent free T4) concentrations that take up to 4 to 6 weeks to overlap the range seen in term infants. The transient rise in serum TSH to the 6-15 mU/L range between 2 and 4 weeks of age in babies born <28 weeks gestation (but not in babies ≥28 weeks gestation) supports the notion that the thyroid gland in babies born <28 weeks gestation requires increased TSH stimulation to normalize thyroid hormone production. Slow recovery of normal thyroid physiology might impact any organ system, e.g., maturation of lung function, but most importantly it could impact neurodevelopment.

Should Iodine Supplementation Be Routine in Preterm Infants?

Most preterm infant formulas do not contain enough iodine to allow these babies to meet the RDA of 30 mcg I/kg/d, and many parenteral nutritional preparations lack iodine completely. A recent Cochrane database review did not show benefit of iodine supplementation in preterm infants on morbidity, mortality, or neurodevelopmental outcome (47). Despite this report, given the known effect of iodine to prevent "endemic cretinism", routine supplementation of preterm formulas and parenteral nutritional preparations would seem important to achieve T4 production matching term infants. Sufficient iodine intake is equally important for nursing mothers. At the same time, care should be taken to avoid exposure to excess iodine.

Do Infants With "Delayed TSH Elevation" Benefit From Detection and Treatment?

Studies show that the majority of preterm babies with delayed TSH elevation will recover to normal TSH levels without treatment. However, the average age at peak TSH elevation is approximately 8 weeks, with a range from 11 to 176 days of life (14). The TSH elevation likely reflects inadequate thyroid hormone production. Although babies recover to normal thyroid function, are there consequences from low T4 levels, present over several weeks? Few studies have been carried out to address this issue. The study from Rhode Island showed

developmental scores were similar to control infants, although the incidence of infants with head circumference <10th percentile was higher in the delayed TSH elevation group (16). It would seem prudent to treat infants with elevated serum TSH and low free T4 levels until recovery to normal thyroid function; since this is difficult to judge without stopping l-thyroxine, most babies are treated until age 2-3 years and then re-evaluated.

Does T4 Treatment Improve Morbidity/ Mortality and Neurodevelopmental Outcome in Preterm Infants <28 Weeks Gestation?

While it is difficult to separate out the effects of co-morbidities from hypothyroxinemia on neurodevelopmental outcome, there are now two randomized, placebo controlled trials in preterm infants <28 weeks gestation that report higher scores in the T4-treated group (42, 46). As noted above, there may be some "physiological" support for this finding, as thyroid function in infants born <28 weeks gestation may be too immature to quickly replace the lost maternal thyroid hormone contribution. That said, the follow-up studies by the Dutch showed that the higher neurodevelopmental scores in the treated group tended to approach the placebo group over time (44). While the more recent trial from Amsterdam, Madrid, and New York did not show benefit of thyroid hormone treatment, the investigators cautioned that "power was insufficient to detect any but very large differences". With these mixed results, as the saying goes, more randomized controlled trials are needed before such treatment becomes standard of care.

AUTHOR CONTRIBUTIONS

SL undertook focused literature review and writing of the manuscript.

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CASE REPORT
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Case Report: Functional Analysis and Neuropsychological Evaluation of Dyshormonogenetic Fetal Goiter in Siblings Caused by Novel Compound Hyterozygous TPO Gene Mutations

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Introduction: It is rare for a euthyroid mother to carry a child with a fetal goiter. However, cases of congenital hypothyroidism (CH) caused by thyroid dyshormonogenesis have been reported. Even though gene mutations associated with fetal goiter have been reported in a few studies, the effects on intellectual development have not been investigated. This study aimed to characterize and investigate the underlying genetic mechanism of CH and neuropsychological development and growth of two siblings with CH-induced fetal goiters.

Case report: Two male siblings from a non-consanguineous marriage with CH and fetal goiter were diagnosed by ultrasonography at 32- and 26-weeks of gestation. This condition was confirmed by cordocentesis in the first pregnancy (TSH: 135 μIU/ml). The mother was euthyroid, and no intra-amniotic levothyroxine treatment was performed. Peripheral blood DNA was screened for TPO mutations. The new deletion p.Cys296Alafs*21 and the p.Arg665Trp mutation, inherited from heterozygous parents, were identified in both patients. Functional analysis showed both mutations reduced the TPO enzyme activity and impaired the membrane localization. The p.Cys296Alafs*21 mutation produces a protein product with a drastically reduced molecular weight. Additionally, a complete clinical and neuropsychological evaluation was also performed. The WISC IV test was employed to provide an overall measure of the siblings' cognitive and intellectual abilities. No growth retardation was detected in either child. In general, both children showed normal neuropsychological development; however, they exhibited slight reduction of Processing Speed Index scores, which are sensitive to neurological and

attentional factors and motor maturation activity. Notably, the younger sibling obtained significantly low scores in the Operational Memory Index, a measure of attention capacity and psychoneurological immaturity.

Conclusion: We described a new TPO compound heterozygosity that severely impaired the TPO activity and membrane localization leading to severe CH and fetal goiter. This is the first report showing the neuropsychological evaluation in patients with dyshormonogenetic fetal goiter. More studies are needed to understand the neurodevelopmental outcomes of neonates with CH-induced fetal goiters.

Keywords: fetal goiter, congenital hypothyroidism, thyroid peroxidase, mutations, neuropsychological evaluation, growth

INTRODUCTION

Congenital hypothyroidism (CH) is the most prevalent (1:2,000–1:4,000 live births) childhood endocrine disease and the most common cause of preventable intellectual disability (1). Most CH patients (80–85%) exhibit thyroid dysgenesis, which encompasses a spectrum of abnormalities, including agenesis (a complete absence of the thyroid gland), hypoplasia (a small gland with a typical location), and ectopy (an abnormally located gland) (2). In 10–15% of the cases, CH is caused by defects in the thyroid hormone biosynthesis or dyshormonogenesis (3). Mutations in thyroid peroxidase (TPO) (4), thyroglobulin (TG) (5), dual oxidase DUOX 1, DUOX maturation factor 1 (DUOXA1) (6), dual oxidase 2 (DUOX2) (7), DUOX maturation factor 2 (DUOXA2) (8), sodium iodide symporter (NIS) (9), and pendrin (SLC26A4) (10) have been associated with dyshormonogenesis.

Secondary to dyshormonogenesis, fetal goiters can also develop. These goiters are rarely large enough to be clinically detected at birth and even more challenging during intrauterine observation. However, advances in ultrasound technology have led to identifying many cases and may suggest fetal thyroid deficiency and hypothyroidism through peripheral gland hypervascularization and paradoxical increased fetal movement (11). It is important to point out that fetal goitrous hypothyroidism can also be caused by iodine deficiency (12), massive iodide exposure (13), and maternal hyperthyroidism therapy (14).

Up to now, mutations in TG, TPO, NIS, and DUOXA2 have been identified in few dyshormonogenetic fetal goiter patients (15–28) as shown in **Supplementary Table 1**. These mutations are usually transmitted in an autosomal recessive fashion; however, some studies have reported monoallelic mutations (29).

The effects of thyroid hormone and its deficit on children's psychomotor and neurological outcomes have been well-documented (30), but not in CH-induced fetal goiter patients. Before fetal thyroid hormone synthesis begins at approximately 11 weeks of gestation (WG), the fetus's brain is protected by the maternal thyroid hormone (31). After that point, fetal thyroid hormones are necessary for healthy development. A previous study screening neonates with CH caused by a total organification defect showed that their thyroid hormone levels were about 40–60% of normal levels and likely of maternal origin (32).

This study aimed to characterize and investigate the underlying molecular genetic mechanism for CH development and neuropsychological progression and growth of two siblings with CH-induced fetal goiters.

CASE REPORT

Clinical Presentation

The present study follows two pregnancies of an initially 32-year-old primigravida. During the first pregnancy, a routine ultrasound scan (USS) at 28 WG did not identify any polyhydramnios or other fetal abnormalities. However, the USS at 32 weeks detected a fetal goiter (**Supplementary Figure 1**). The mother was subsequently referred for endocrinological evaluation. The mother stated that she does not take medication and that she presented thyroid hyperplasia two years earlier. As shown in **Table 1**, subsequent cordocentesis confirmed fetal hypothyroidism (TSH: 135 μIU/mL; FT4: 0.57 ng/dL). At 34 WG, the fetus was delivered by cesarean without any complications due to spontaneous premature labor. The male newborn weighed 2.145 kg and had Apgar scores of ten at 1 and 5 min. The newborn presented weak suction and vomiting and remained in the intensive care unit for five days. Serum thyroid hormones were measured two days after birth, and hypothyroidism was confirmed. The neonate was subsequently treated with levothyroxine (LT4).

During the second pregnancy, the USS at 22 WG revealed no abnormalities. Like the first pregnancy, four weeks later (26 weeks), a fetal goiter was detected (**Supplementary Figure 1**). It was decided that no intervention would be administered at the time. Due to the progressive increase of the goiter volume, delivery was induced at 35 WG. The male child was delivered without complication and weighed 2.255 kg presenting Apgar scores of nine at 1 and 5 min. Two days after birth, congenital hypothyroidism was confirmed (TSH: 83.89 μIU/mL), and LT4 treatment was initiated. Thyroid hormone status of the patients and parents and LT4 doses over time are presented in **Table 1**.

Both siblings and the family showed good adherence to LT4 treatment; however, the patients still have goiter detected by USS. They showed good clinical conditions and puberty stages according to Tanner (33). Considering the height and body

TABLE 1 | Thyroid hormone status of Patients 1 and 2 and of their parents and LT4 dose over time.

	TSH μIU/mL (0.3–5.0)	FT4 ng/dL (0.8–2.0)	TG ng/mL (1.35–35)	anti-TPO IU/mL (<9)	LT4 μg/day
Patient 1: Fetal goiter detected at 32 WG; birth at 34 WG					
32 WG Cordocentesis	135.0	0.57	–	3	–
At birth*	–	0.94	–	<10	–
2 days after birth	28.36	1.07	–	<10	25
2 weeks after birth	3.11	1.44	26.59	–	25
7 months	24.41	0.925			50
1.1 years old	2.02	1.71			50
1.9 years old	1.33	2.22	–	–	50
2.4 years old	8.68	1.68			75
3.2 years old	0.35	2.47	–	–	75
5 years old	2.52	1.94	–	–	62.5
10 years old	3.52	1.42	–	–	88
14.9 years old	6.69	1.13	–	–	150
15.8 years old	1.18	1.29	–	–	150
16.6 years old	6.98	1.36	–	–	150
Patient 2: Fetal goiter detected at 26 WG; birth at 36 WG					
2 days after birth	83.89	1.02	–	–	25
4 days after birth	12.68	1.70	530.6#	–	25
3 months after birth	2.91	1.88	–	–	25
6 months after birth	2.54	1.78	–	–	25
9 months after birth	11.49	1.29			50
1 year old	0.02	2.51	–	–	37.5
5.2 years old	9.57	1.28	–	–	62.5
10 years old	8.80	1.06	–	–	75
10.3 years old	1.64	1.48	–	–	75
11 years old	1.41	1.18	–	–	75
11.8 years old	2.65	1.33	–	–	75
Mother[&]	1.99	0.97	–	5	N
Mother[#]	0.93	1.10	7.91	1	N
Father	1.24	1.05	12.59	3	N

*WG, weeks of gestation. * Not enough material for TSH testing; N, not in use; [&] in the third trimester of the first pregnancy with anti Tg:1 IU/mL (ref <40). [#] after the second pregnancy.*

mass indices, no growth retardation was observed according to World Health Organization (5–19 years old) (34) (**Supplemental Figure 2**), and there were no motor problems detected in either child during the medical follow-up. Currently, Patient 1 is 16 years old and in his second year of high school. He has a height of 175 cm and weight of 55.7 kg, Height/Age and BMI/Age z scores of 0.08 and −1.19, respectively, and bone age of 17 years. He takes 150 mcg of levothyroxine per day. Patient 2 is 11 years old. He is in elementary school. He is 148.5 cm tall and weighs 39 kg. His calculated z scores for Height/Age and BMI/Age are 0.17 and 0.16, respectively, and his bone age is 10 years. Patient 2 is also treated with levothyroxine (75 mcg/day). In May 2021, laboratory thyroid tests of the siblings' serum were TSH 6.98 μcIU/mL and 2.65 μcIU/mL (0.27–5.00) and FT4 (1.36 ng/dL and 1.33 ng/dL (0.75–2.00) for Patients 1 and 2, respectively. In Patient 1, a slight increase in TSH level was observed, with normal levels of FT4 (**Table 1**). It was then decided to maintain the levothyroxine dose and to repeat the tests in two months.

Mutation Detection and Functional Assessment

The sequences analyses revealed that the genomic DNA of the two siblings contained three TPO mutations: p.Gln660Glu (C.1978C>G) and p.Arg665Trp (c.1993C>T) in exon 11 and a new deletion p.Cys296Alafs*21 (c.886delT) in exon 8. Thep.Arg665Trp mutation was present in the mother's DNA, and the p.Gln660Glu (4) and p.Cys296Alafs*21 (35) mutations were found in the father's DNA (**Figure 1**). The p.Gln660Glu mutation is on the same allele as the deletion mutation; thus, it is unlikely to contribute to the disease. Thus, the presence of both the p.Arg665Trp and the p.Cys296Alafs*21 mutations indicates compound heterozygosity in the siblings' DNA.

The pathogenicity of the p.Arg665Trp (Arg665Trp-TPO) and p.Cys296Alafs*21 (delT886-TPO) mutations was evaluated in HEK293 cells. The delT886-TPO mutation alters the protein reading frame and introduces a new stop codon at residue 316, resulting in a 35 kDa protein product. In comparison, the Arg665Trp-TPO missense mutant and TPO-WT migrate at a molecular weight of 103 kDa (**Figure 2A**). We also measured the enzymatic activities of TPO-WT, Arg665Trp-TPO, and delT886-TPO. As shown in **Figure 2B**, the TPO activities of Arg665Trp-TPO and delT886-TPO are significantly lower than TPO-WT levels.

Next, we assessed TPO localization by immunofluorescence. Under cell permeabilizing conditions (**Figures 3A–D**), cytoplasmic TPO-specific immunostaining was observed in the wild-type and mutant TPO transfected cells. These results confirm TPO transfection and expression. Under non-permeabilizing conditions (**Figures 3E–H**), TPO-WT was localized along the cell membrane. Notably, we observed reduced membrane staining in cells transfected with delT886-TPO and Arg665Trp-TPO, indicating that these mutant proteins

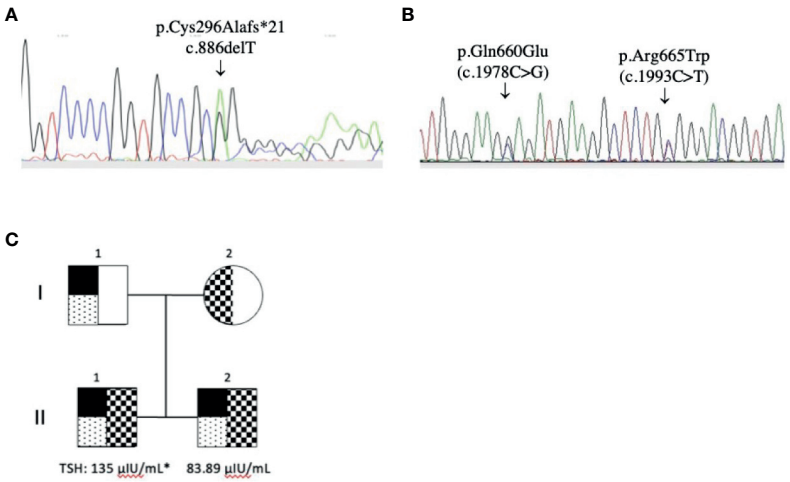

FIGURE 1 | TPO gene mutations. **(A)** p.Cys296Alafs*21 (c.886delT) in exon 8 (reverse sequence) and **(B)** p.Gln660Glu (C.1978C>G) in exon 11 and p.Arg665Trp (c.1993C>T) in exon 11 (reverse sequence); **(C)** Pedigrees of the family and THS values at diagnosis (* cordocentesis). The p.Cys296Alafs*21 (c.886delT) and the p.Gln660Glu (C.1978C>G) mutation was detected in both patients (II.1 and II.2) and the father (I.1). The p.Arg665Trp (c.1993C>T) mutation was detected in both patients and the mother (I.2).

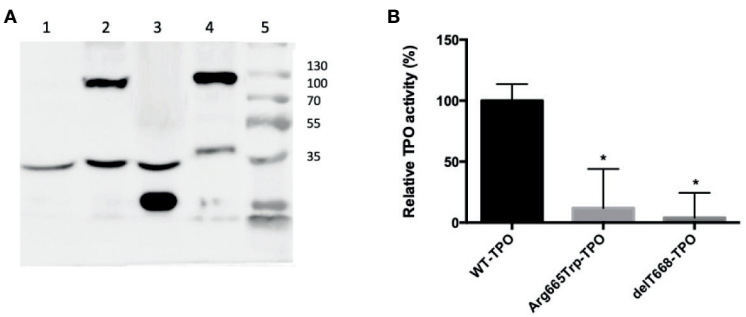

FIGURE 2 | **(A)** Western blot of HEK293 extracts from cells transfected with wild-type or mutant TPOs. Lane: 1) pCDNA 3.1; Lane 2) Arg665Trp-TPO, 103 kDa; Lane 3) delT668-TPO, 35 kDa; Lane 4) TPO-WT; 103 kDa; Lane 5) molecular weight marker Thermo Scientific Page Ruler (Thermo Scientific, Carlsbad, CA). All strains expressed the endogenous control alpha-tubulin (53 kDa). **(B)** Extracellular enzymatic activity of HEK293 cells expressing wild-type or mutant TPOs. Enzymatic activity was assessed using the Amplex UltraRed reagent. $\lambda_{EXCITATION}$ = 530 nm and $\lambda_{EMISSION}$ = 560 nm. The values are expressed as a percentage of the TPO-WT transfected cells' activity (*$p < 0.05$).

are not correctly inserted into the membrane (**Figures 3F, G**). It is also worth mentioning that no immunostaining was detected in cells transfected with empty pcDNA vector under either condition. The full methods are in the **Supplementary File**.

Neuropsychological Evaluation

A clinical neuropsychologist evaluated the two brothers for attention (divided, sustained, and focused), cognitive flexibility, short- and long-term memory (verbal and visual), intellectual processes (reasoning, abstract thought, and critical thinking), motor functions (movements, laterality, and others), visual functions (perception, discrimination, and visuospatial and visuoconstructive organization), praxis, general intelligence, learning ability, and language (expressive, receptive and written). The children were also evaluated for the presence of Attention Deficit Hyperactivity Disorder (ADHD). Herein, the fourth edition of the Wechsler Intelligence Scale for Children (WISC IV) test to assess the siblings' intelligence was used. These evaluations were performed at 11 years of age for Patient 1 and 6 years for Patient 2. A summary of these results is presented in **Table 2**. In general, the children exhibited behavior consistent with their age, sex, and education. Moreover, the evaluations showed that both boys had adequate age-related logical reasoning, conceptual verbal formation (abstract thinking), memory and verbal fluency, semantic knowledge, intellectual curiosity, and ability to deal with abstract symbols. Quantitative analyses of the WISC-IV results and comparisons of each patient with himself using the factorial indexes, which are considered more accurate measures of intelligence, showed that both patients exhibited lower Processing Speed Index (related to the

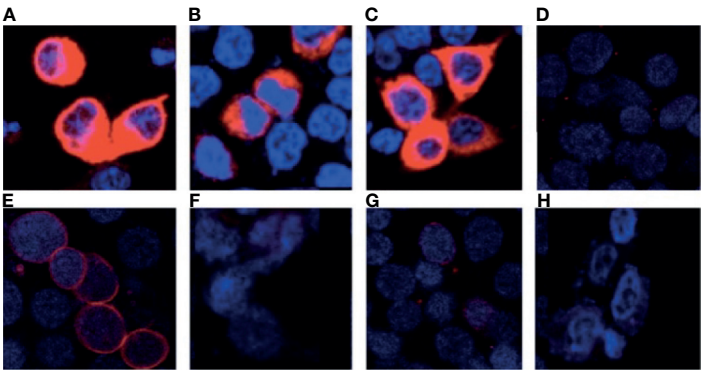

FIGURE 3 | Cellular localization of wild-type and mutant TPOs. Immunofluorescence, using anti-TPO RPE5379- (Abcam) and AlexaFluor 594 (Invitrogen), under permeabilized conditions, Panel **(A)** TPO-WT, Panel **(B)** delT668-TPO, Panel **(C)** Arg665Trp-TPO, and **(D)** pcDNA and impermeabilized conditions Panel **(E)** TPO-WT, Panel **(F)** delT668-TPO, Panel **(G)** Arg665Trp-TPO, and Panel **(H)** pcDNA transfected HEK293 cells. Images were acquired with a Leica TCS SP8 confocal microscope at 63× magnification.

TABLE 2 | Summary of the neuropsychological evaluation results of the dyshormonogenetic fetal goiter patients using WISC-IV Tests.

Characteristics	Patient 1	Patient 2
Executive functions		
Abstraction and judgment	Above average	Low Average
Sustained attention/concentration	Average	Low Average
Alternate attention	High average	Below average
Selective attention	Average	Low Average
Verbal and non-verbal memory process		
Non-verbal (visual) memory	Below average	High average
Verbal memory	Above average	High average
Language		
Verbal comprehension, naming, and vocabulary	Average	Average
Verbal fluency	Average	Average
Constructive ability		
Visual reproduction	Below average	–
Praxia	Low average	Low average
Humor and behavior evaluation		
Stress	Normal	Positive
Hyperactivity-TDAH	Negative	Hyperactivity and impulsivity*
General intellectual operation (QI)	Normal	Normal

*Scores were categorized as well above average, above high average, average, below average, or well below average compared to their age reference group at each assessment. *To be confirmed in a future evaluation.*

speed of mental processing and motor graph), and motor visual organization scores. Additionally, Patient 2 had a significantly lower Operational Memory Index (OMI), according to the WISC-IV. The OMI provides a measure of the child's attention. Due to its sensitivity, it has been utilized for detecting ADHD; however, to confirm the ADHD diagnosis, new evaluations are required between 7 and 12 years of age. Although Patient 2 is currently 11 years old, the parents refused consent for revaluation.

DISCUSSION

Fetal goiter is a rare thyroid disorder with an incidence of about 1:40,000 live births. Despite being a perinatal complication with low incidence, it can cause airway obstruction and polyhydramnios and increase the fetus' risk of other morbidities and/or mortality (18, 36). This study investigated the genetic causes of CH and fetal goiter development and their impact on neurological functions. Herein, we identified mutations in the TPO gene of two brothers from a non-consanguineous marriage who exhibited fetal hypothyroidism and are currently undergoing LT4 treatment.

The high TSH and TG levels and the patients' fetal goiters led us to investigate TPO gene mutations by sequencing patient DNA. Herein, we identified three mutations in both patients. These mutations include p.Gln660Glu (4) and p.Cys296Alafs*21 (a new deletion) inherited from the father, and p.Arg665Trp (35) inherited from the mother.

Studies have shown that TPO protein, an essential enzyme for thyroid hormone synthesis, is localized to the apical membrane of the thyrocyte (37). TPO activity depends on the proper conformation, cellular localization and an intact catalytic site (38). The new deletion, which introduces a stop codon at amino-acid 316 and produces a 35 kDa protein, exhibits significantly reduced TPO activity (**Figure 2**). The deletion results in the loss of the catalytic site (exons 8–10), the transmembrane domain (exon 15), and the extracellular domain (**Supplementary Figure 2**). The immunofluorescence experiments demonstrate that this mutant is localized in the cytoplasm and exhibits reduced membrane localization (**Figure 3**). It was previously reported that the TPO mutant p.Gln660Glu displays reduced activity and membrane localization (39). It is unlikely that the p.Gln660Glu mutation contributes to the disease since it is not present in the protein as it is located downstream of the truncating mutation p.Cys296Alafs*. Thus, our results indicate compound heterozygosity involving

p.Cys296Alafs*2 and p.Arg665Trp, mutations present in the paternal and maternal alleles, respectively.

It should be pointed out that the p.Arg665Trp mutation, previously identified in two patients with severe dyshormonogenesis, one with a history of neonatal goiter, was found to have reduced activity in the guaiacol oxidation assays and no membrane localization (35, 40). These results are consistent with the present study's data, showing that Arg665Trp-TPO exhibits drastically reduced enzymatic activity and plasma membrane localization when expressed in HEK-293 cells (**Figures 2** and **3**). In this sense, our results provide further evidence demonstrating the severity of the p.Arg665Trp mutation.

Fetal goiter is usually diagnosed during the second or third trimester (11), which coincides with the detection at 32 and 26 WG for Patients 1 and 2 in our study. This late manifestation is because the maternal thyroid hormone supports fetal development during the first trimester, with maternal T3 regulating neuronal proliferation and the onset of neuronal cerebral cortex migration (41). During the second trimester, the fetal thyroid hormones progressively contribute more to neurogenesis, neural migration, myelination, axonal growth, dendritic arborization, and glial differentiation. Finally, in the third trimester, maternal and fetal thyroid hormones are required for central nervous system maturation (42). It has been reported that maternal hypothyroidism negatively impacts the neuropsychological development of children (43), an outcome probably due to abnormal cortical development during the first trimester (44).

On the other hand, fetal hypothyroidism appears to affect cerebral areas later in development, influencing language, associative memory, auditory processing, attention, and executive processing (45). Additional evidence associating intrauterine brain damage with fetal hypothyroidism comes from children with severe CH due to agenesia or with a thyroid gland and very low neonatal FT4. These children face a higher risk for subtle irreversible neurological deficits despite early treatment after birth (46–48) and exhibit cognitive problems with memory, attention, and visuospatial processing that can persist into early adolescence (49). Furthermore, a summary of several studies demonstrated that children diagnosed with CH by neonatal screening and who received optimal therapy presented IQ reductions of about 0.5 SD (45).

In our study, the neuropsychological assessment indicated that in general these patients with fetal goiter exhibited a normal neuropsychological development, with some high or below average scores. For example, a deficit in working memory was identified in Patient 2. This type of deficit is typically related to the management of day-to-day information difficulties. Moreover, children with this deficit can have difficulties planning, ranking, establishing priorities, distinguishing importance, and engaging in activities requiring the manipulation of temporarily stored information. They usually need more time and put forth more effort to carry out tasks. In the school context, it is common to have difficulties in mathematics operations that involve multiple steps, remembering homework instructions and simultaneously processing multiple information sources (50). A decrease in the information processing index was observed in both patients, characterized by a slow visual motor function and diminishing perceptual-motor and attention functions. This index provides a measure of the ability to decode symbols and process new information. It is sensitive to neurological factors, motor maturation, and attentional factors. Thus, children with these deficits require more time to learn the same amount of information than children of their age and are more likely to be tired since additional effort is needed to perform tasks (50).

While it is easy to speculate that the siblings' reduced neurological skills are related to fetal hypothyroidism, it would be premature to make this conclusion. Moreover, prenatal fetal goiter treatment has been proposed to reduce goiter volume, prevent comorbidities, and provide adequate thyroid status at birth, but this therapy remains controversial. A recent study confirmed the feasibility of a conservative intrauterine LT4 treatment; however, the medications (LT4 and/or T3), recommended doses, and administration methods have not been defined. The authors state that prenatal treatment risks and benefits must be considered case by case (29). Notably, a 2020–2021 consensus updated the guidelines for the diagnosis and management of congenital hypothyroidism (CH) (51). They strongly recommended intraamniotic T4 injections in a euthyroid pregnant woman with large fetal goiter related to hydramnios and/or tracheal occlusion.

Considering the long-term medical follow-up, both siblings exhibited good clinical conditions and normal growth. They showed good adherence to LT4 treatment and adequate hormonal status along with the increase of the LT4 dose. Previous studies have also shown normal growth development of fetal goiter patients under adequate treatment (15, 21).

In conclusion, this study discovered a novel compound heterozygous mutation, including a previously unknown deletion in the TPO gene. This mutation led to severe impairment in TPO activity and membrane localization and appeared to be associated with severe intrauterine CH and fetal goiter. Under adequate treatment, the long-term follow-up showed a normal growth progression of both patients. This is also the first report investigating the neuropsychological factors in CH-induced fetal goiter patients. More studies are needed to state the neurodevelopmental outcomes of dyshormonogenetic fetal goiter.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/genbank/, BankIt2432519 human MW660360.

ETHICS STATEMENT

This study was approved by the UNIFESP Ethical Committee (CAAE 59240816.2.0000.5505). Written informed consent

was obtained from a parent of the patients for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

TR was the endocrinologist that diagnosed the fetal goiter in both patients and performed the follow-up. MS performed the mutagenesis and most of the immunofluorescence images. MF performed the DNA sequencing of the patient's DNA. ZD performed the neuropsychological evaluation. VF performed the TPO activity evaluation. MR was responsible for the cell culture and transfection experiments. IR was the coordinator of the project. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2021.671659/full#supplementary-material

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Next-Generation Sequencing Analysis Reveals Frequent Familial Origin and Oligogenism in Congenital Hypothyroidism With Dyshormonogenesis

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Context: Congenital hypothyroidism (CH) is related to dyshormonogenesis in 15% to 40% of the world population and associated with homozygous or heterozygous variants in the main genes of the hormone synthesis pathway. Emerging diagnostic tools, such as next-generation sequencing (NGS), have been used to efficiently explore panels of genes and identify complex mechanisms of pathogenesis.

Objective: We explored 19 candidate genes known to be causative for permanent or transient CH to evaluate the role of complex gene variations in CH phenotype.

Patients, Design and Setting: Using the NGS approach, we studied 65 newborns with thyroid dyshormonogenesis (TDH). New variants were assessed *in silico* for pathogenicity.

Results: Among the 65 infants, 56.9% presented a variant in one or more genes of the thyroid hormone synthesis axis. We identified homozygous or compound heterozygous variants in the *TG*, *DUOX2*, *TPO*, or *SLC5A5* genes in 10 infants and heterozygous variants in *DUOX2*, *TG*, *TPO*, and *TSHR* in 19 others. In seven cases, a heterozygous variant in the *TG* gene was the unique anomaly detected, but related to disturbed hormonal balance. Oligogenic variants were found in eight infants associated with severe CH and goiter in five of them.

Conclusion: The systematic exploration of genes involved in thyroid hormone synthesis by NGS in TDH showed high diagnostic relevance. Oligogenic inheritance could be related to phenotypic heterogeneity and a high frequency of goiter.

Keywords: high throughput molecular screening, familial origin, oligogenicity, congenital hypothyroidism, thyroid dyshormonogenesis

INTRODUCTION

Congenital hypothyroidism (CH) is the most common inborn endocrine disorder detected at birth by mass biochemical newborn screening. Epidemiology for CH has recently changed with an increased incidence close to 1/2,600 in France, mainly related to an increase in CH with thyroid dyshormonogenesis (TDH) representing 37% to 49% of CH cases relative to regional origin (1–3). In the south of France, 47% of CH are due to TDH and 53% to thyroid dysgenesis (TD). Genetic exploration of CH has often been restricted to a small number of genes because of a cost and time-consuming process to explore them by Sanger technology. Apart from a monogenic pattern of inheritance, the role of oligogenicity in disease development remains poorly explored. Recent studies have used next-generation sequencing (NGS) technology for better genotyping of newborns with suspected CH (4–9). Despite differences in the methodological approach, all studies highlighted an increased proportion of familial origin single or multiple pathogenic variants leading to thyroid dyshormonogenesis.

In France, the nationwide neonatal screening program of CH, implemented since 1978, identifies without ethnic *a priori* assumptions all children with high TSH levels. They are explored in accordance with the European Society for Paediatric Endocrinology (ESPE) consensus by biology, ultrasonography, and scintigraphy to distinguish aetiologies between TD and TDH. Analysis of this large and multi-ethnic unselected population could specify the landscape of familial aetiology in TDH development while considering the frequency of transient CH. The present NGS study established a targeted thyroid gene panel in a subset of 65 children screened at birth and confirmed to have CH. The aim of our study was to explore the frequency of complex molecular mechanisms for TDH related to phenotypic heterogeneity. We focused on TDH due to its increased incidence in our region (+7.9% per year) compared to the stable incidence of TD.

PATIENTS AND METHODS

Patients

Sixty-five patients were included in the study (30 females and 35 males); all except one were issued from non-related families living in the Midi-Pyrenees' region (south west of France, 3 million inhabitants). In 10% (7/65), different ethnic origins were present (Turkish, Maghreb countries).

All patients had early hypothyroidism as defined by ESPE consensus guidelines for congenital hypothyroidism screened at birth (regional neonatal screening), confirmed at the age of 10 days, and explored by ultrasonography (10). Secondary to genetic sampling, scintigraphy was performed to confirm aetiologies and for three infants to redirect toward TD (F37 for ectopia and F6, F14 for hypoplasia). Ectopy is a rare event in our population, as only 16% of TD presented ectopia during the same period of this study. For F2 patient, radionuclide scintigraphy revealed severely reduced uptake by the thyroid gland suggesting a functional defect of NIS. Based on serum FT4 levels, CH was defined as severe when FT4 was <5 pmol/L, moderate when FT4 was 5 to 10 pmol/L, and mild when FT4 was 10 to 15 pmol/L. TSH resistance was referred as FT4 in the normal range with a high level of TSH.

Infants were treated from diagnosis by levothyroxine (5–15 μg/kg/d) and re-evaluated after two or three years as proposed by consensus: 44 (67%, 44/65) were confirmed as persistent TDH; the 21 remaining infants had transient CH, but for seven of them, the biological profile showed persistent high levels of TSH and normal FT4. Relative to FT4 values, treatment was stopped for infants with transient CH. For 27 children (41.5%, 27/65), clinical goiter was suspected and confirmed by ultrasonography.

For all families except five (F22, F31, F33, F36, and F 37), samples were obtained from family members to check for inheritance and co-segregation with phenotype. Written informed consent for patients or relatives was obtained for NGS and/or Sanger analyses and data were included in a biocollection (number: DC-2015-2450).

DNA Extraction and Sequencing

Genomic DNA was isolated from peripheral blood using the QIAamp DNA blood kit according to the manufacturer's instructions (Qiagen, Hilden, Germany). A total of 10 ng of DNA per sample was used for library preparation, using the DNAprep with enrichment protocol (Illumina; San Diego, CA) and a custom NGS panel including in 19 thyroid-related genes (**Supplemental Table 1**). Sequencing was performed on the Nextseq550 platform (Illumina) using the Mid Output kit v2.5. For a sequence variant to be considered valuable, a sequencing coverage of 30× reads was used as a minimum requirement in the present study. Sequence data were processed using custom bioinformatic software and compared to NextGENe independent analysis (Softgenetics, State College, PA, USA) to align reads to the HG19 reference genome. Copy number variants (CNVs) were detected using the COV'COP tool (11) and confirmed by MLPA analysis (MRC-Holland, Amsterdam, Netherlands). All reported variants, described using the HGVS nomenclature, were explored using public and license databases, including HGMD professional, gnomAD, and ClinVar, as well as literature searches. Minor allele frequency <5% was considered. The majority of variants were rated according to the American College of Medical Genetics and Genomics (ACMG) guidelines (12) and VarSome ACMG implementation (http://varsome.com). For variants with no pathogenic reporting, we classified them based on allele frequency in corresponding ethnic population, cosegregation with thyroid balance within the upper limit (TSH value) for the carrier parent and non-published data. Amino acid predictions were performed using the MutationTaster (http://www.mutationtaster.org), SIFT (http://sift.jvci.org), and PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2) software tools and additional supporting evidence for pathogenicity was stated for variants of uncertain significance (VUS). We considered variants as deleterious when concordant annotations by two of these software tools using different algorithms were present. For intronic variants close to the splicing site, we used the ESEFinder2.0 software tool.

Variants detected by the Illumina platform were confirmed by targeted Sanger sequencing on an ABIXL3500 apparatus using the BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems; Thermo Fisher Scientific, Inc.).

The data for this study have been deposited in the European Nucleotide Archive (ENA) at EMBL-EBI under accession number PRJEB42793.

Statistical Analysis

The statistical significance was assessed using the Mann-Whitney *U* test. Differences were considered significant at $P < 0.05$. All analyses were performed using StatView, version 5.0 (SAS Institute, Gary, NC, USA).

RESULTS

Spectrum of Genetic Variants

Among the 65 children, 37 (56.9%) presented a constitutive variant from different ACMG classes: 3 (VUS), 4 (probably pathogenic), and 5 (pathogenic) in one or two genes of the thyroid hormone biosynthesis pathway, according to VarSome results and, for some variants, to our own pathogenicity evidence as referred in *Patients and Methods* section. Functional studies will ultimately be required to prove the pathogenicity of novel variants. We showed that for the majority of patients (78.4%; 29/37), variants belonging to classes 4 and 5 have been previously reported in databases and/or in the literature. Biallelic variants in *TG*, *TPO, DUOX2,* and *SLC5A5* genes were found in 27% (10/37) of patients, whereas monoallelic variants in *TSHR*, *TPO, DUOX2,* and *TG* were identified in 51.3% (19/37). Oligogenism was identified for 21.6% (8/37) of the infants, mainly involving variants in the *TG* and *TPO* or *DUOX2* genes, except for homozygotic twins (F32, *DUOX2/TPO*). Considering mono or biallelic variants in a unique gene independently of oligogenic inheritance (29/37), the *TG* gene was most commonly associated with TDH in our cohort (37.9%; 11/29), followed by a fairly equivalent percentage of variants in the *TPO* (24.1%; 7/29) and *DUOX2* (20.7%; 6/29) genes. For *TG*, six variants (F1, F4, F24, F28, F31, and F33) led to truncated TG before or in the carboxy-terminal acetyl cholinesterase-like domain, which is crucial for conformation and intracellular trafficking (13, 14).

According to ACMG class 3, VUS corresponded to monoallelic variants in the *TG* (F3, F4, F9, F13, and F29), *TPO* (F27), and *DUOX2* (F9) genes. For F3 and F4, VUS in *TG* were associated with pathogenic *TG* variants. For F9, two VUS were associated (in *TG* and *DUOX2*) and related to neonatal goiter. The latest two VUS in the *TG* gene (F13, F29) were related to moderate CH in a 27-week premature girl without goiter (F13) and to mild CH in a child presenting with psychomotor delay (F29). Finally, for F27, the splicing variant was related to homozygous deletion of exon 17 of the *TPO* gene, confirmed by MLPA analysis (data not shown).

We did not detect any variants in the *DUOX1*, *DUOXA1,* and *GNAS1* genes, according to the low frequency of these events in familial TDH (15, 16). No pathogenic variants in other genes (coding and non-coding regions) explored in our study and related to dysgenesis and dysthyroidism were identified.

Genotypical data and inheritance for the 37 children positive for ACMG classes 3 to 5 of the constitutive variants are shown in **Table 1**.

Phenotype-Genotype Correlation

Among the 37 children presenting constitutive variants, 21/37 (55.5%) were male with an increased male/female sex ratio of 1.31 (21/16) compared to the global sex ratio of 1.18 (35/30) or to that of the NGS negative group of 1.15 (15/13). No extra-thyroidal phenotype has been noticed for all the children. Goiter was significantly associated with positive cases (56.7%, 21/37) compared to infants negative for variants but with proven CH, (14.2%, 4/28). Goiter was also significantly associated with homozygous, compound heterozygous and oligogenic variants regardless of the severity of CH (94% 17/18). Types of variants related to CH severity and goiter for the 37 infants are presented in **Figure 1**.

Pathogenic variants or VUS were associated with permanent TDH for 89.1% (33/37) and with transient hypothyroidism in 10.8% (4/37), all of the latter associated with *DUOX2* variants. For 75% of the *TSHR*-positive cases (F6, F14, F15, F34), no goiter was detected, but hypoplasia was noticed in two infants after scintigraphy. Two cases corresponded to the same heterozygous variant Arg450His with a difference in the biological spectrum from TSH resistance for F6 to severe hypothyroidism for F15.

Comparing patient phenotypes using the ESPE criteria for CH severity based on FT4 levels, biallelic and oligogenic variants were associated with severe CH (73.3%, 11/15) and goiter 90.9%, 10/11), while heterozygous variants in the *TG*, *TPO, DUOX2,* and *TSHR* genes were associated with infants presenting moderate and mild CH (63.2%, 12/19) with a low risk of goiter (25%, 3/12).

We also identified a new homozygous deletion in the TPO gene in one infant (F27) presenting moderate CH with goiter. Among the diverse splicing mRNA TPO variants already described, none were related to the unique absence of exon 17 (17). Even if the structure of TPO remains to be determined, a recent modelling study suggested that homozygous loss of the intracellular domain of this enzyme is responsible for cell trafficking and could impact the conformation of its active site (18).

Exploring for the role of heterozygous *TG* VUS in CH (F13 and F29), we showed that for F13 with CH in a 27-week premature girl, the mother presented with clinical adult-onset goiter and moderate hypothyroidism associated with the same heterozygous variant in the *TG* gene. For F29, the father of a boy with mild CH had a history of hypothyroidism and goiter with adult onset, related to the same VUS. Clinical and inheritance data for the 37 infants are shown in **Table 2**. Clinical and biological data for the 28 remaining infants without identified variant are listed in **Supplemental Table 2**.

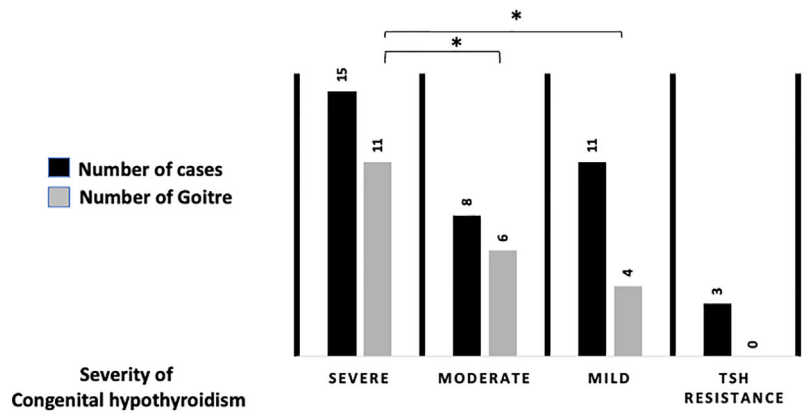
DISCUSSION

Early TDH diagnosis and treatment are essential for normal brain development and physical growth, but genetic and carrier

TABLE 1 | Genotypical data and inheritance for the 37 children diagnosed for TDH.

ID	Genes	exon/Intron	Nucleotide	Protein (zygosity)	dbSNP number	gnomAD MAF	Pathogeny	Maternal Genotype	Paternal Genotype	SIFT score	Polyphen-2 score	MutationTaster prediction	Improved classification according to ACGM guidelines	Causative ACGM level	HGMD- First report
F11	*DUOX2*	E22	c.2895_2898del	p.Phe966Serfs*29 (Het)	rs530719719	0.002	P	WT	Het	0	1	Disease-causing	PVS1+PP3+PP5	5	Moreno, 2002, N Engl J Med
F12	*DUOX2*	E22	c.2895_2898del	p.Phe966Serfs*29 (Het)	rs530719719	0.002	P	Het	WT	0	1	Disease-causing	PVS1+PP3+PP5	5	Moreno, 2002, N Engl J Med
F21	*DUOX2*	E22	c.2895_2898del	p.Phe966Serfs*29 (Het)	rs530719719	0.002	P	Het	WT	0	1	Disease-causing	PVS1+PP3+PP5	4	Moreno, 2002, N Engl J Med
F30	*DUOX2/DUOX2*	E5	c.498C>A	p.Ser166Arg (Homo)	rs370438048	0.0001	LP	Het	Het	0	1	Disease-causing	PM1+PM2+PP3+PP4	4	N/A
F36	*DUOX2*	I26	c.3515+5 G>T	p.? (Het)	rs375943962	0.00	LP	N/A	N/A	0	1	Disease-causing	PM2+PP3+PP4+PP5	4	N/A
F37	*DUOX2*	E20	c.2635G>A	p.Glu879Lys (Het)	rs774556391	0.00	LP	N/A	N/A	0	1	Disease-causing	PM1+PM2+PP3+PP5	4	Maruo, 2008, J Clin Endocrinol Metab
F32-1	*DUOX2/TPO*	E29 (*DUOX2*) E8 (*TPO*)	c.3799C>T c.1199T>A	p.Arg126Trp (Het oligo) p.Val400Asp (Het oligo)	rs752437461 N/A	0.00 N/A	LP LP	Het WT	WT Het	0 0	1 1	Disease-causing Disease-causing	PM1+PM2+PP3+PP5 BP1+PM2+PP3+PP4+PP5	4 4	Wang, 2014,Clin Endocrinol N/A
F32-2	*DUOX2/TPO*	E29 (*DUOX2*) E8 (*TPO*)	c.3799C>T c.1199T>A	p.Arg126Trp (Het oligo) p.Val400Asp (Het oligo)	rs752437461 N/A	0.00 N/A	LP LP	Het WT	WT Het	0 0	1 1	Disease-causing Disease-causing	PM1+PM2+PP3+PP5 BP1+PM2+PP3+PP4+PP5	4 4	Wang, 2014,Clin Endocrinol N/A
F2	*SLC5A5/ SLC5A5*	E13	c.1593C>G	p.Tyr531* (Homo)	rs121909177	0.00	P	Het	Het	0	1	Disease-causing	PVS1+PM2+PP3+PP5	5	Pohlenz, 1998, J Clin Invest
F13	*TG*	E21	c.4481C>T	p.Pro1494Leu (Het)	rs146498231	0.0008	VUS	Het	WT	0	0.75	Disease-causing	BP1+PM2+PM3	3	Makretskaya, 2018,PLoS One
F16	*TG*	I30	c.5686+1G>A	p.? (Het)	rs374620255	0.00	P	Het	WT	0	0.9	Disease-causing	PVS1+PM2+PP3	5	Hermanns, 2013, J Pediatr Endocrinol Metab
F18	*TG*	E29	c.5485C>T	p.Arg1829Trp (Het)	rs148982115	0.0002	LP	WT	Het	0	0.9	Disease-causing	PM2+PP3+PP4+PP5	4	N/A
F24	*TG*	E11	c.2787del	p.Lys929Asnfs*37 (Het)	N/A	N/A	LP	WT	Het	0	1	Disease-causing	PVS1+PM2	4	N/A
F29	*TG*	E44	c.7640T>A	p.Leu2547Gln (Het)	rs2979042	0.0001	VUS	WT	Het	0	0.95	Disease-causing	BP1+PP3+PP4+PP5	3	Nicholas, 2016, J Clin Endocrinol Metab
F31	*TG*	E22	c.4588C>T	p.Arg1530* (Het)	rs121912646	0.0001	P	N/A	N/A	0	1	Disease-causing	PVS1+PM2+PP3+PP5	5	Targovnik, 1993,J Clin Endocrinol Metab
F33	*TG*	E30	c.5676G>A	p.Trp1892* (Het)	N/A	N/A	P	N/A	N/A	0	1	Disease-causing	PVS1+PM2+PP3	5	N/A
F10	*TG/DUOX2*	E9 (*TG*) E31 (*DUOX2*)	c.1958G>A c.4156G>A	p.Gly653Asp (Het oligo) p.Gly1386Ser (Het oligo)	rs2069548 rs139584933	0.010 0.0001	LP LP	WT Het	Het WT	0 0	0.99 1	Disease-causing Disease-causing	PM2+PP3+PP4+PP5 PM2+PP3+PP4+PP5	4 4	de Filippis, 2017, Hum Mol Genet Tan, 2016, Horm metab Res
F20	*TG/DUOX2*	E3 (*TG*) E31 (*DUOX2*)	c.199G>A c.2895_2898del	p.Gly67Ser (Het oligo) p.Phe966Serfs*29 (Het oligo)	rs116340633 rs530719719	0.010 0.002	P P	Het WT	WT Het	0 0	1 1	Disease-causing Disease-causing	BP1+PP3+PP4+PP5 PVS1+PP3+PP5	3 5	de Filippis, 2017, Hum Mol Genet Moreno, 2002, N Engl J Med
F7	*TG/DUOX2*	E10 (*TG*) E20 (*DUOX2*)	c.2381G>T c.2597T>G	p.Gly794Val (Het oligo) p.Met866Arg (Het oligo)	rs1180105954 rs200948626	0.00 0.0001	LP LP	Het WT	WT Het	0 0	1 1	Disease-causing Disease-causing	PP1+PP3+PP4+PP5 PM1+PM2+PP3+PP4+PP5	4 4	N/A Muzza, 2013, J Clin Endocrinol Metab
F9	*TG/DUOX2*	E27 (*TG*) E25 (*DUOX2*)	c.5299_5301del c.3367G>A	p.Asp1767del (Het oligo) p.Ala1123Thr (Het oligo)	rs112749206 rs113632824	0.0003 0.0001	VUS VUS	Het WT	WT Het	0 0.09	1 1	Disease-causing Disease-causing	BP1+PP3+PP4+PP5 BP1+PP3+PP4+PP5	3 3	N/A Kim, 2013,Int J Ped Endocrinol
F1	*TG/TG*	E47	c.8131A>T	p.Lys2711* (Homo)	N/A	N/A	P	Het	Het	0	0.99	Disease-causing	PVS1+PM2+PP3+PP5	5	N/A
F28	*TG/TG*	E7	c.886C>T	p.Arg296* (Homo)	rs121912648	0.0004	P	Het	Het	0	1	Disease-causing	PVS1+PM2+PP3+PP5	5	Van de Graaf, 1999, J Clin Endocrinol Metab
F3	*TG/TG*	E3	c.229G>A	p.Gly77Ser (Comp Het)	rs1422698837	0.0007	VUS	Het	WT	0	1	Disease-causing	BP1+PP3+PP4+PP5	3	Van de Graaf, 1999,J Clin Endocrinol Metab
		E41	c.7123G>A	p.Gly2375Arg (Comp Het)	rs137854434	0.0002	LP	WT	Het	0	1	Disease-causing	BP1+PM2+PP3+PP4+PP5	4	Van de Graaf, 1999, J Clin Endocrinol Metab
F4	*TG/TG*	E3	c.229G>A	p.Gly77Ser (Comp Het)	rs1422698837	0.0007	VUS	WT	Het	0	1	Disease-causing	BP1+PP3+PP4+PP5	3	Van de Graaf, 1999, J Clin Endocrinol Metab
		E4	c.416G>A	p.Trp139*(Comp Het)	rs141306917	0.0001	P	Het	WT	0	1	Disease-causing	PVS1+PM2+PP3+PP5	5	Hishinuma, 2006, J Clin Endocrinol Metab
F23	*TG/TPO*	E9 (*TG*) E13 (*TPO*)	c.1958G>A c.2386G>A	p.Gly653Asp (Het oligo) p.Asp796Asn (Het oligo)	rs2069548 rs773759871	0.015 0.00	LP P	Het WT	WT Het	0 0	0.99 1	Disease-causing Disease-causing	PM2+PP3+PP4+PP5 PVS1+PM1+PM2+PM5 +PP3	4 5	de Filippis, 2017, Hum Mol Genet N/A
F8	*TG/TPO*	E10 (*TG*) E14 (*TPO*)	c.2610G>T c.2513G>A	p.Glu870His (Het oligo) p.Cys838Tyr (Het oligo)	rs2229843 N/A	0.0042 N/A	LP LP	WT Het	Het WT	0 0	0.96 0.99	Polymorphism Disease-causing	PM2+PP3+PP4+PP5 PM1+PM2+PP3+PP4	4 4	N/A N/A
F22	*TPO*	E9	c.1399G>A	p.Val467Met (Het)	rs373267637	0.0002	LP	N/A	N/A	0.01	0.94	Polymorphism	PM2+PP3+PP4+PP5	4	N/A
F25	*TPO*	E9	c.1399G>A	p.Val467Met (Het)	rs373267637	0.0002	LP	WT	Het	0.01	0.94	Polymorphism	PM2+PP3+PP4+PP5	4	N/A
F5	*TPO*	E14	c.2422del	p.Cys808Alafs*24 (Het)	rs763662774	0.0001	P	WT	Het	0	1	Disease-causing	FVS1+PM2+PP3+PP5	5	Bakker, 2000, J Clin Endocrinol Metab
F19	*TPO/TPO*	E11 E14	c.1978C>G c.2395G>C	p.Gln660Glu (Comp Het) p.Glu799Gln(Comp Het)	rs121908088 N/A	0.0002 N/A	P LP	Het WT	WT Het	0 0	1 1	Disease-causing Disease-causing	BP1+PM2+PP3+PP5 BP1+PM1+PM2+PM5 +PP3	5 4	Santos, 1999, Clin Endoc Bikker, 1995, Hum Mut
F26	*TPO/TPO*	E8	c.1197G>C	p.Ser398Thr (Homo)	N/A	N/A	P	Homo	WT	0	0.94	Disease-causing	BP1+PM2+PM3+PP3+PP5	5	N/A
F27	*TPO/TPO*	E17	c.2749-2_2802del	p.? (Homo)	N/A	N/A	VUS	Het	Het	0	1	Disease-causing	BP1+PP3+PP4+PP5	3	N/A
F35	*TPO/TPO*	E5	c.387del	p.Asn129Lysfs*80 (Homo)	rs766399662	0.00	P	Het	Het	0	1	Disease-causing	FVS1+PM2+PP3+PP5	5	Rivolta, 2003, Hum Mut
F14	*TSHR*	E2	c.202C>T	p.Pro68Ser (Het)	rs142063461	0.0004	P	Het	WT	0	1	Disease-causing	BP1+PM2+PP3+PP4+PP5	5	Tenenbaum-Rakover, 2009, J Clin Endocrinol Metab
F15	*TSHR*	E11	c.1349G>A	p.Arg450His (Het)	rs189261858	0.0003	P	Het	WT	0	1	Disease-causing	BP1+PM2+PP3+PP4+PP5	5	Tenenbaum-Rakover, 2015, Thyroid
F34	*TSHR*	E11	c.1612G>A	p.Ala538Thr (Het)	rs151264748	0.00	LP	WT	Het	0	1	Disease-causing	BP1+PM2+PP3+PP4	4	N/A
F6	*TSHR*	E11	c.1349G>A	p.Arg450His (Het)	rs189261858	0.0003	P	Het	WT	0	1	Disease-causing	BP1+PM2+PP3+PP4+PP5	5	Tenenbaum-Rakover, 2015, Thyroid

WT, wild type; homo, homozygous; Het, heterozygous; Comp Het: compound heterozygous; Het Oligo, oligogenism; P, pathogenic; LP, likely pathogenic; VUS, variant of uncertain significance; TG, thyroglobulin; TPO, thyroperoxidase; TSHR, TSH receptor; SLC5A5, sodium/iodide symporter (NIS); DUOX2, dual oxidase 2; N/A, not applicable.



Homozygous	5	4	1	1	1	1		
Compound Heterozygous	1	1	2	2				
Oligogenism	5	5	1	1	2	2		
TSHR heterozygous	1		1	1			2	
TPO heterozygous					2		1	
TG heterozygous	1		3	1	3			
DUOX2 Heterozygous	2	1			3	1		

FIGURE 1 | Phenotype/genotype correlation for the 37 patients according to severity of Congenital Hyperthyroidism (ESPE guidelines), NGS results and related to type and number of variants. *p < 0.05.

identification encourage familial counselling and can allow for delaying the decision to treat for 3 years. Over the past two decades, the increased incidence of TDH associated with the high heterogeneity of its biological spectrum has been investigated. Relative to the technical limitations of direct sequencing for diagnosis, recent studies have demonstrated the efficiency of next-generation sequencing technology for CH screening (4, 6, 8, 19, 20). Our study exploring 65 patients identified by the French neonatal screening program in one reference centre showed that, for predominantly non-consanguineous families of different ethnic origins, familial TDH was frequent in France (56.9%). Irrespective of biological CH severity, we also observed that goiter was mainly associated with familial TDH (56.7%) compared to infants negative for ACMG variant classes 3 to 5, as a relevant clinical sign to ask for a genetic investigation. As previously described, we identified that homozygous variants in *TG*, *TPO,* and *SLC5A5* and oligogenicity were associated with goiter and severe CH whereas monoallelic variants in *TSHR*, *TG*, *DUOX2,* and *TPO* were related to milder phenotypes (21, 22). Inactivating heterozygous TSHR variants were present in 10.8% (4/37 cases) of our TDH cases with a heterogeneous clinical presentation: CH severity was inconstant, especially for the hot spot variant Arg450His leading to both subclinical (F6) and severe CH (F15). These results were in accordance with previous recommendations for systematic molecular analysis of the *TSHR* gene for CH with TSH resistance presentation (23). Thus, genetic exploration of TDH should be now considered as necessary as that for thyroid dysgenesis after neonatal screening.

In our study, monoallelic *DUOX2* variants were as frequent as monoallelic *TPO* variants, but were also more frequently involved through an oligogenic mechanism. *DUOX2* variants are associated with high phenotypic variability from transient to permanent CH and sometimes there is a difference in CH severity for the same variant (24). We observed the same variability from severe to mild or transient CH for infants in our study without a convincing hypothyroidism being found in the carrier parent. The presence of two DUOX isoforms could constitute an efficient redundant mechanism to maintain sufficient H_2O_2 supply for iodide organification. Thus, transient CH could be related to the difference in thyroid hormone requirements from the neonatal period to adulthood with sufficient H_2O_2 supply by DUOX1 only after the infantile period (5, 25, 26).

The contribution of oligogenic variants was frequent (21.6%) in our study, with diverse CH severity. Our results are consistent with the recent study of de Filippis and colleagues presenting frequent oligogenic inheritance (26.2%) in CH patients with either gland-in-situ or dysgenesis (6). In such cases, heterozygous variants in several genes can lead to cross-loss of enzyme activity (27, 28). Thus, monoallelic variants in the *TG* gene suggest that the oligogenic mechanism could be related to previous questionable CH cases with apparently unique heterozygous variants in the *TG* gene on direct sequencing (29). By exploring oligogenic inheritance, we showed that this mechanism was responsible for TDH with a significant association with severe CH and goiter. This oligogenic model could also explain differences in the penetrance and expressivity of CH in some families.

Our NGS design was able to detect CNV, intronic and proximal regulatory variants in the chromosomal region of the

TABLE 2 | Clinical and biological data for the 37 children diagnosed for TDH.

ID	Sex	Serum TSH (confirmation)	Serum FT4	Thyroid Evaluation	CH Severity	Zygosity	Gene	Permanent/ transient
F11	F	109	3.4	Goitre	Severe	Het	*DUOX2*	Transient
F12	F	92	12	Eutopic	Mild	Het	*DUOX2*	Transient
F21	M	74	15	Eutopic	Mild	Het	*DUOX2*	Transient
F30	M	>300	3.4	Goitre	Severe	Homo	*DUOX2/DUOX2*	Permanent
F36	F	76	13	Goitre	Mild	Het	*DUOX2*	Transient
F37	F	640	2.6	Ectopia	Severe	Het	*DUOX2*	Permanent
F32-1	M	227	3.5	Goitre	Severe	Oligo	*DUOX2/TPO*	Permanent
F32-2	M	327	3.5	Goitre	Severe	Oligo	*DUOX2/TPO*	Permanent
F2	M	>150	1.7	Goitre	Severe	Homo	*SLC5A5/ SLC5A5*	Permanent
F13	F	12	9.1	Eutopic	Moderate	Het	*TG*	Permanent
F16	M	>100	4.4	Eutopic	Severe	Het	*TG*	Permanent
F18	M	12	7	Eutopic	Moderate	Het	*TG*	Permanent
F24	M	150	5.6	Goitre	Moderate	Het	*TG*	Permanent
F29	M	34	14.3	Eutopic	Mild	Het	*TG*	Permanent
F31	M	26	14.2	Eutopic	Mild	Het	*TG*	Permanent
F33	F	95	11.2	Eutopic	Mild	Het	*TG*	Permanent
F10	M	128	7	Goitre	Moderate	Oligo	*TG/DUOX2*	Permanent
F20	F	300	4.4	Goitre	Severe	Oligo	*TG/DUOX2*	Permanent
F7	M	13	15	Goitre	Mild	Oligo	*TG/DUOX2*	Permanent
F9	M	62	10	Goitre	Mild	Oligo	*TG/DUOX2*	Permanent
F1	F	>500	4.3	Goitre	Severe	Homo	*TG/TG*	Permanent
F28	M	>180	3.7	Goitre	Severe	Homo	*TG/TG*	Permanent
F3	M	N/A	8.6	Goitre	Moderate	HetComp	*TG/TG*	Permanent
F4	M	173	5.1	Goitre	Moderate	HetComp	*TG/TG*	Permanent
F23	M	>100	1.6	Goitre	Severe	Oligo	*TG/TPO*	Permanent
F8	F	700	1.2	Goitre	Severe	Oligo	*TG/TPO*	Permanent
F22	F	N/A	11.9	Eutopic	Mild	Het	*TPO*	Permanent
F25	F	29	13.6	Eutopic	Mild	Het	*TPO*	Permanent
F5	M	13	18.2	Eutopic	TSH resistance	Het	*TPO*	Permanent
F19	F	>100	2	Goitre	Severe	HetComp	*TPO/TPO*	Permanent
F26	F	>150	2.9	Eutopic	Severe	Homo	*TPO/TPO*	Permanent
F27	F	N/A	8.7	Goitre	Moderate	Homo	*TPO/TPO*	Permanent
F35	M	24.2	12.6	Goitre	Mild	Homo	*TPO/TPO*	Permanent
F14	F	44	20.6	Hypo	TSH resistance	Het	*TSHR*	Permanent
F15	M	447	2.2	Eutopic	Severe	Het	*TSHR*	Permanent
F34	F	61	8.6	Goitre	Moderate	Het	*TSHR*	Permanent
F6	M	50	16.6	Hypo	TSH resistance	Het	*TSHR*	Permanent

Severity of disease is defined on FT4 levels at the age of 10 days according to ESPE consensus criteria in mild, moderate or severe (respectively in clear, gray, or black); severe, < 5 pmol/L; moderate, 5–10 pmol/L; mild, 10–15 pmol/L. Serum TSH at confirmation, IU/ml; FT4, pmol/L.

Hom, homozygous; Het, heterozygous; HetComp, compound heterozygous; Oligo, oligogenism; TG, thyroglobulin; TPO, thyroperoxidase; TSHR, TSH receptor; SLC5A5, sodium/iodide symporter (NIS); DUOX2, dual oxidase 2; N/A, not applicable.

genes explored. None of these anomalies was detected in our families. This modern genetic testing has demonstrated that complex mechanisms can be frequently involved in the phenotypic heterogeneity of CH, even if the presence of additional variants on non-explored chromosomes or epigenetic regulation could not be excluded (30). We recommend increasing the diagnostic efficiency of CH by screening a large panel of genes involved in thyroid genesis, hormone synthesis and action from the TDH diagnosis. In accordance with recent recommendations from the ENDO-European Reference Network (30), we confirm that targeted-NGS or WES analyses for documented families should be used to improve CH diagnosis and treatment. Thus, except for the *DUOX2* heterozygous variant, infants with TDH and positive for deleterious variants did not need a diagnostic re-evaluation between two- and three-years age.

DATA AVAILABILITY STATEMENT

The data for this study have been deposited in the European Nucleotide Archive (ENA) at EMBL-EBI under accession number PRJEB42793.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by DC-2015-2450 Biological Resource Center of Toulouse Hospital. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

All authors provided contributions to study conception and design, acquisition and interpretation of data, and drafting the article. Most contributions in design of the study: IO-P and FS. Data collection: IO-P, TE, MB, SG and AC. Analysis of data: FS and VJ. Manuscript writing: IO-P and FS. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2021.657913/full#supplementary-material

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Congenital Hypothyroidism and the Deleterious Effects on Auditory Function and Language Skills: A Narrative Review

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Congenital hypothyroidism (CH) is an endocrine disease commonly found in newborns and is related to the absence or reduction of thyroid hormones (THs), which are essential for development since intrauterine life. Children with CH can develop hearing problems as THs are crucial for the auditory pathway's development and maturation. Sensory deprivations, especially in hearing disorders at early ages of development, can impair language skills, literacy, and behavioral, cognitive, social, and psychosocial development. In this review we describe clinical and molecular aspects linking CH and hearing loss.

Keywords: thyroid, congenital hypothyroidism, hearing loss, auditory system, hypothyroidism

INTRODUCTION

The development of auditory pathways depends on the presence of adequate serum levels of thyroid hormones (TH) and their action on TH receptors (1–3). These hormones regulate proteins and enzymes responsible for the structural formation of the inner ear, being crucial for the proper performance of auditory function (4).

In fact, TH deficiency or TH defect action can cause severe changes in the development of the auditory system (2, 5). Clinical situations of reduction or absence of normal serum levels of TH, such as congenital hypothyroidism (CH), are frequently associated with hearing loss (6, 7). However, the incidence of hearing loss (HL) in individuals with CH is still uncertain, and it could affect ~20% of patients (8–10), occurring isolated or associated with vertigo and tinnitus (11).

It is well-known that when sensory deprivation events occur in the first months of life, a period considered critical for the maturation of biological functions, there is a high potential for subsequent

Abbreviations: CH, congenital hypothyroidism; T_4, Hormone thyroxine; hypothalamic TRH, Thyrotropin-Releasing Hormone; NNSP, National Neonatal Screening Program; NS, Newborn screening; PAX8, Paired box gene 8; *SECISBP2*, Selenocysteine-insertion sequence binding protein 2; T_3, Triiodothyronine; TH, thyroid hormones; TSH, Thyroid Stimulating Hormone; TR, Thyroid hormone receptor; *THRα*, thyroid hormone receptor alpha; THRβ, Thyroid hormone receptor beta; TSHR, Thyroid Stimulating Hormone receptor; DIO2, Type 2 deiodinase; OHC, Outer hair cells.

significant delays in language, cognition, academic, emotional, and social development (12, 13). Therefore, the early detection and intervention of hearing problems, even in subclinical stages, allow individuals with auditory dysfunction to obtain sociolinguistic performances close to normal hearing (12, 14).

Given these facts and considering the scarcity of literature on the subject, the present study sought to achieve a narrative review on the probable dysfunctions of the auditory pathways connected to CH and TH deprivation in early neonatatal period, and its adverse impacts on social performance and language acquisition and development.

Clinical Aspects of Congenital

CH is an endocrine disease commonly found in newborns (15) with a worldwide occurrence of approximately 1:2,000 to 1:4,000 live births. It mainly affects females in the proportion of roughly 2:1 (16). CH is a public health issue, which can be detected with newborn screening (NS). The lack of an early diagnosis and adequate treatment can result in neurological and motor development changes and irreversible mental retardation (17). The main objective of neonatal screening is to promote the detection of congenital diseases before the symptomatic phase, enabling early treatment (18). CH does not usually present symptoms at birth, or there are only subtle manifestations of the disease, making clinical diagnosis difficult (19). The neonatal screening program recommends a TSH cut-off level of 10 mUI/L (7, 20). Newborns with high TSH in the test are referred for evaluation and confirmation of the diagnosis (21). Confirmation of the diagnosis of CH is made with laboratory exams, showing TSH greater than 15 mUI/L and total or free T_4 with normal or low values (7, 17).

CH is normally classified as permanent or transient, whose etiology is classified into primary, secondary, and tertiary. The permanent condition requires lifelong treatment, as the hormonal deficiency is persistent. On the other hand, transient CH regains typical TH production in the initial months or years of life. In permanent primary cases, thyroid dysgenesis (TD) corresponds to 85% of cases, whereas dyshormonogenesis (DH) represents 10 to 15% of cases (22). In secondary CH, the lesion is in the pituitary; and in the tertiary form, the dysfunction is in the hypothalamus. The last two cases are extremely rare. Central CH (secondary or tertiary) is commonly associated with other pituitary hormonal deficiencies (15, 17). The absence of stimuli from pituitary TSH (Thyroid Stimulating Hormone) or hypothalamic TRH (Thyrotropin-Releasing Hormone) is the cause of deficient hormone production in the central CH (23).

The etiology and clinical phenotype of CH become essential in determining the severity, outcomes, and treatment of the disease, as patients may need therapy with higher doses and close monitoring, especially during early periods of life (24). THs are essential for adequate neurodevelopment since intrauterine life (25). Their absence leads to dysfunction of specific brain areas, affecting regions such as the posterior parietal, inferior temporal lobes, caudate nucleus, and hippocampus, which are responsible for, respectively, spatial location, object identification, attention, and memory (26, 27).

Action of Thyroid Hormones on Auditory Function

THs play an essential role in developing the inner ear during the embryonic period (28). Since the fetal period, the T_3 is essential for auditory development, when the embryo in the first trimester depends exclusively on maternal THs, beginning its hormonal synthesis in the second half of the gestational period (2, 3). Triiodothyronine (T_3) is mediated by the thyroid hormone receptor (THR), whose action on cochlear sensory cells is caused by the differential expression of thyroid hormone receptor alpha (THRα) and thyroid hormone receptor beta (THRβ) (21).

In the rodent cochlea, THRs are expressed in the sensory epithelium and other tissues from mid-gestation into the postnatal period and function as transcription factors playing important roles in control target genes relevant for auditory development and function, and the abnormal regulation of genes controlled by THRs has been assumed to be the origin of neurosensory deafness associated with CH (29).

The *THRα* gene is widely expressed throughout the spiral organ of corti, while the *THRβ* gene has its expression prominently in the greater epithelial ridges of sensitive hair cells (5, 30, 31). This gene expression pattern points out that the spiral organ is a direct-action site for TH and explains the scientific evidence of morphological and functional abnormalities of the structures that form the cochlea in cases of hypothyroidism (28, 32–37). Indeed, the THRs' expression is timely coordinated in order to have a very precise signaling necessary for proper THR-dependent differentiation events, comprising complete inner sulcus, sensory epithelium, spiral ganglion, cochlea, and auditory nerve maturation (38).

Table 1 summarizes mouse models of TH action or production defects. Actually, THRα1 is considered non-essential for hearing, while defects on THRβ, in mice, present deafness linked to cochlear alterations. On animal models, THRβ-null mice show threshold elevations ranged from a few decibels to complete loss of auditory responsiveness. An isoform-specific importance ranking is observed, because only THRβ1 signaling defect is associated with retardation in the expression of the fast-activating potassium conductance in inner hair cells, whereas deletion of the THRβ2 isoform does not lead to anormal cochlear function (38).

Nonetheless, deletion of both THRβ1 and THRα1 produces exacerbated defects that simulate those provoked by hypothyroidism (38). In reality, human genetic alterations associated with loss of TRβ function, a condition named resistance to TH, also result in deafness (39).

The critical developmental time period of the middle and inner ears occurs in parallel to the natural elevation of TH serum plasma levels. Thyroxine (T_4), liberated by the thyroid gland into the circulation, must be metabolically activated or inactivated by iodothyronine deiodinases, and 3,5,3'-triiodothyronine (T_3) is the main ligand of the THRs. Therefore, TH adequate intracellular levels are accomplished after action of deiodinase type 2 (D2) and deiodinase type 1 (DI) encoded by Dio2 and Dio1, respectively (29).

TABLE 1 | Mouse models for understanding the relevance of genes involved in thyroid development, hormone biosynthesis, and thyroid hormone action on hearing function.

Gene	Molecular mechanism	Thyroid phenotype	Hearing function
Pax8$^{-/-}$	Inactivation of the *Pax8* gene	CH, Athyreosis	Deafness, degeneration of outer hair cells Deafness, sensorineural hearing loss
Tshr$^{hyt/hyt}$	Autosomal recessive mutation in the TSHR gene	CH, Thyroid hypoplasia	Deaf-mutism, abnormality in the outer hair cell morphology Deafness, sensorineural hearing loss
TRβ$^{-/-}$	Inactivation of the *TR*β gene	Resistance to thyroid hormone	Deafness, sensorineural hearing loss
TRβ$^{tm1/tm1-}$	*TR*β gene point mutation reducing the affinity of TR to TH		Deafness
TRα1$^{-/-}$*β*$^{-/-}$	Compound *TRα1 and β genes*	Resistance to thyroid hormone	Deafness
SECISBP2	Gene indirectly disrupt T3 signaling by inhibiting translation of deiodinases		Hearing loss Otitis media
SLC26A4	Gene codifica o transportador de ânions Pendrin.	Goiter Pendred syndrome Defective organification of iodide in the thyroid gland	Non-syndromic deafness Sensorineural hearing loss Enlarged vestibular aqueduct in the inner ear
DIO 2 $^{-/-}$	Deletion of Dio2		Deafness

PAX8, paired box gene 8; SECISBP2, selenocysteine-insertion sequence binding protein 2; TSHR, thyroid stimulating hormone receptor (TSHR); DIO2, type 2 deiodinase; THRα, thyroid hormone receptor alpha; THRβ, thyroid hormone receptor beta; CH, congenital hypothyroidism; SLC26A4, solute carrier family 26 member 4.

Other evidence that suggests strong influences of TH in the cochlea is related to the expression of the *SLC26a5* gene, which encodes the prestin protein. This protein is considered the outer hair cells (OHC) engine in the cochlear amplification process (40), which is reduced, immature, and with reduced distribution under hypothyroidism conditions (41–43). The gene expression encoding the K^+ channels, *KCNQ4*, responsible for the endolymphatic potential formation, has also been discussed in the literature. Therefore, it has been shown that these ion channels are significantly reduced and poorly distributed under conditions of thyroid hypofunction (44). **Figure 1** illustrates the molecular structures inherent in external hair cells, which are dependent on adequate serum levels for thyroid hormones in the body.

Hormone deficiency can cause reductions in β-tectorin protein in the tectorial membrane, which explains structural abnormalities of the tectorial membrane and cochlear function. The OHCs are susceptible to serum thyroid hormone levels (36). Thus, serum levels of thyroid hormones circulating in the bloodstream can affect cell differentiation, which reduces the amount of organelles in the cytoplasm (37). These changes may be accompanied by abnormalities of the afferent dendrites and delayed growth of the efferent terminals that make direct connections with the OHC (4).

In the case of neural and central structures, studies in animal models have shown abnormalities in myelination and reduction of axons in the anterior commissure and corpus callosum (36). There is also a reduction in the number of microtubules in the neural cytoplasm and an altered distribution of apical dendrites of the pyramidal neurons (37). Added to this, there are records of a decline in the deoxyglucose levels of the metabolism marker in regions such as the cochlear nucleus, superior olivary complex, nuclei of the lateral lemniscus, inferior colliculus, medial geniculate body, and auditory cortex. Therefore, it is possible to state that TH deficiency will significantly affect the auditory pathway (42).

Consequences of CH on Auditory System

Hearing is one of the essential senses for human communication, and it is where the individual develops speech. In addition, it is through hearing that the process of acquisition and development of oral language occurs. The auditory system consists of a peripheral portion (outer, middle, and inner ears), which captures sounds and transforms them into electrical impulses, and a central portion (brain auditory pathways), responsible for the analysis and interpretation of what is heard (45). Any complication in one of these portions can result in hearing loss (46), compromising not only communication but also receptive and expressive language, literacy, school performance, and the child's psychosocial development (45).

The functionality of the thyroid gland is crucial for the development of the auditory system (1). THs are vital for auditory pathway morphogenesis and maturation (3), and the deficiency of these hormones jeopardizes the development of hearing (47). Therefore, CH can result in hearing loss (27), and even with early treatment, small hearing changes can be observed in individuals with CH (48). This happens due to the cochlea's susceptibility to metabolic disorders, resulting from its intense activity and low energy reserve (49). THs act in both systems (peripheral and central) in the auditory system, and they are responsible for forming key structures of the inner ear, such as the cochlear duct, organ of Corti, and tectorial membrane (50). Therefore, the shortage or lack of THs brings losses to these structures.

Audiological changes noted in CH patients are diverse. However, losses with sensorineural, bilateral, and symmetrical characteristics are often found, with degrees varying from mild to moderate (8, 9). Actually, the risk of hearing loss may be associated with the severity of CH (43). In the researched literature, hearing changes in CH are characterized as peripheral or central, of insidious occurrence, with impaired auditory abilities (cognitive functions related to hearing). These

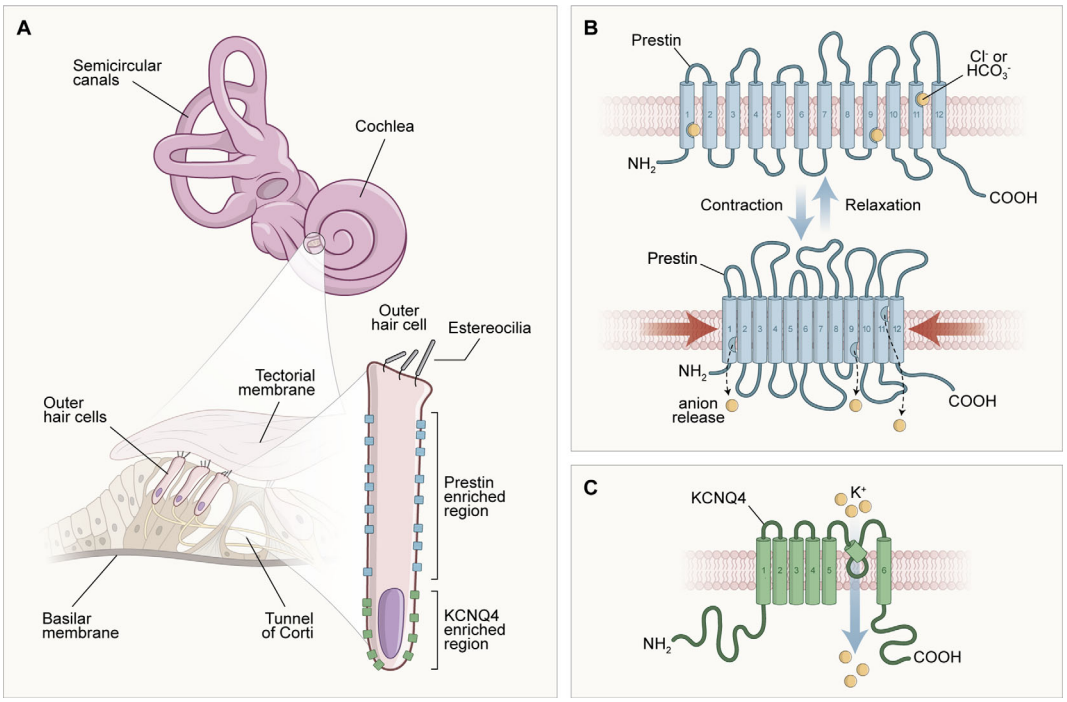

FIGURE 1 | Scheme of the morphological configuration and special distribution of transmembrane proteins responsible for the endolymphatic potential (KCNQ4) generation and cochlear electromotility (prestin). In **(A)**, the structures of the inner ear are illustrated in different parts: at the top of the image, there are the cochlea and semicircular canals, structures that compose the inner ear macroscopically; to the left of the image, in a microscopic analysis, there is the spiral organ (Tunnel of Corti) formed of the tectorial membrane, supporting and sensory cells; and to the right of the image, the morphology of the outer hair cell can be observed, delimiting the molecular location of the transmembrane proteins prestin (lateral) and KCNQ4 (basal). In **(B)**, it is possible to observe the unique arrangement of the prestin protein and its physiology. Prestin is involved with the motor function of outer hair cells (OHC). This activity occurs when OHCs are depolarized through the influx of K+ positive electrical charges after a sound stimulation, creating a positive intracellular environment that favors the displacement of Cl- anions from the prestin's binding sites into the cytoplasm. This electrical charge movement causes a shortening of prestin and, consequently, a reduction in the size of the OHC, characterizing the active mechanism and the bio-electromotility of the OHC that occurs in the absence of calcium and ATP. In **(C)**, after depolarization, an OHC enters the repolarization and hyperpolarization stage due to the exit of cations in the basal portion of the potassium channels formed by the transmembrane protein KCNQ4, which contributes to the generation of the endolymphatic potential.

skills are essential to the development of oral and written language and social-emotional progress. Additionally, they affect the individual in periods considered critical to developing global skills and full stage of experimentation and interaction with the environment, compromising the quality of life. In this context, when a hearing disorder is detected early, even during the neonatal period, early intervention through speech therapy and indication of hearing aids, if necessary, may be required and performed, preventing future harm to the child.

Impact of Hearing Loss at Early Ages

In cases where TH deficiency occurs in early periods, as in CH, the risk of hearing loss in children is increased (8, 10). This data is significantly worrying when thinking about the harm that the reduction or absence of action of TH in the crucial periods of neurological development and maturation can bring. The central nervous system is one of the most affected (51), and it can alter the processing of the acoustic signal up to the cortex, causing difficulties in auditory skills (52) that will result in problems with behavioral, language, and social difficulties.

The crucial periods for the development of children's hearing and oral language occur in early childhood. Nerve structures are already specialized in the brain of newborns with auditory cortical areas formed and ready to receive acoustic stimuli from the external environment. Consequently, the first contact with sounds is provided, instigating the mother tongue's acquisition and increasing the linguistic repertoire (53, 54). Hence, when newborns have alterations in their auditory pathways that limit them to having an adequate sound sensation during the first 3 years of life, their linguistic and social potential will be low and reduced (53, 54). In the absence or deficit of sound stimuli at critical times, without adequate intervention, the child may present vital educational, social, and emotional delays (12).

The literature also shows that some language deficits, fine motor skills, visuospatial processing, attention and memory, and hearing disorders can persist in patients with CH even with early treatment (47, 48). Even moderate or mild hearing loss can alter

the hearing perception of voiceless phonemes (55, 56), making the understanding of soft speech unintelligible, even in a quiet environment (57). As a result, phonological discrimination, phonological awareness, and phonological memory are compromised, consequently interfering in the learning processes of these children, directly affecting their quality of life and their families (58, 59).

CONCLUSION

THs are essential for brain and intellectual development, as well as for peripheral and central auditory functions that extend from the fetal period to 2 years of age, a period considered critical for typical development. Therefore, CH can be considered a potential risk factor for changes in acoustic signals' processing mechanisms along the auditory pathway, which manifests itself as cognitive, language, and socioemotional delays.

AUTHOR CONTRIBUTIONS

CLOA: conception, writing. CA: Editing, review. HR: Conception, writing. All authors contributed to the article and approved the submitted version.

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Update on Neonatal Isolated Hyperthyrotropinemia: A Systematic Review

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The purpose of this paper was to systematically summarize the published literature on neonatal isolated hyperthyrotropinemia (HTT), with a focus on prevalence, L-T4 management, re-evaluation of thyroid function during infancy or childhood, etiology including genetic variation, thyroid imaging tests, and developmental outcome. Electronic and manual searches were conducted for relevant publications, and a total of 46 articles were included in this systematic review. The overall prevalence of neonatal HTT was estimated at 0.06%. The occurrence of abnormal imaging tests was found to be higher in the persistent than in the transient condition. A continuous spectrum of thyroid impairment severity can occur because of genetic factors, environmental factors, or a combination of the two. Excessive or insufficient iodine levels were found in 46% and 16% of infants, respectively. Thirty-five different genetic variants have been found in three genes in 37 patients with neonatal HTT of different ethnic backgrounds extracted from studies with variable design. In general, genetic variants reported in the *TSHR* gene, the most auspicious candidate gene for HTT, may explain the phenotype of the patients. Many practitioners elect to treat infants with HTT to prevent any possible adverse developmental effects. Most patients with thyroid abnormalities and/or carrying monoallelic or biallelic genetic variants have received L-T4 treatment. For all those neonates on treatment with L-T4, it is essential to ensure follow-up until 2 or 3 years of age and to conduct medically supervised trial-off therapy when warranted. TSH levels were found to be elevated following cessation of therapy in 44% of children. Withdrawal of treatment was judged as unsuccessful, and medication was restarted, in 78% of cases. Finally, data extracted from nine studies showed that none of the 94 included patients proved to have a poor developmental outcome (0/94). Among subjects presenting with normal cognitive performance, 82% of cases have received L-T4 therapy. Until now, the precise neurodevelopmental risks posed by mild disease remain uncertain.

Keywords: newborn screening, levothyroxine, subclinical hypothyroidism, systematic review, neonatal hyperthyrotropinemia

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Abbreviations: CH, congenital hypothyroidism; DUOX2, dual oxidase 2; HTT, hyperthyrotropinemia; NBS, newborn screening; SCH, subclinical hypothyroidism; SNV, single-nucleotide variant; TPO, thyroid peroxidase; TRAbs, TSHR (TSH receptor) antibodies.

INTRODUCTION

The state of mild elevated venous TSH (e.g., ≥6–20 mU/L) beyond 21 days of life with thyroid hormone concentrations within the normal range is termed or described as isolated hyperthyrotropinemia (HTT) or subclinical hypothyroidism (SCH) (1–3). The term SCH is more commonly used after infancy, when peripheral thyroid hormone levels are within the normal range but TSH is mildly elevated.

Before newborn screening (NBS) for congenital hypothyroidism (CH), most cases of mild CH and HTT remained unidentified due to their unapparent clinical course. Over a decade, the TSH cutoff value established by NBS programs has been adjusted downward, resulting in an increasing trend in the diagnosis of infants with mild CH and HTT in TSH-based NBS programs.

In general, it is difficult to obtain a true prevalence of the cases of neonatal isolated HTT for various reasons. First, certain studies do not report FT4 levels and confusion arises regarding the differentiation of mild CH from HTT, which might represent a continuum along a scale of thyroid dysfunction (4). Some studies do not distinguish between transient mild CH or borderline CH, and transient HTT, and describe all such cases as "transient TSH elevation" or transient hypothyroidism. Second, published studies have employed varying criteria for defining this condition: NBS programs have applied different TSH cutoff values, and studies have used initial (dried) whole blood spot TSH, first and second blood spot TSH levels, cord blood (dried or serum) TSH, or serum TSH (obtained at NBS or recall examination). Usually, the day of extraction is not the same for all NBS programs and the cutoff point is not adapted to the reality of time collection.

The criteria for assessing CH severity can be posed in terms of clinical, biochemical, and radiological features. Biochemically, CH severity can be judged as mild, moderate, or severe based on the serum FT4 concentration (<5, 5 to <10, and 10 to 15 pmol/L, respectively) (2). While in CH thyroid hormone levels are in general decreased, FT4 levels are, by definition, within the normal range in isolated HTT.

Neonatal isolated HTT can be transient or persistent. Transient neonatal HTT is usually defined as an abnormal transient elevation of neonatal serum TSH with normal T4 values. Transient neonatal HTT should be differentiated from false-positive NBS tests, defined as an abnormal screening test value, with normal results of serum tests taken immediately afterward (usually at 2 weeks of age). Most newborns suspected of CH because of elevated borderline neonatal TSH levels would have normal, or nearly normal, TSH, and normal FT4 after a few days, at the recall examination. Newborns with very short-lasting HTT should be classified as false-positive at NBS because the initial neonatal abnormalities can be attributed to a variety of transitory causes that have no significant adverse clinical consequences (5). Specimens collected in the first 24 to 48 h of life may lead to false-positive TSH elevations when using any screening test approach (1). During the first few hours of life, newborns experience a physiological increase in TSH levels in response to the environment, followed by a progressive decrease in its levels. The occurrence of high neonatal TSH (TSH ≥ 5 mU/L) decreases with the number of days from birth to sampling (6). It has been also demonstrated that the concentration of neonatal TSH decreases with age until it stabilizes at between 11 and 15 days of life (7).

Transient HTT has been mainly attributed to either iatrogenic iodine overload or iodine deficiency during fetal and early postnatal life (8). Transient mild CH and transient HTT are also usually proposed to be associated with maternal ingestion of goitrogenic substances which reach the fetus *via* placental transfer, or by maternal–fetal transfer of thyrotropin receptor (TSHR)-blocking antibodies, which are IgG immunoglobulins (8–10).

Neonatal HTT may also result from prematurity and/or low birth weight. TSH concentrations were found to be higher in small-for-gestational-age infants (11). A high incidence of HTT in premature neonates has also been reported, particularly in those small-for-gestational-age (12). Although the reason for the increased TSH levels is unknown, its origin could be multifactorial as many hypotheses have been proposed (11). TSH elevation in infants with Down syndrome is also highly prevalent during the neonatal period (3). A Turkey study has demonstrated HTT in 32 of the 80 newborns with Down syndrome (13).

Most reported cases of neonatal HTT occur as a transient form, while the persistent form is less well understood (14). Distinguishing between transient and persistent forms of HTT at the time of diagnosis is difficult and generally requires a large time frame (4). In general, the decisive consistent distinguishing factor between transitory and persistent forms of HTT is a reassessment of thyroid function at the time of treatment withdrawal, occurring in general after 2 or 3 years of age.

Finally, there is also controversy regarding the need for levothyroxine (L-T4) therapy in neonatal HTT (3). Many practitioners elect to "play safe" and treat infants with HTT to prevent any possible adverse developmental effects. Some studies have reported cases of mild CH or isolated HTT progressing to overt (more severe) hypothyroidism. As the developing brain has a critical dependence on thyroid hormone for the first 2 or 3 years of life, it is prudent to assure normal thyroid hormone levels during this period.

The purpose of this paper was to systematically review the published literature to compile—and, when possible, quantify—the existing data on neonatal HTT. Our aim has been primarily to address the following questions. First, how does the reported frequency of HTT vary depending on the mode of identification, and what other factors it depends on? Second, what is the most common cause of neonatal HTT? Third, what have we learned about management and clinical progression from follow-up and re-evaluation studies? Finally, is there any convincing evidence about the impact on cognitive development in these infants that supports L-T4 replacement therapy?

METHODS

The literature search was done on studies up to August 8, 2020, on the PubMed database from the National Library of Medicine using the following keywords and terms: (congenital OR neonatal OR newborn) AND ("subclinical hypothyroidism"

OR "compensated hypothyroidism" OR hyperthyrotropinemia OR hyperthyreotropinemia OR hyperthyrotrophinaemia OR hyperthyrotropinaemia). Searches were limited to studies on humans that were published in English. Eligible studies had no minimum number of participants and there were no country restrictions. All the studies that investigated the prevalence, L-T4 management, re-evaluation of thyroid function during infancy or childhood, etiology including genetic variation, thyroid imaging test results, bone maturation, and/or developmental outcome were considered in this study. Data on L-T4 therapy were carefully extracted from each study when available. Articles were screened using the following inclusion criteria: 1) must contain information on neonatal HTT, preferably with data on TSH and FT4 levels, and a clear phenotype can be inferred (it is possible to discriminate HTT from CH, especially from mild CH); and 2) HTT must have been diagnosed during the neonatal period or early infancy (1–3 months of age). For large cohorts, included subjects should have less than 1 year of age at diagnosis and on average no more than 3 months of age. Patients with increased serum TSH levels, evaluated at recall examination because of a positive NBS or for other reasons (e.g., a first-degree relative of an index case), were included.

A formal variant classification was assigned according to the recommendations from the American College of Medical Genetics (ACMG). The variant interpretation was performed with Varsome (hg19) (15). Variants labeled as uncertain significance (VUS) according to Varsome were further revised and reclassified if necessary, according to functional studies and evidence from Variant Effect Predictor (VEP, Ensembl).

Cases presenting with mild CH or apparent low levels of T4/FT4 were excluded. Cases positive at NBS with normal serum TSH at reexamination were excluded. Studies were also excluded when it was not possible to identify if participants had increased serum TSH at confirmation of diagnosis (exceptions were cases receiving L-T4 treatment, since it may be inferred that TSH levels were confirmed in serum). Excluded were also studies conducted on cohorts with maternal or neonatal disease (except for thyroid disease) or genetic syndromes (except for resistance to TSH); studies conducted entirely on premature, small-for-gestational-age, low birth weight, or Down syndrome subjects; studies reporting exclusively synonymous single nucleotide variants (SNVs) and common sequence variants ("genetic polymorphisms"); and review articles and studies reporting only *in vitro* experiments.

RESULTS

Literature Search and Case Inclusion

The PubMed search strategy resulted in 439 hits. Reviews and experimental papers were used to perform a further search, which revealed an additional 66 records. The initial screen was based on title, abstract, and occasional whole-text scan. After the in-depth screening, 170 relevant citations remained for further review. All these articles were thoroughly read and evaluated. Eligible studies were independently reviewed by two reviewers; 109 were consistently excluded by both authors, while 15 additional studies were excluded after discussion and agreement between reviewers. Nine studies including subjects positive at NBS with normal serum TSH at reexamination were excluded (**Table S1**). In those studies, reexamination was performed beyond 2 weeks of life (range 10–90 days), and therefore, it is not possible to address if serum TSH was elevated at 14 days of life and became normal shortly afterward. One study was excluded because of wide inclusion criteria, and five studies were excluded since increased TSH values detected at NBS were not further confirmed by serum analysis (**Table S1**). Finally, 46 citations were used to build the nine summary tables included in this systematic review (**Tables 1**, **2** and **Tables S2–S8**). A flowchart of the article selection process is shown in **Figure 1**.

The Changing Prevalence of Neonatal HTT

Nine studies reported on the prevalence of HTT in a total of 2,715,031 infants. The overall prevalence of HTT was estimated at 0.06% (1,551/2,715,031), but prevalence varied widely among studies (range 0.001%–0.1%, **Table 1**). The estimated prevalence of HTT in both Europe and East Asia is also 0.06% (**Table 1**). Finally, the computed HTT: CH ratio is 1.2:1 (1,532:1,288; data available from seven studies).

Clues to the Etiology of Transient HTT

We examined the potential causes of transient HTT such as either iodine overload or iodine deficiency, maternal ingestion of goitrogenic substances, and maternal–fetal transfer of TSHR-blocking antibodies.

Six studies comprising 77 subjects evaluated serum and/or urinary iodine concentration in neonatal HTT (**Table S2**). Cutoff values for determining abnormal iodine concentrations were not available in some studies; 46% (19/41) and 16% (8/50) of infants had increased and decreased iodine levels, respectively.

Placental transfer of maternal IgG antibodies against the thyroid TSHR is another putative cause of transient neonatal HTT. We found only five studies investigating the presence of anti-TSHR antibodies (TRAbs) and/or TSHR stimulatory antibodies in the mother and/or in the infant (**Table S3**). Information about maternal thyroid disease was also collected. Two studies have demonstrated the presence of TRAbs in cases of neonatal HTT (Azzopardi P 2010), or in the mothers of those infants (Evans C 2011), suggesting transplacental transfer of maternal antibodies (31, 32). Two additional studies have also reported transient HTT due to maternal autoimmune thyroid disease with the presence of anti-TSHR antibody activity in both maternal and infant serum (Tamaki H 1989, Schwingshandl J 1993) (33, 34).

No studies reporting on the association between neonatal HTT and maternal ingestion of goitrogenic substances were found in this systematic review of the literature.

Thyroid Imaging in Neonatal HTT

Newborns with isolated HTT may have mild changes in thyroid morphology and/or genetic abnormalities. **Table S4** and **Figure 2** compile data of 304 subjects extracted from 28

TABLE 1 | Prevalence of neonatal hyperthyrotropinemia.

Id	Country	Period	Condition	Description	Prevalence (%)	HTT : CH	Patients receiving L-T4
Fu C. 2017 (16)	China	2009–2016	HTT	Increased TSH (>10 mU/L) and normal FT4 at recall (days 7–28)	911/1,238,340 (0.07)	911:731	0/911
Kumorowicz-Czoch M. 2011 (17)	Poland	2000–2006	HTT	Increased TSH (>9.1 mU/L) and normal FT4 at recall (day ~26)	4/233,120 (0.002)	4:58	4/4, trial-off at ~4 years
Corbetta C. 2009 (18)	Italy	1999–2005	HTT	Mildly increased TSH (5.0–9.9 mU/L) and normal/high FT4 at recall	578/629,042 (0.09)	578:435	0/578
Nishiyama S. 2004 (9)	Japan	2000–2002	HTT	Mildly increased TSH (cutoff value na) and normal serum FT4 at recall	24/37,724 (0.06)	24:6	14/24
Tyfield L.A. 1991 (19)	England	1981–1987	HTT	Increased TSH (>10 mU/L) and normal T4 at recall (day ~30)	3/185,723 (0.002)	3:45	0/3
Miki K. 1989 (20)	Japan	1975–1983	t-HTT	Increased TSH (>17 mU/L) at recall (2–8 wk) but normal at 2–9 mo, TH in the normal range, + other criteria	16/281,468 (0.006)	na	5/16, follow-up
Sava L. 1984 (21)	Italy	(30 mo)	t-HTT	Increased TSH (>8 mU/L) and normal T4 at recall (day ~32), but normal TSH at wk 3–6	11/7,953 (0.1)	11:4	0/11
Czernichow P. 1981 (22)	Belgium	1979–1980	t-HTT	Increased TSH (>40 mU/L) and normal TH at recall (days 15–30), TSH decreases to normal after 5 mo	3/10,261 (0.03)	na	0/3
Miyai K. 1979 (23)	Japan	1975–1978	t-HTT	Increased TSH (>12 mU/L) and normal TH at 2 mo, TSH decreases to normal after 7–9 mo	1/91,400 (0.001)	1:9	0/1
Summary estimates					1,551/2,715,031 (0.06)	1,532:1,288	23/1,551 (1.5%)

t-HTT, transient HTT; TH, thyroid hormones; mo, months; wk, weeks; na, not available.

TABLE 2 | Developmental outcome in neonatal hyperthyrotropinemia.

Id	Condition	Observations	*N* cases	L-T4	Test and/or assessment	Impaired developmental outcome	Age (years)*
Rovelli R. 2010 (24)	HTT	No preterm, asphyxia, or congenital disease	3	3/3	Mental development (Griffith's scale)	0/3	1–2
Demirel F. 2007 (25)	HTT	na	36	36/36	Denver developmental screening test	0/36	3
de Roux N. 1996 (26)	HTT	1/4 preterm	4	3/4	Intellectual development	0/4	na
Nishiyama S. 2004 (9)	HTT	Full-term	15	12/15	Psychomotor development	0/15	2
Tomita Y. 2003 (27)	p-HTT	GA 36–40 wk, BW 2,090–3,580 g	14	14/14	Japanese Denver developmental screening test	1/14 (DS)	~3–6
Vigone M.C. 2005 (28)	p-HTT	na	1	1/1	Mental development (Griffith's scale)	0/1	4
Mizuno H. 2009 (29)	p-HTT	Full-term, BW 2,374–3,450 g	4	4/4	Intelligence quotient	0/4	6
Tyfield L.A. 1991 (19)	p-HTT	Uneventful pregnancy and neonatal period	3	0/3	Developmental status regarded by parents and pediatricians	0/3	5–6
Miki K. 1989 (20)	t-HTT	Full-term, normal BW	16	5/16	Psychomotor development (Tsumori and Isobe scale) or intelligence (Wechsler scale)	1**/16 (deafness)	2–7
Summary estimates			96	–	–	2/96	1–7

Data in column "L-T4" are expressed as a ratio of the number of LT4-treated patients to the total number of cases. Data in column "Impaired developmental outcome" are expressed as a ratio of the number of participants with "abnormal" results to the total number of cases.

p-HTT, persistent HTT; t-HTT, transient HTT; GA, gestational age; BW, birth weight; DS, Down syndrome; wk, weeks; na, not available.

**Age at assessment.*

***Untreated patient (Miki K. 1989).*

included studies (33 datasets) reporting thyroid imaging results. Thyroid morphology and/or function was more frequently assessed by ultrasonography and Technetium-99m (99m-Tc) scintigraphy and less commonly by radioiodine scan; 27% (83/304) of included subjects showed thyroid gland abnormalities. Of 83 subjects with abnormal results, 34% (28/83) showed enlarged thyroid gland or increased radionuclide uptake, while 65% (54/83) subjects showed anatomical abnormalities (hypoplasia, hemiagenesis, or ectopy), decreased radionuclide uptake, or no uptake.

As expected, the occurrence of thyroid abnormalities was found to be higher in the persistent form of HTT

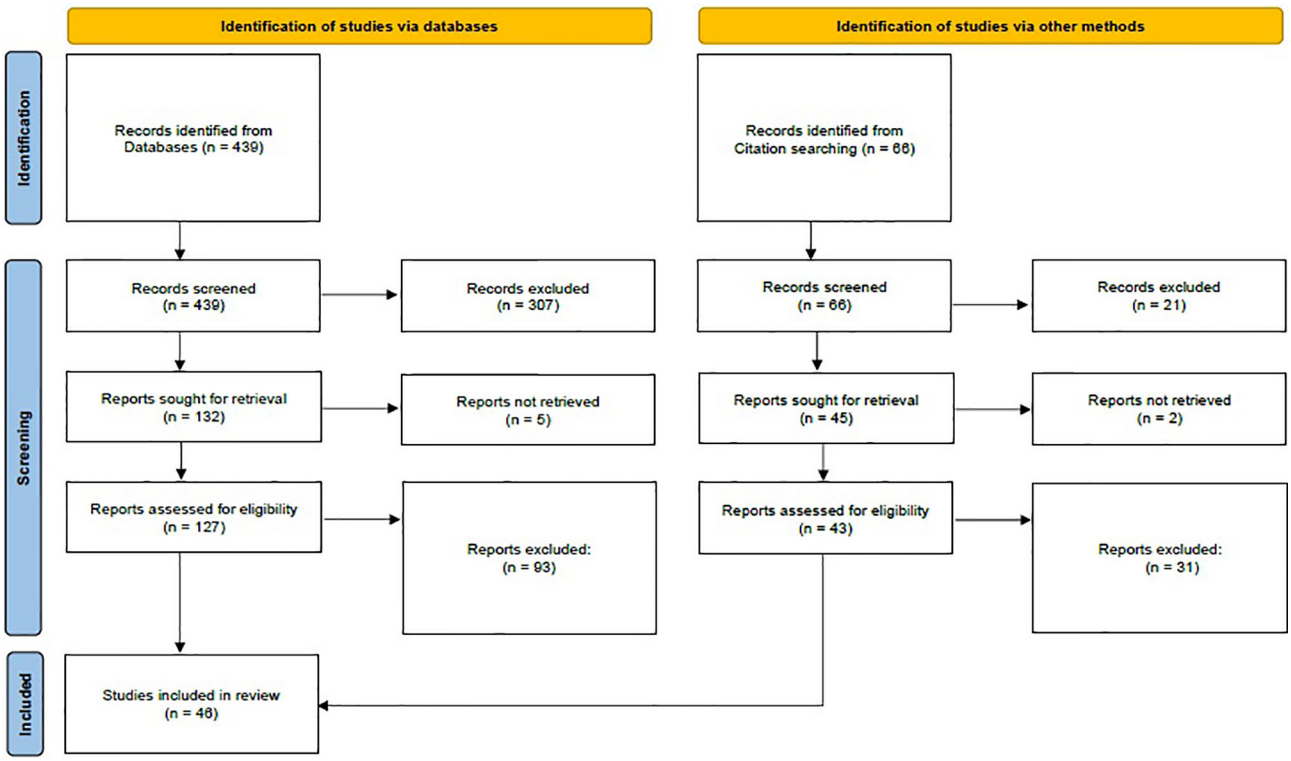

FIGURE 1 | PRISMA flow diagram. Adapted from Page et al. (30).

(persistent: 19/81, 23% *vs.* transient: 2/51, 4%, **Table S4**); however, it should be also considered that persistent forms are more likely to be examined by thyroid imaging than transient conditions. Note that, for some datasets, detailed information related to conditions was not available (**Table S4**). On the other hand, among subjects with abnormal thyroid imaging tests, different frequencies of persistent and transient forms of neonatal HTT were found (persistent: 19/21 *vs.* transient: 2/21, **Figure 2**).

The number of patients with abnormal imaging tests receiving L-T4 therapy was estimated from datasets where information about L-T4 treatment was available. Interestingly, between those subjects with abnormal thyroid imaging tests, 77 of 88 (87.5%) patients have received L-T4 therapy (one dataset was excluded from computation because treatment information was not available, see **Table S4**).

Finally, the study of Nishiyama S 2004 (9) was included in this systematic review but not incorporated into **Table S4** for the reason that it is not possible to obtain the exact number of subjects having abnormal imaging tests. Thyroid volumes measured using ultrasonography were found to be increased with respect to normal controls in 15 infants with neonatal HTT.

Insights Into Genetic Variation in Neonatal HTT

Table S5 compiles 37 cases extracted from 18 studies reporting genetic variants in neonatal HTT. Data were carefully extracted from studies with variable designs. Information about thyroid imaging tests, phenotype later in life, family background (first-degree relatives presenting with increased TSH levels), ethnicity, consanguinity, and study design was collected.

To date, genetic variation has been found in three genes in patients with isolated neonatal HTT (**Table S5**): *TSHR*, thyroperoxidase (*TPO*), and dual oxidase 2 (*DUOX2*). Overall, 35 different genetic variants have been found in 37 cases (**Table S5**).

Cases were found to be heterozygous, homozygous, or compound heterozygous. Biallelic carriers were more frequently reported than monoallelic (heterozygous) carriers (25 *vs.* 12 cases). Intriguingly, an Israeli group has reported a case of neonatal isolated HTT who required L-T4 therapy carrying two heterozygous missense variants in the *TSHR* gene and one heterozygous missense variant in the *TPO* gene (35).

Until now, 26 different *TSHR* sequence variants have been documented in 33 cases with neonatal-onset HTT (**Table S5**). Twenty-five SNVs, both missense and nonsense, as well as a deletion (frameshift mutation) in the coding sequence, have been identified in 33 patients of different ethnic backgrounds (**Table S5**). **Table S6** shows the interpretation of the *TSHR* sequence variants. Of those 25 SNVs, 20 were interpreted as pathogenic or likely pathogenic. T655*(delAC) causes the formation of a nonsense codon leading to the production of a receptor that lacks the entire transmembrane domain 7 and the intracellular (C-terminal) tail. According to the variant interpretation from **Table S6**, it can be speculated that in almost all patients the phenotype may be

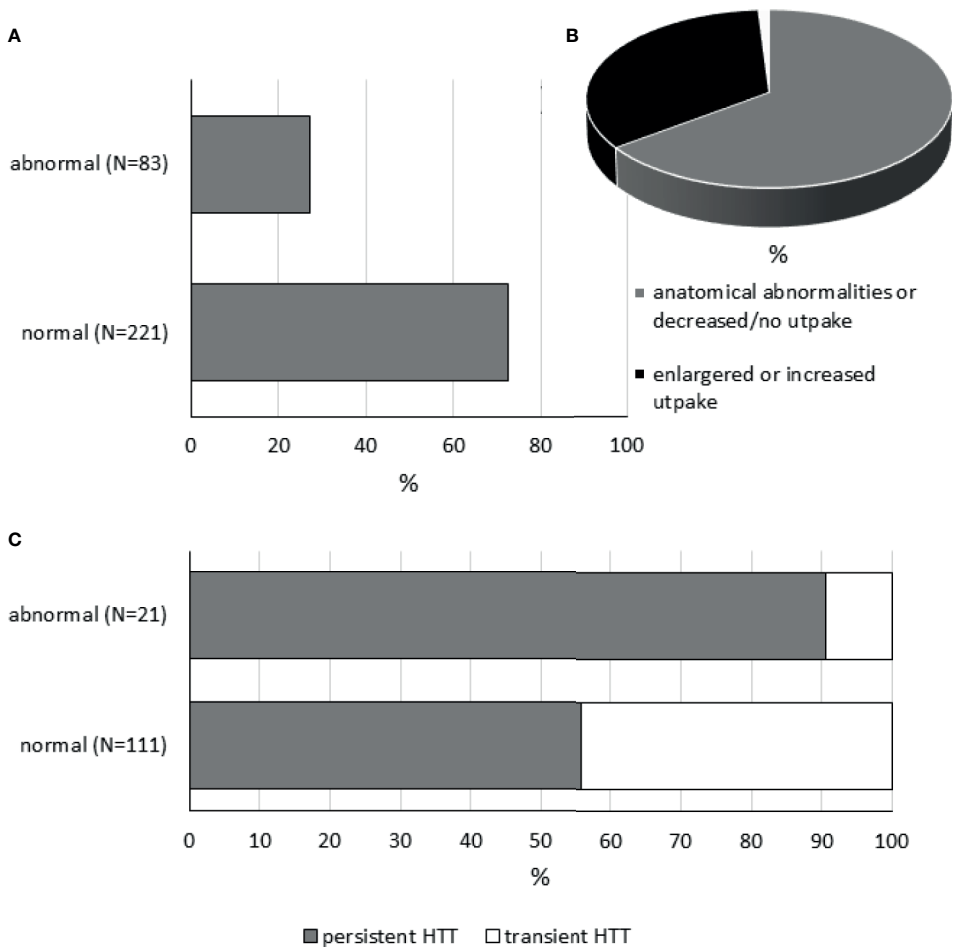

FIGURE 2 | Thyroid imaging tests in neonatal HTT. Panel **(A)** shows the percentages of cases with normal and abnormal findings. Panel **(B)** is a pie chart showing the proportion of patients with anatomical abnormalities (hypoplasia, hemiagenesis, or ectopic thyroid gland) or decreased radionuclide uptake or no uptake (in gray, *N* = 54), enlarged thyroid gland, or increased radionuclide uptake (in black, *N* = 28), or data not available (in white, *N* = 1). Panel **(C)** shows the percentages of persistent and transient conditions in subjects with normal or abnormal findings.

explained by the genotype. More specifically, 27 of 33 (82%) patients were carriers of (mono or biallelic) pathogenic (or "likely pathogenic") variants in the *TSHR* gene (**Figure 3**). In six cases (see **Table S4**), homozygous or heterozygous variants of uncertain significance could not account for pathogenicity.

Homozygous *TSHR* variants were found in nine cases, six Japanese patients carrying R450H, two members of consanguineous kindreds, and one subject born to consanguineous parents (**Table S5**).

Among all *TSHR* variants, R450H was most frequently reported. R450H was observed in Japan in 12 cases (6 compound heterozygous and 6 homozygous cases, **Table S5**).

Variants are distributed throughout the *TSHR* gene with no evident hot spot. As shown in **Table S6**, 54% of the reported sequence variants are distributed in the extracellular domain.

Although the number of studies reporting genetic studies is scarce, some conclusions can be drawn about the characteristics of subjects carrying *TSHR* sequence variants (**Table S5** and **Figure 3**). First, L-T4 therapy is frequently initiated during the neonatal period in cases carrying monoallelic or biallelic variants. More specifically, L-T4 therapy was started in 97% of cases (29/33, in 3 cases information related to L-T4 treatment was not available). Second, thyroid imaging may not be correlated to genetic variation. Abnormal imaging tests were found only in 5 of 28 (18%) cases carrying sequence variants in the *TSHR* gene (in 5 cases information about thyroid imaging tests was missing). And third, mildly altered thyroid tests (slightly increased TSH and/or abnormal TRH test) are commonly (94%, 17/18) observed during childhood or adolescence in these patients (in 15 cases information was not available).

Genetic variation in *TPO* and *DUOX2*, key genes required for thyroid hormone synthesis, has been reported in neonatal HTT (**Table S5**). Kotani and colleagues described a partial iodide organification defect caused by likely pathogenic sequence variants in the *TPO* gene (36). *DUOX2* pathogenic sequence variants may also cause mild to moderate forms of hypothyroidism, including transient CH and transient HTT

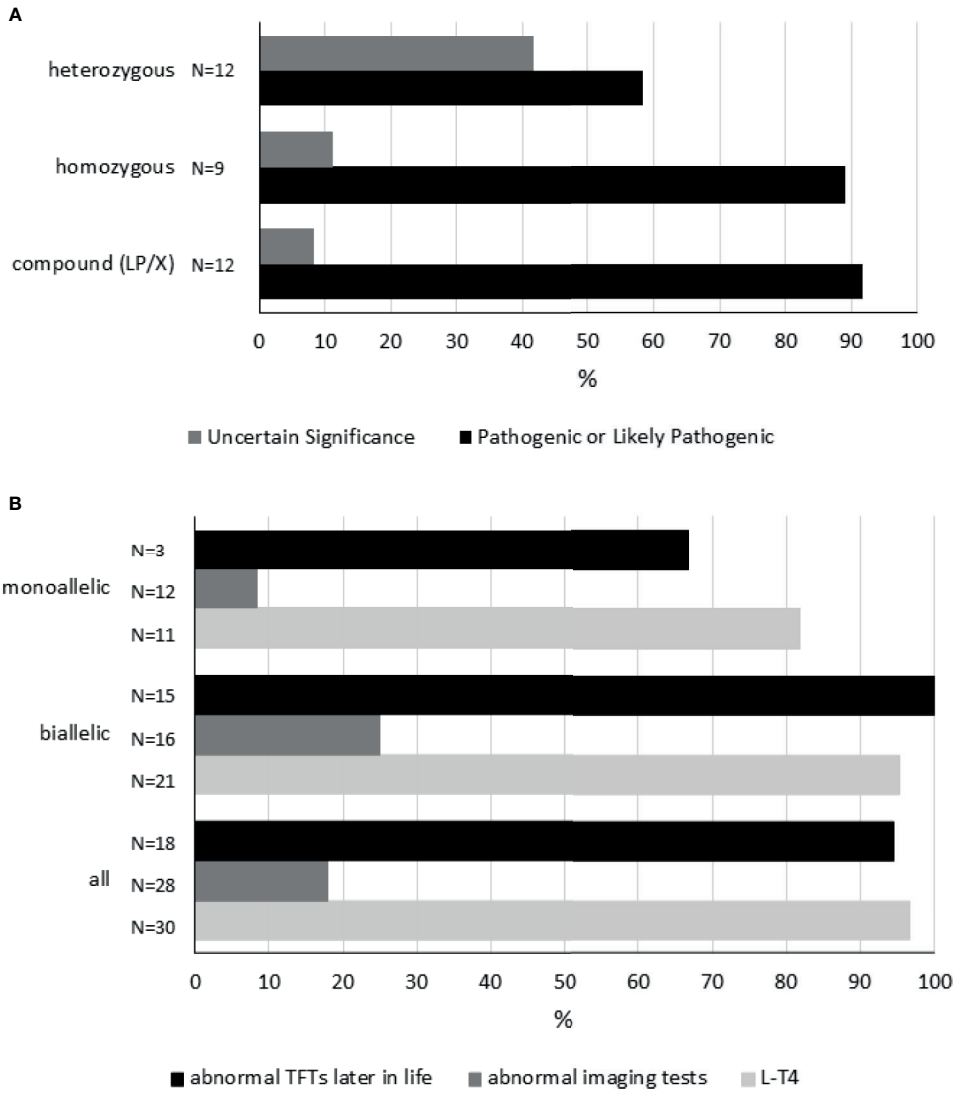

FIGURE 3 | Genetic variation in the *TSHR* gene. Panel **(A)** shows each reported genotype [compound heterozygous (LP/X), homozygous and heterozygous] representing a cluster. As disease severity will depend on the combination of variants in every locus, variants were interpreted and classified (see **Table S6**). Within each cluster, the percentages of each combination of sequence variants are shown. "Pathogenic" and "Likely Pathogenic" were grouped together. Panel **(B)** shows 1) the percentage of subjects receiving L-T4 therapy, 2) the percentage of cases showing abnormal imaging tests, and 3) the percentage of individuals having abnormal TFTs later in life according to genotype. Compound heterozygous (LP/X) and homozygous genotypes were grouped together as biallelic. TFTs, thyroid-function tests. For details, please see **Table S5**.

(37). In this systematic review, six different *DUOX2* (missense, nonsense, and frameshift) variants were found in infants with neonatal-onset HTT without goiter, consistent with resistance to TSH, rather than dyshormonogenesis (**Table S5**).

Management: To Treat or Not to Treat?

Table S7.1 compiles 10 follow-up studies (including 476 subjects) where some or all participants were treated with L-T4 and re-evaluation of thyroid function was performed after withdrawal of treatment, usually at ~2–3 years of age. Information about L-T4 discontinuation (before trial-off medication) to prevent overtreatment and progression to overt hypothyroidism was collected when available. Data on thyroid status at re-evaluation and attempts of L-T4 withdrawal were also extracted, including information on successful and unsuccessful attempts.

More than half of the participants (60.5%, 288/476) were treated with L-T4 (**Table S7.1**). In general, treatment is started in infants with persistent mild TSH elevation and/or abnormal TRH test results.

During treatment, certain patients experienced elevated levels of thyroxine. In some cases, therapy was stopped, and patients

showed full recovery within the first year of life. Levothyroxine cessation before trial-off was reported in four studies, with an estimated overall percentage of L-T4 discontinuation of 11% (32/281, **Table S7.1**).

After the patient reached 2–3 years of age (range 1–6 years), L-T4 treatment was ceased to conduct thyroid function tests. In those cases where TSH levels were within the normal range, patients were followed up without L-T4 therapy. However, according to **Table S7.1**, TSH levels were found to be elevated following cessation of therapy in 101 of 229 cases (44%).

In general, all patients had a confirmatory trial-off levothyroxine; however, in certain infants, discontinuation was never tried, especially in patients having elevated TSH levels while receiving therapy. Overall, therapy withdrawal was not attempted in 17.5% of cases (40/229, **Table S7.1**).

A matter of debate is whether to reassume L-T4 treatment once blood TSH is mildly elevated following the withdrawal of treatment in childhood. There remains a role for clinical judgment in the management of these cases. A persistently abnormal TSH was not always used as a rule of thumb to resume medication. It has been reported cases of increased TSH but a normal response to TRH test where treatment was not restarted (Demirel F 2007) (25), and cases of increased TSH where patients were followed until normal TSH concentration was reestablished (Tomita Y 2003) (27). Withdrawal of treatment was judged as unsuccessful, and medication was restarted, in 78% of cases (60/77, 7 datasets).

Figure 4 shows a flowchart illustrating the approach to the management of neonatal HTT based on the reviewed literature.

Clinical Evolution in Children With Neonatal-Onset HTT

Different cohort studies have been conducted to assess thyroid function progression in neonatal isolated HTT; however, most published studies are retrospective with low sample sizes. Overall, data collected from 14 studies (**Tables S6.1, 6.2**) indicate that the percentage of patients with persistent elevation of TSH levels during early childhood is not negligible (45%, 125/279).

On the Topic of Growth Rate and Cognitive Development

Some studies have addressed the question of growth rate and cognitive development in children diagnosed during the neonatal period with HTT. In general, normal bone maturation was reported in all children. Data collected from nine studies showed that 2 of 74 (3%) cases with neonatal-onset HTT showed delayed bone age or ossification (**Table S8**). In contrast, evidence on an association between neonatal HTT and cognitive development is scarce and contradictory. We have compiled studies evaluating cognitive development. Data about perinatal outcomes (prematurity, birth weight, congenital disease, and related information) were extracted when available. Data collected from nine studies have shown that 2 out of 96 (2%) cases had abnormal cognitive outcomes during infancy or childhood (**Table 2**). However, after excluding two cases where the impairment of cognitive development would be related to congenital defects (deafness, Down syndrome), none of the remaining 94 subjects showed adverse developmental outcomes (0/94). Among subjects with normal cognitive performance, 82% (77/94) of cases received L-T4 therapy. Results should however be interpreted with caution since some studies have not assessed cognitive and/or motor abilities throughout standardized scales, and detailed information about diagnostic tools was not always available (**Table 2**).

DISCUSSION

The purpose of this paper was to systematically review the published literature to compile—and, when possible, quantify—the existing data on neonatal HTT. We have collected evidence extracted from 46 research articles in nine tables (**Tables 1**, **2** and **Tables S2–S8**). We aimed to address specific questions which are discussed below.

How Does the Reported Frequency of HTT Vary Depending on the Mode of Identification, and What Other Factors Does It Depend on?

It has been demonstrated that several factors may affect the concentration of TSH, including maternal thyroid diseases and drugs, perinatal outcomes (including the type of delivery and birth conditions), and the methods and timing of TSH determination (38). Gender is a known factor influencing neonatal TSH level. The percentage of neonatal HTT is higher among male newborns, in contrast with the higher number of female newborns with CH in general (6, 16, 39, 40).

A proportion of transiently raised TSH may be attributable to analytical assay difficulties. A "maternal factor", by means of maternal IgG antibodies, has been repeatedly reported to interfere in some old radioimmunoassays of TSH (22, 41–45). There is also some variation in the prevalence between geographical areas and countries. The disparity in the reported prevalence may also be in part to population genetics and ethnicity. The risk of high neonatal isolated TSH has been previously shown to be dependent on maternal ethnicity (6). A cohort study conducted in Skopje, the capital of Macedonia, also reported ethnic differences in the incidence of high serum TSH levels in neonates (46). Probably, an underestimation of the prevalence of HTT may occur in countries where the thyroid NBS is practiced by measuring the total T4 level, which is expected to be within the normal range in HTT (8). Finally, there is some discussion about adapting the threshold according to the time of year (6). Seasonal aggregation has been described for recall rates in Iran, with recall occurring significantly more in winter than in other seasons (47, 48).

Because neonatal HTT is identified on the basis of the amount of a hormone that has a continuous distribution in the general population, prevalence estimates certainly should scale as the cutoff is decreased (49). Within an individual NBS program, undoubtedly, an increase in recall rates and prevalence of HTT is expected by lowering cutoffs. Through a systematic review of the literature, we attempted to understand the nature of the variation in the reported frequency of HTT. Given that definitions and

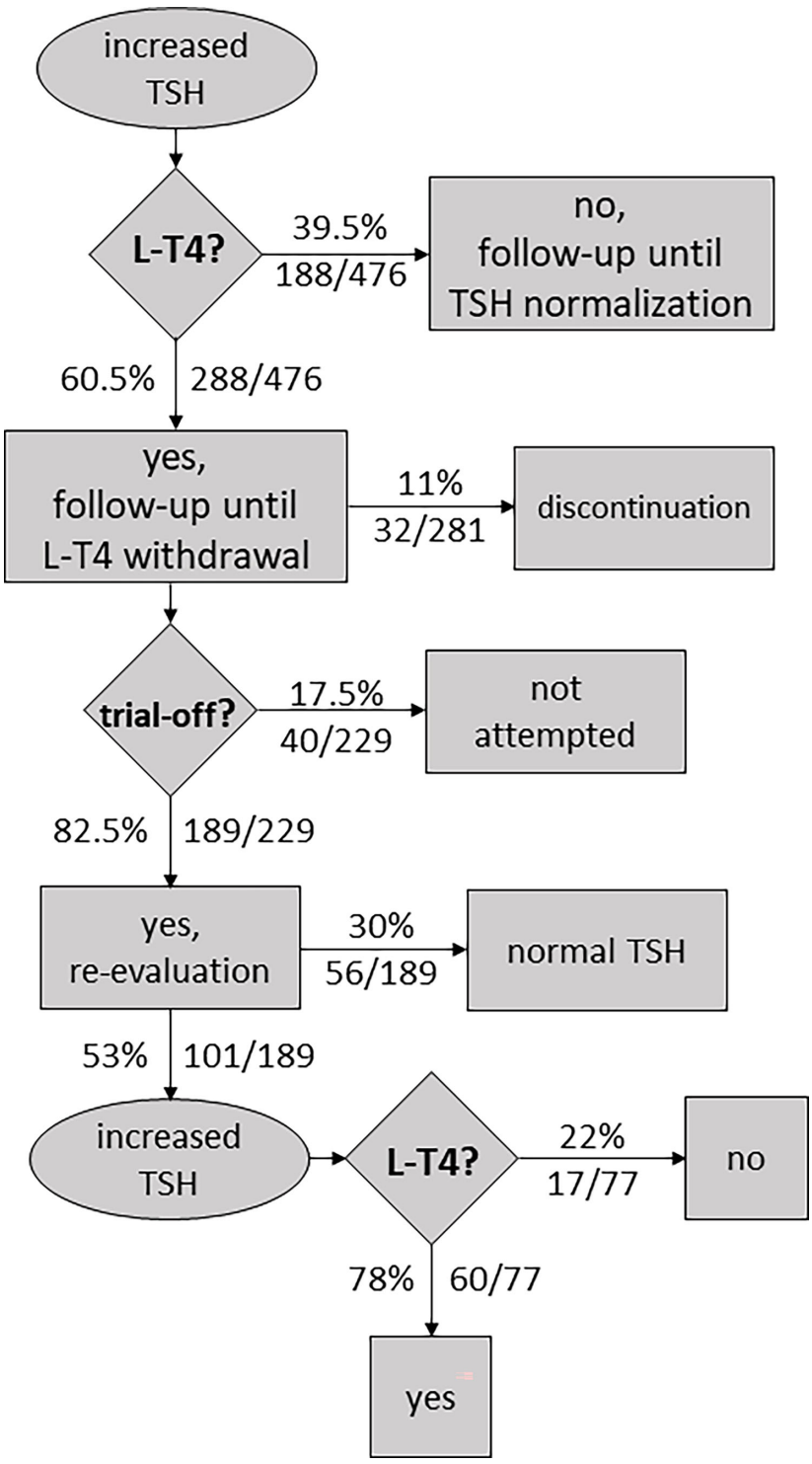

FIGURE 4 | Flowchart illustrating an approach to the management of neonatal hyperthyrotropinemia based on the reviewed literature.

diagnostic criteria vary widely among NBS programs and studies, there are significant differences between the reported incidence rates of this condition, beyond the expected variation associated with different cutoff values (**Table 1**).

Certainly, there is still some doubt about the value of detecting cases of neonatal HTT through NBS programs. Lain and collaborators have outlined the arguments both for and against lowering TSH cutoffs at NBS, along with a section focused on the

economic implications (50). The whole purpose of population-based biochemical screening is to identify medically actionable conditions, affected by treatment at a presymptomatic stage (51). While overt CH meets this goal, the evidence for neonatal HTT is vague, and whether mild abnormalities in thyroid function in the newborn have an impact on cognitive development remains controversial. Ultimately, NBS programs need to balance the goal of detecting such newborns versus the economic cost burden and parental anxiety. Accordingly, differing viewpoints are expected from a public health perspective and from the clinical pediatric endocrinologist.

Some actions can be taken to decrease the reexamination rate and the incidence of transient HTT. Iodine excess in babies is considered to be a factor that causes increases in recall rates and in the frequency of transient HTT, ultimately increasing screening costs (52). In this systematic review, excessive iodine levels were more frequently observed than insufficiency, and in general, maternal iodine availability acted as a disease modifier. Excessive and insufficient iodine levels were found in 46% and 16% of infants, respectively (**Table S2**). The fetus and newborn can be exposed to high maternal iodine concentrations either by crossing the placenta perinatally or by secretion of iodine into breast milk postnatally (9). To avoid excess consumption of iodine during gestation and lactation, pregnant women should be checked and advised for their iodine intake. Direct iodine overload in the newborn may be also caused by either disinfectant agents or contrast medium from practices performed during the perinatal period (9). The use of compounds without iodine for antisepsis should be recommended in the peripartum period (52). On the other hand, iodine insufficiency in pregnancy would be avoided with iodine supplements or iodized salt. Deficient maternal iodine values are associated with high TSH values. It has been demonstrated that women who give birth to infants with false-positive results at NBS have lower iodine concentrations as compared with mothers who give birth to infants with normal cord blood TSH levels (53).

Among the etiologies for transient HTT, TSHR-blocking antibodies seem not to significantly increase the incidence rate. Different generations of competitive binding TRAb tests for the detection of TSHR-binding inhibitory immunoglobulin have been used in the serological diagnosis of thyroid disease, principally in Graves' disease. Of note, the TSHR stimulatory antibodies can only be detected by cell-based bioassays and should be differentiated from the TSHR-blocking antibodies (34). The presence of TSHR-blocking antibodies is investigated by the measurement of TRAbs or thyrotrophin-binding inhibitor immunoglobulins. On the other hand, *in vitro* assays can demonstrate the presence of thyroid-stimulation-blocking antibodies, serum immunoglobulins that had the ability to inhibit thyroid adenylate cyclase stimulation. The diagnosis of transient HTT should be suspected especially if the mother has autoimmune thyroid disease. In fact, a higher recall rate in NBS in infants of mothers with hypothyroidism and autoimmune thyroiditis has been reported, and most of them returned to normal during follow-up (45, 54). A family history of neonatal high thyrotropin with normal thyroid function which resolves spontaneously deserves the measurement of TSHR antibodies. Although TSHR-blocking antibodies are a rare cause of transient HTT, autoimmune thyroid disease is quite prevalent in the general population.

Finally, exposure to antithyroid drugs would substantially contribute to the incidence rate of transient HTT, given the high prevalence of maternal hyperthyroidism; however, no studies reporting on the association between neonatal HTT and maternal ingestion of goitrogenic substances were found.

Identifying the mechanisms behind transient HTT would make it possible to discriminate at an early stage between the transient and the persistent forms and, thus, reduce medication and follow-up expenses.

Is Genetic Variation the Most Common Causation or Origination of Persistent Neonatal HTT?

A continuous spectrum of thyroid impairment severity can occur because of either genetic or environmental factors, or both (5).

In this systematic review, 35 different genetic variants have been found in three candidate genes (**Table S5**). In the 37 included cases, biallelic carriers are more frequently described than monoallelic carriers of genetic variants.

Resistance to TSH is a genetic disease characterized by hyposensitivity to a biologically active TSH molecule. This definition excludes autoimmunity with TSHR-blocking antibodies mimicking the phenotype of resistance to TSH (55, 56). The phenotype is determined by the degree to which the function of the "mutant" TSH receptor is diminished (29). Affected individuals have elevated serum TSH in the absence of goiter, with the severity ranging from isolated HTT to severe CH (55). As expected, resistance to TSH is commonly caused by sequence variants in the *TSHR* gene (56).

Until now, 26 different *TSHR* sequence variants have been documented in 33 cases with neonatal-onset HTT (**Table S5**), half of them distributed in the extracellular domain (**Table S6**). The human *TSHR* gene encodes for a G-protein-coupled receptor with a classical seven-transmembrane domain interacting with G proteins and an extracellular domain. The TSHR extracellular domain is unusually large and encoded by exons 1 to 9 and part of exon 10 (both transmembrane and intracellular domains are encoded entirely by exon 10). Eighty-two percent of the cases were found to be carriers of pathogenic (or "likely pathogenic") variants in the *TSHR* gene (**Tables S5, S6**), and thus, genetic variants may explain the phenotype of the patients.

Different researchers have tried to elucidate the genetic variation that may be involved in the etiology of isolated neonatal HTT. Calaciura et al. have examined a cohort of children with neonatal HTT or false-positive results at NBS and found that 3 of 45 children were carriers of heterozygous variants in two candidate genes, *TPO* and *TSHR* (57). In another study, simple or compound *TSHR* heterozygous variants were detected in up to 30% (34/111) of children with SCH, half of whom were positive at NBS (17/34), with a high prevalence of first-degree family history for SCH (58).

Of note, some published studies were not able to detect genetic variation in candidate genes in cases with neonatal-onset HTT (10, 59–61). Worth mentioning, neonatal HTT may also be found in the

context of complex syndromes, like pseudohypoparathyroidism (8, 62–64). Nowadays, there is no estimate of the frequency of pathogenic variants in neonatal HTT.

Genetic approaches based on systematic sequencing and analysis of customized and well-designed panels of genes would further outline the etiology of isolated neonatal HTT. Such studies and long follow-up of patients are needed. Furthermore, the genetic analysis would also permit familial genetic counseling (65).

Before proceeding to molecular analysis or in areas where genetic studies are not available, it seems reasonable to corroborate TSH levels in first-degree relatives of newborns presenting with HTT. According to data in **Table S5**, it can be estimated that more than 50% (17/33) of the first-degree relatives of both biallelic and monoallelic carriers of genetic variants also show increased TSH values.

Since there are no estimates of the frequency of pathogenic variants in neonatal HTT, it is still unknown if the genetic variation is the most common causation of persistent neonatal-onset HTT.

Abnormal thyroid imaging tests were found only in 5 of 28 cases carrying sequence variants in the *TSHR* gene, so it can be speculated that thyroid abnormalities are a different etiology of neonatal HTT (**Table S5**). In this systematic review, the overall frequency of thyroid abnormalities in neonatal HTT was estimated at 27%; however, this percentage decreases to 11% (27/248) after excluding one outlier study (Oren A 2016, **Table S4**) (40).

What Have We Learned About Management From Follow-Up Studies?

The last consensus about the biochemical criteria used in the decision to start treatment for CH suggests that if the serum TSH concentration is 6–20 mU/L beyond the age of 21 days in a healthy neonate with an FT4 concentration within the age-specific reference interval, either start L-T4 treatment immediately and retest, off-treatment, at a later stage, or withhold treatment but retest 1 to 2 weeks later (3). In this "gray area," treatment versus observation is based on clinical judgment due to lack of evidence in favor or against treatment. The management of infants with TSH elevations between 6 and 10 mU/L that persist after the first month of life is especially controversial (1).

It is not an easy decision to treat infants based on a single laboratory abnormality. Consistent with the mild nature of neonatal HTT, LT-4 treatment is started at an older age and at a lower dose compared with classic CH (40). The majority of neonates diagnosed with HTT have apparently normal thyroid glands, such that imaging studies do not help in the treatment decisions (66). Undertaking thyroid imaging diagnostic tests should not delay the decision on observation versus treatment beyond 4 weeks of age.

The available evidence is still insufficient to establish whether genetic analyses might represent a helpful diagnostic tool for a tailored management of patients with HTT, and whether it would allow discrimination between conditions that may require L-T4 therapy and follow-up. In any case, at present, genetic studies usually take more than a few weeks, and hence, such studies may not be helpful in treatment decision-making.

It deserves mentioning that most patients with thyroid abnormalities (77/88, **Table S4**) and/or carrying monoallelic or biallelic genetic variants in the *TSHR* gene have received L-T4 treatment (29/33, **Table S5**).

Treatment should be introduced on an individual basis, in discussion with the family. The concerns and anxieties raised in the parents by both repeated examinations and treatment should be carefully considered (57). Withholding treatment and carefully monitoring thyroid function is a rational approach. **Table 1** shows that, in general, conservative management without any medical treatment is applied. Nonetheless, monitoring of thyroid function in infants presenting with HTT is certainly important as some studies have demonstrated cases of patients becoming biochemically hypothyroid, with low T4 and rising TSH levels, requiring treatment with L-T4 (14). Abnormal confirmatory TSH values should be followed up until 2 or 3 years of age, even in patients with an apparent properly adjusted set point for pituitary–thyroid feedback. Unfortunately, few NBS programs routinely follow up detected cases beyond the diagnosis.

After discussing the concern with the parents and paying close attention to avoid overtreatment, some physicians would decide to give L-T4 to infants to maintain both TSH and thyroid hormone levels within the normal range (57). A low dose of L-T4 for HTT infants with frequent monitoring after initiation of treatment seems reasonable. Some authors have reported elevated FT4 levels at some point during the treatment period, and occasionally, L-T4 treatment is stopped due to iatrogenic hyperthyroidism or thyrotoxicosis (8, 25, 67, 68). Therefore, in all those patients for whom L-T4 replacement therapy is started, it is imperative to ensure follow-up until 2 or 3 years of age and to conduct medically supervised trial-off therapy when warranted. This may reduce iatrogenic hyperthyroidism, medical costs, and parental anxiety associated with HTT management (8).

In **Figure 4**, we have presented the approach to the management of neonatal HTT based on the reviewed literature. A matter of concern is whether it is appropriate to resume L-T4 treatment if the TSH levels are slightly elevated following treatment withdrawal. Overall, withdrawal of treatment was judged as unsuccessful, and medication was restarted in 78% of the cases (**Table S7**). Because increased TSH levels seem to be almost always compensated and do not worsen over time, most pediatricians would take the decision to cease treatment and not to resume it despite the presence of SCH beyond 2 or 3 years of age. Children should remain under observation to ensure suitable levels of FT4 throughout childhood.

Are There Factors Associated With Long-Term Clinical Evolution?

The prediction of the long-term evolution of these infants is still difficult. The evolution of thyroid function cannot be predicted by thyroid imaging tests. The occurrence of abnormal imaging tests was found to be higher in the persistent than in the transient form of HTT. However, the overall frequency of abnormal imaging tests in neonatal HTT is low (**Table S4**).

The clinical spectrum of thyroid dysfunction caused by sequence variants in the *TSHR* gene is wide. However, mildly

altered thyroid tests are commonly observed during childhood or adolescence in these patients (**Table S5**).

The variable phenotypic expression can be seen in cases with the same genotype. Mizuno et al. performed a clinical investigation of Japanese patients who had HTT as neonates, in whom a homozygous R450H sequence variant had been demonstrated. They found that although patients may not exhibit obvious hypothyroidism in infancy, resistance to TSH will become apparent with time (29). The elevation of circulating TSH would sometimes represent a compensatory mechanism in the presence of partial refractoriness to TSH action (69). Thyroid hormones are critical for growth and metabolic functions, and thyroid functional requirements increase in certain moments of life such as the neonatal period and puberty. It is always possible that more stringent environmental challenges, such as mild iodine deficiency, can stretch the limited resources of these subjects and manifest as overt hypothyroidism (35).

Follow-up is necessary to characterize a transient or persistent disorder. For children and adolescents who have had neonatal HTT, an overall frequency of SCH of 45% was estimated (**Table S7**).

The significance of SCH during childhood is still a matter of debate. This condition suggests a compensated state of primary thyroid failure requiring increased levels of TSH to maintain normal levels of thyroid hormones. Hashimoto's thyroiditis is by far the most common cause of SCH. However, in many cases, etiology cannot be identified. Idiopathic SCH has been described in children with neonatal isolated HTT.

Prospective studies with long-term close follow-up of children with neonatal-onset HTT are missing. An Italian group has reexamined a cohort of children with neonatal HTT or false-positive results at NBS. SCH was found in 28 of 44 children at the age of 2–3 years. SCH persisted in 14 of 44 (32%) children at an average age of 8 years; however, most of those children reversed to a normal thyroid function during advanced childhood (5). The reason for this normalization with increasing age is unknown. When environmental factors are auspicious, even partially impaired thyroid function may provide sufficient thyroid hormones, and the child will reach normal TSH values. The authors further suggest that these children might be prone to develop hypothyroidism if environmental conditions will become less favorable at a later stage (5).

Is There Any Convincing Evidence About the Impact on Cognitive Development in These Children That Supports L-T4 Replacement Therapy?

The most interesting question that remains to be answered is undoubtedly whether L-T4 treatment improves the neurodevelopmental outcome. In general, studies have shown normal cognitive development in children with neonatal-onset HTT; nonetheless, conclusions are limited by small sample size, poor adjustment for confounding factors, or absence of clinic-based diagnostic tools.

Observational studies cannot demonstrate causality. As far as infants are concerned, further neurodevelopmental studies are required, including psycho-intellectual evaluation of treated versus untreated infants. While randomized controlled double-blind trials comparing thyroid hormone treatment and placebo (or observation only) on neurodevelopmental would be the best way to definitively answer the question about the benefit of treatment, those studies are unlikely to be performed for ethical reasons. Until now, the precise neurodevelopmental risks posed by mild disease remain uncertain.

As far as we know, this is the first systematic review on neonatal HTT. Within the limitations of this study, it should be mentioned that, in general, our estimations are derived from data extracted from heterogeneous studies. Between-study variability originates mainly from study design, inclusion criteria, and cutoff values for TSH. The most significant limitation found in the existing published data, as previously discussed, is the lack of consensus in definitions and differentiation between transient and persistent HTT.

According to the reviewed literature, HTT is present in neonates with an estimated incidence of 0.06%. Although it is still unknown which is the most common causation of persistent neonatal HTT, if any, infants suspected of persistent neonatal HTT should be closely followed up until 2 or 3 years of age. Long-term follow-up is also needed to detect moments in life when thyroid functional requirements increase, or environmental conditions become less advantageous. Here, it is tempting to consider whether the condition presenting with circulating TSH values fluctuating above the upper limit of the normal range should be termed as permanent HTT. Perhaps, a distinction should also be made between terms persistent and permanent.

It is not a simple decision to treat these infants. We have presented a flowchart for the management of neonatal HTT based on published literature. Although the optimal approach to this condition is still controversial, this systematic review should provide helpful guidance. Until now, the precise neurodevelopmental consequences caused by mild thyroid disease remain uncertain. Controlled studies are needed to determine whether the impact on cognitive outcome in these children supports L-T4 replacement therapy. Meanwhile, accurate assessment of developmental status, close follow-up of affected individuals, and a future systematic review on this specific topic will provide valuable information to improve disease management.

AUTHOR CONTRIBUTIONS

MT designed the study. MT and AC screened articles, determined eligibility, and performed data extraction. MT and AC drafted the final manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2021.643307/full#supplementary-material

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REVIEW
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Monocarboxylate Transporter 8 Deficiency: From Pathophysiological Understanding to Therapy Development

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Genetic defects in the thyroid hormone transporter monocarboxylate transporter 8 (MCT8) result in MCT8 deficiency. This disorder is characterized by a combination of severe intellectual and motor disability, caused by decreased cerebral thyroid hormone signalling, and a chronic thyrotoxic state in peripheral tissues, caused by exposure to elevated serum T3 concentrations. In particular, MCT8 plays a crucial role in the transport of thyroid hormone across the blood-brain-barrier. The life expectancy of patients with MCT8 deficiency is strongly impaired. Absence of head control and being underweight at a young age, which are considered proxies of the severity of the neurocognitive and peripheral phenotype, respectively, are associated with higher mortality rate. The thyroid hormone analogue triiodothyroacetic acid is able to effectively and safely ameliorate the peripheral thyrotoxicosis; its effect on the neurocognitive phenotype is currently under investigation. Other possible therapies are at a pre-clinical stage. This review provides an overview of the current understanding of the physiological role of MCT8 and the pathophysiology, key clinical characteristics and developing treatment options for MCT8 deficiency.

Keywords: MCT8 deficiency, monocarboxylate transporter 8, Allan-Herndon-Dudley syndrome (AHDS), thyroid hormone transport, thyroid hormone signaling

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INTRODUCTION

Throughout life, thyroid hormone plays an indispensable role in many processes in almost all tissues of the human body. During prenatal and early postnatal life, adequate thyroid hormone signalling is crucial for normal neurodevelopment (1). Furthermore, thyroid hormone regulates key metabolic processes (e.g. mitochondrial respiration) in various tissues, including the liver, kidneys and muscles (2, 3).

Thyroid hormone is the common name for both the prohormone thyroxine (T4), the major product of the thyroid, and the biologically active triiodothyronine (T3). Intracellular thyroid hormone signalling is governed by three major processes: 1) transport of thyroid hormone across the cell membrane, facilitated by specific thyroid hormone transporter proteins, 2) conversion of T4 into T3 or the inactive metabolite reverse (r)T3 and further degradation into other inactive thyroid hormone metabolites by deiodinating enzymes types 1-3 (DIO1-3), and 3) genomic action of T3

upon binding to thyroid hormone receptor (TR) α and β (4). Together, these mechanisms allow for a precise and tissue-specific regulation of intracellular thyroid hormone signalling. This is pivotal for proper development and function of many tissues and crucial for the overall homeostasis of the hypothalamus-pituitary-thyroid (HPT) axis (2, 3). Hence, alterations in any of these mechanisms can result in tissue-specific thyroid hormone signalling defects. In human, defects in all these mechanisms have been identified and such disorders generally impair cellular thyroid hormone signalling (5–12).

To date, the most specific thyroid hormone transporter identified is monocarboxylate transporter (MCT) 8, encoded by *SLC16A2* on chromosome Xq13.2 (13). Pathogenic variants in this gene result in a clinical syndrome of severe intellectual and motor disability and increased serum T3 concentrations, leading to thyrotoxic symptoms in peripheral tissues, together known as MCT8 deficiency [also known as Allan-Herndon-Dudley Syndrome (AHDS); OMIM number 300523] (8, 9, 14). Patients generally have poor head control, remain non-verbal, are wheelchair bound and severely underweight and often die at a young age (15). Since the identification of the first series of patients with MCT8 deficiency, many efforts have been undertaken to better understand this rare disorder and to develop potential therapeutic strategies (**Figure 1**). However, important pathophysiological questions remain unanswered to date and treatment options are limited.

PHYSIOLOGICAL ROLE OF MCT8

The *SLC16A2* gene comprises two transcriptional start sites, resulting either in an MCT8 protein of 613 amino acids (referred to as the 'long' isoform) or 539 amino acids (referred to as the 'short' isoform). Although the functional properties of both isoforms are highly similar, the short isoform is generally considered physiologically relevant, since this is the only isoform identified in human tissue to date. *In vitro* overexpression studies have demonstrated a role for the extended N-terminus, specific to the long MCT8 isoform, in ubiquitin-dependent proteasomal degradation, thereby potentially regulating expression of MCT8 protein (16, 17). Yet, there is no definitive proof for the existence of a long MCT8 protein isoform under physiological conditions, which led to the recent change of the reference sequence from the long isoform (NM_006517.3; NP_006508.1) to the short isoform (NM_006517.5; NP_006508.2). It should be emphasized that most literature on MCT8 available to date used the long translational isoform to assign the position of variants. To avoid confusion amongst researchers and clinicians, we recently proposed to continue using the long isoform of MCT8 (counting from the first translational start site) in the nomenclature of *SLC16A2* variants, according to the majority of variants described in literature (18, 19).

MCT8 is capable of mediating the flux of thyroid hormone across the cell membrane through facilitated diffusion, independent of pH or a Na^+ gradient (18). Major substrates of MCT8 are the iodothyronines T3 and T4; to a lesser extent, it is also capable to mediate transport of the inactive metabolites rT3 and 3,3-diiodothyronine (3,3'-T2) (20). Both uptake and efflux of thyroid hormone across the cell membrane are facilitated by MCT8 (21). Interestingly, transport of thyroid hormone metabolites lacking the αNH2 group [e.g. 3,3',5-triiodothyroacetic acid (Triac) and 3,3′,5,5′-tetraiodothyroacetic acid (Tetrac)] is not dependent on MCT8 (22, 23). Silychristin, a flavonolignan found in some traditional European and Asian medicinal compounds, is capable of specifically and effectively blocking MCT8-mediated thyroid hormone transport in different *in vitro* and *ex vivo* models (24, 25). Based on studies using the inhibitors bromsulphthalein (BSP) and desipramine, Jomura et al. suggest that MCT8 can be

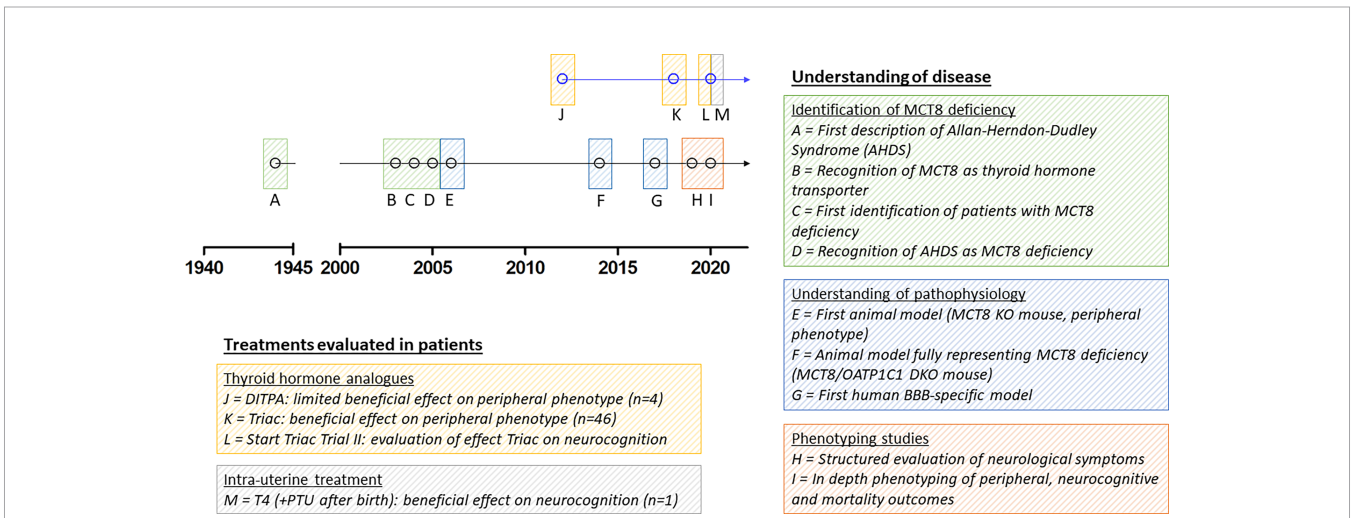

FIGURE 1 | Schematic representation of seminal discoveries in understanding of disease and treatment effects in patients. Due to limited space, we were unable to include all other relevant discoveries on MCT8 deficiency in the figure. MCT8, monocarboxylate transporter 8; AHDS, Allan-Herndon-Dudley Syndrome; KO, knock-out; OATP1C1, organic anion transporter 1C1; DKO, double knock-out; BBB, blood-brain barrier; DITPA, diiodothyropropionic acid; T4, thyroxine; PTU, propylthiouracil.

involved in efflux of the anti-epileptic drug phenytoin across the blood-brain barrier (BBB) (26). However, as these inhibitors are not MCT8-specific and with BSP being an inhibitor of the majority of thyroid hormone transporting organic anion transporter proteins (OATPs) (18), additional evidence is warranted to support this hypothesis.

MCT8 is ubiquitously expressed throughout the human body. Both *MCT8* mRNA and protein are most prominently expressed in the liver, but are also found in significant quantities in the thyroid, kidneys, pituitary and brain (27). In particular, MCT8 is expressed in different neural cells, including subtypes of neurons, astrocytes, oligodendrocytes and tanycytes (28, 29). Expression of MCT8 in tanycytes may potentially play an important role in the negative feedback of HPT axis homeostasis (30). During all stages of intra-uterine development, MCT8 expression is observed in vascular structures within the foetal brain and, later in development, in their surrounding astrocytes (31). Moreover, MCT8 expression is observed in radial glial cells, leptomeningeal cells and blood vessels in the subarachnoid space of murine models (at 14 to 38 gestational weeks) (31). These findings are indicative of a prominent role for MCT8 at the BBB and, to a lesser extent, also at the blood-cerebrospinal fluid barrier (BCSFB), and underlie the current paradigm that MCT8 is crucial for thyroid hormone transport across the BBB in particular. Interestingly, recent detailed protein expression studies from Wilpert et al. on murine brain tissues demonstrated strong expression of MCT8 in the brain barriers and many subpopulations of neurons (including cortical and cerebellar neurons) at a young age (postnatal day 6, representative for the foetal phase in human), but a sharp decrease in MCT8 expression in neurons upon aging, whereas expression in the BBB and BCSFB did not change upon aging. In adult post-mortem and fresh human brain tissues from 4 older individuals (50 – 82 years), a similar pattern of strong MCT8 expression in endothelial cells and minimal to no expression in neuronal tissues was found (32). Together, these findings indicate the presence of MCT8 in the majority of brain tissues during (prenatal) development, whereas its presence may be restricted to the brain barriers later in adult life. Yet, it is hitherto unknown how MCT8 functionally contributes to intracellular thyroid hormone homeostasis in different cell types at different developmental stages.

Despite its ubiquitous expression in human tissues other than brain (so called peripheral tissues), the physiological role of MCT8 in these tissues is less well-defined. Importantly, along with MCT8, various alternative thyroid hormone transporters are ubiquitously expressed throughout the peripheral tissues (members of the L-type amino acid transporter and OATP family, as well as Na+-taurocholate cotransporting polypeptide and the recently identified thyroid hormone transporter SLC17A4) (18). Hence, other peripheral tissues have a variable dependence on MCT8 for adequate thyroid hormone homeostasis (33, 34).

MCT8 is highly expressed on the basolateral side of follicular epithelial cells of the murine thyroid, as demonstrated by different *in situ* hybridization, immunohistochemistry and immunoblot analyses (35, 36). Similar observations were made in the human thyroid (37).

PATHOPHYSIOLOGY OF MCT8 DEFICIENCY

The relevance of adequate MCT8-mediated thyroid hormone transport becomes apparent in patients harbouring mutations in the *SLC16A2* gene. Such mutations result in a phenotype of severe intellectual and motor disability and signs of peripheral thyrotoxicosis, including negative clinical sequelae as tachycardia and being severely underweight. As *SLC16A2* is located on the X chromosome, mostly men are affected; one female with MCT8 deficiency due to unfavourable X-inactivation has been described (38). A detailed description of the clinical characteristics of this disease is provided in section *Disease Characteristics* of this review.

Different animal models have been exploited to better understand the pathophysiology of MCT8 deficiency. The first animal model generated for these purposes was the Mct8 knockout (KO) mouse (39, 40). In mice, which are commonly studied to delineate tissue-specific intracellular thyroid hormone signalling processes, the expression pattern of MCT8 is largely comparable to the human situation (18). However, in contrast to the human BBB, the BBB of mice (and other rodents) co-expresses the T4-specific transporter Oatp1c1, enabling T4 transport into the brain and subsequent local conversion into T3 by Dio2 (41). This observation is highly relevant in studying cerebral thyroid hormone homeostasis, as murine models do not well resemble the human physiological situation. Although Mct8 KO mice well resemble the serum thyroid hormone fingerprint of patients with MCT8 deficiency, no overt neurological abnormalities were identified. Therefore, results obtained in rodent brains should be translated with caution to the human situation (42). Hence, Mct8 KO mice are generally considered to be an adequate model for the peripheral thyrotoxic state observed in patients with MCT8 deficiency, but should not be used when evaluating (treatment effects on) the neurocognitive phenotype (42). Subsequently, an Mct8/Oatp1c1 double KO (DKO) mouse model was generated, which indeed mimicked both the peripheral thyrotoxic as well as the neurocognitive phenotype of MCT8 deficiency. The latter was illustrated by pronounced locomotor abnormalities, altered Purkinje cell dendritogenesis, a reduction in cerebral T3 and T4 content to 10%, decreased cerebral expression of the thyroid hormone sensitive gene *Hairless* and reduced cerebral levels of myelin basic protein (MBP), indicating hypomyelination (41). More recent studies suggest that Mct8/Dio2 DKO mice may also mimic human MCT8 deficiency, as inactivation of Dio2 prevents the formation of sufficient intracerebral T3 concentrations even in presence of functional Oatp1c1 (43). In addition to murine models, zebrafish and chicken models are available and resemble parts of the phenotypic spectrum of MCT8 deficiency (44, 45).

The functional contribution of MCT8 in the BBB only became recently apparent when Vatine et al. successfully obtained MCT8 deficient vascular endothelial and neural cells from patient-derived induced pluripotent stem cells (iPSCs) (46). In these MCT8 deficient neural cells, reduced thyroid hormone uptake capacity was found. However, the ability of these MCT8 deficient iPSCs to differentiate to neural cells was grossly unaltered. This latter observation suggests that, despite the absence of functional MCT8, sufficient intracellular T3 concentrations are reached in these cells. Utilizing iPSCs to model the BBB demonstrated the relevance of MCT8 in this barrier, with transport of T3 across the modelled BBB being significantly reduced in absence of MCT8 (46). Defective MCT8 in specific neuronal populations or in neural stem cells from patient derived iPSCs was not studied. Complementary studies of Mayerl et al. on adult hippocampal neurogenesis in murine neural stem cells showed that this process is largely regulated by thyroid hormone signalling in a cell-autonomous fashion, with MCT8 functioning as an important gate keeper (47). Upon both global deletion and adult neural stem cell-specific deletion of MCT8, differentiation of hippocampal neuroblasts was reduced. Similarly, recent studies of Vatine et al. demonstrated that oligodendrocyte precursor cells (OPCs) are not able to generate myelinating oligodendrocytes in the context of reduced cerebral thyroid hormone signalling, as observed in MCT8 deficiency (29). It should be noted that these studies used different models representing different neural cell types and different stages of neuronal development, limiting direct comparison of their results. López-Espíndola et al. performed post-mortem studies on the cerebral cortex and cerebellum of a human foetus with MCT8 deficiency (30 gestation weeks) and an 11-year old patient with MCT8 deficiency (48). The foetal brain already showed delayed cortical and cerebellar development (including altered Purkinje cell dendritogenesis), hypomyelination (indicated by low levels of MBP) and impaired maturation of the axons, indicating that the brain suffers from a severe state of low thyroid hormone signalling during pregnancy. Similar features were identified in the brain of the 11-year old patient, thus excluding spontaneous recovery of the neural aberrations with increasing age. Cerebral cortex T3 and T4 concentrations were reduced by 50%. The observed features were in line with the features found in Mct8/Oatp1c1 DKO mice (41). Interestingly, the reduction in cerebral thyroid hormone content was less prominent when compared to Mct8/Oatp1c1 DKO mice. This remarkable difference is currently not well understood; amongst other factors, differential expression of so far unidentified thyroid hormone transporters or other protecting mechanisms could underlie this observation. Together with the studies of Wilpert et al., these studies point towards a prominent role of MCT8 in the transport of thyroid hormone across the brain barriers, and the role of MCT8 in neural cells appears to vary between cell types and developmental stages.

Over the years, multiple hypotheses have been postulated on the pathophysiology of the distinct thyroid hormone fingerprint in MCT8 deficiency. It was first reasoned that, due to reduced cerebral T3 uptake, a progressive buildup of T3 in serum stimulates DIO1 activity in peripheral tissues, thus further aggravating the peripheral thyrotoxicosis by enhancing T4 to T3 conversion. In Mct8/Pax8 DKO and Pax8 KO mice, both being completely athyroid, injection of T3 resulted in similar serum T3 concentrations in both groups (35). In contrast, injection of T4 resulted in lower serum T4 concentrations in Mct8/Pax8 DKO mice compared to Pax8 KO mice, indicating increased deiodinase activity independent of the circulating T3 concentrations. Hence, a reduced cerebral thyroid hormone uptake does not explain the characteristic high T3:T4 ratio observed in MCT8 deficiency. As MCT8 is expressed in human tanycytes and thyrotropin releasing hormone (TRH)-expressing neurons (28, 49), it was hypothesized that MCT8 may have an important role in controlling the HPT axis, and in particular the negative feedback system. Moreover, the co-expression of MCT8 and DIO2 in folliculostellate cells of the human pituitary indicates that MCT8 might also control the HPT axis on pituitary level, with folliculostellate cells acting in a paracrine fashion (49). In Mct8 KO mice the thyroidal secretion of T4 is decreased, whereas increased release of T3 is observed (35, 36). It should be noted that the ratio of thyroidal T3 *vs* T4 secretion and intrathyroidal deiodinase activity are different between rodents and humans (50). Taken together, it is likely that MCT8 plays a role in thyroid hormone homeostasis at all levels of the HPT axis (**Figure 2**). This, however, does not completely explain the high serum T3:T4 ratio that is observed in all patients with MCT8 deficiency (see *Disease Characteristics*), as the increased peripheral thyroid hormone metabolism remains not well understood in this hypothesis.

A third hypothesis focusses on deiodinase activity in peripheral tissues. Dio1 has a role in rT3 clearance and converts the prohormone T4 into biologically active T3. Dio2 has an activating function by catalysing T4 to T3 conversion, whereas Dio3 primarily has a role in inactivation of thyroid hormone (conversion of T4 to rT3 and T3 to T2) (4). In the liver and kidneys of Mct8 KO mice, strongly increased activity of Dio1 was observed, whereas increased Dio2 activity was found in the brain, pituitary and skeletal muscles (39, 40). In these mice, increased liver T3 content and increased expression of Dio1 were observed, and resulted in clinical features of increased thyroid hormone signalling in the liver (e.g. decreased serum cholesterol concentrations), in line with observations made in patients with MCT8 deficiency (15, 39). By utilizing Mct8/Dio1 DKO mice, Liao et al. demonstrated that the alterations in Dio1 activity are primarily responsible for the high T3:T4 ratio in Mct8 KO mice (51). Interestingly, proximal tubule cells that normally express MCT8, showed increased Dio1 expression in the absence of MCT8. Moreover, upon peripheral injection of radiolabelled T3 and T4 in Mct8 KO mice, increased accumulation of radioactivity was observed in kidney, but not in liver homogenates (40). Also, liver-specific KO of Dio1 in Mct8 KO mice did not result in normalization of the circulating thyroid hormone levels, suggesting that the increased hepatic Dio1 expression is not causing the abnormal thyroid hormone levels (52). Together, these findings hint towards a crucial role for MCT8 in renal T4 efflux, thereby maintaining global thyroid hormone homeostasis (**Figure 2**). Based on these findings, it is currently hypothesized that, due to deficient MCT8-mediated

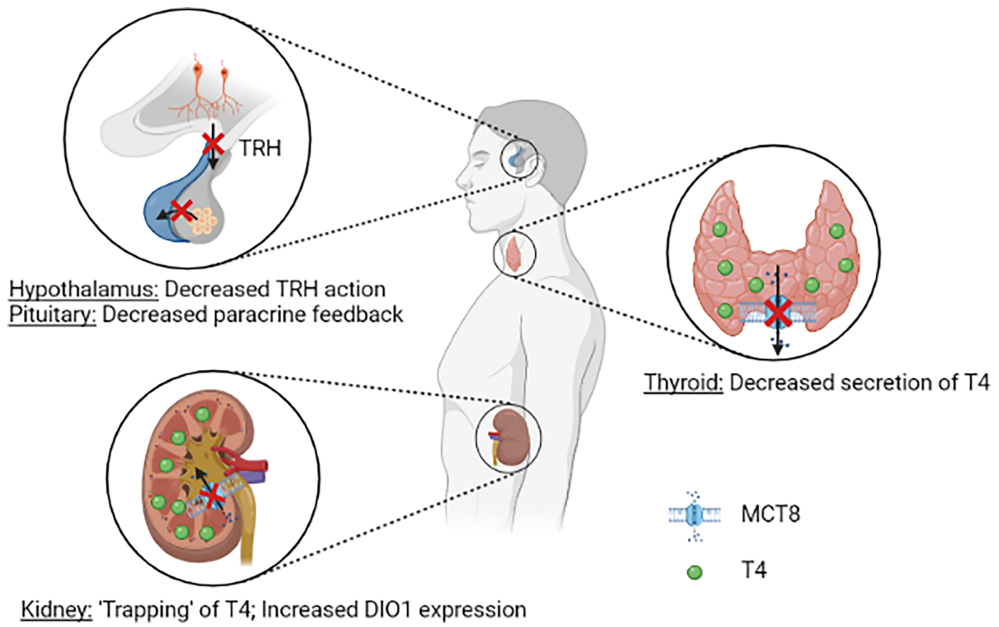

FIGURE 2 | Schematic representation of the current understanding of potential pathogenic mechanisms underlying the abnormal serum thyroid hormone concentrations in patients MCT8 deficiency. Loss of MCT8 function may lead to altered thyroid hormone sensitivity in the hypothalamus and pituitary (upper left panel). Loss of MCT8 function results in decreased thyroidal T4 secretion (right panel). Loss of MCT8 function may results in decreased excretion and 'trapping' of T4 in the kidneys, and subsequent increase in DIO1 expression. TRH, thyrotropin releasing hormone; T4, thyroxine; MCT8, monocarboxylate transporter 8; DIO1, deiodinase type 1.

thyroid hormone excretion, T4 is trapped in the kidneys, which results in an upregulation of Dio1. Subsequent to this increased expression, T4 is rapidly conversed to T3 by Dio1 and released to the circulation by other transporters, resulting in increased serum T3 concentrations. However, further studies are warranted to confirm if, and decipher how the kidneys contribute to the distinct fingerprint of thyroid function tests in MCT8 deficiency.

DISEASE CHARACTERISTICS

In 1944, Allan, Herndon and Dudley first described families with several members suffering from an X-linked form of neurodevelopmental delay, resulting in a lack of speech and impaired walking ability. Hence, it was coined Allan-Herndon-Dudley Syndrome or AHDS (53). The underlying pathogenic mechanism of AHDS remained unclear up until 2004. Soon after the identification of MCT8 as a specific thyroid hormone transporter (13), the first patients with a mutation therein were identified, presenting a remarkably similar clinical phenotype as those males described by Allan, Herndon and Dudley (8, 9). In 2005, Schwartz et al. provided definite proof that the families originally described by Allan, Herndon and Dudley indeed carried mutations in MCT8 (14). Thus, the terms MCT8 deficiency and AHDS both comprise the same syndrome and are both commonly used in the field. With the current tendency to avoid eponyms, MCT8 deficiency is currently preferred to describe the disease.

Initial reports mainly focused on the neurodevelopmental phenotype, resulting from defective thyroid hormone transport into the brain. Over time, it was increasingly recognized that, in contrast to the brain, other tissues of the human body (hereafter called peripheral tissues) are in a chronic thyrotoxic state. Due to the abundance of alternative thyroid hormone transporters in peripheral tissues, these tissues adequately sense the high circulating T3 concentrations.

When studying the genetic basis of MCT8 deficiency, it is critical to discriminate deleterious mutations from benign (rare) variants (54). Approximately 150 different mutations in about 250 different families have been described in literature and a prevalence of 1:70,000 males has been suggested (15, 55). They can be classified in three major groups: large deletions resulting in an incomplete MCT8 protein, insertions/deletions/nonsense mutations resulting in a frameshift or premature truncation, and missense mutations resulting in a single amino acid change (18). Whereas the pathogenic character of the first two categories can be predicted with considerable certainty, not all missense variants impair MCT8 thyroid hormone transport function. Evaluation of residual thyroid hormone transport capacity of these variants in *in vitro* systems or patient-derived fibroblasts is therefore indispensable to confirm the pathogenic nature of identified variants (56–58). Recent studies suggested that C-terminal missense variants beyond amino acid residue Met574 (long isoform) are well-tolerated and likely to not results in a

phenotype (59). Additional studies are warranted to further delineate the relationship between the different mutations and the phenotypic variability.

In the years following its discovery, understanding of the phenotype of MCT8 deficiency largely relied on reports of individual cases and studies including small cohort of patients with MCT8 deficiency. With the number of identified patients strongly increasing with rising awareness amongst physicians in recent years, the opportunity arose to study larger cohorts of patients (15, 60). Following structured evaluation of two cohorts, better understanding of different aspects of MCT8 deficiency was obtained. The first cohort, described by Remerand et al., comprised of 24 patients in whom specifically the neurological features were further detailed (60). In line with previously reports, they showed that the neurocognitive phenotype of MCT8 deficiency comprises severe intellectual and motor disability. Hypotonia, spasticity and dystonia are key clinical features (observed in 100%, 71% and 75%, respectively). The majority of patients had poor head control, did not develop speech and did not attain the ability to sit or walk. Magnetic resonance imaging (MRI) depicted hypomyelination in the majority of patients (19 out of 24 patients) and global brain atrophy in approximately 50% of patients. Similar observations were made earlier, in 13 MRI-scans of 6 patients (61). Moreover, detailed diffusion tensor imaging, available in three subjects, demonstrated a lack of definition in the anteroposteriorly directed association tracts, whereas the commissural white matter tracts (indicating the corpus callosum) and the corticospinal tracts appeared relatively normal. A recent review of the available literature suggested that MRI-scans of patients show gradual improvement of myelination throughout life, indicating a state of delayed myelination rather than hypomyelination (62). In contrast, post-mortem microscopic evaluation of the brain of an 11-year old patient demonstrated deficient myelination [also see *Pathophysiology of MCT8 Deficiency* (48)]. Strikingly, in the cohort of Remerand and colleagues, 8 out of 24 (33%) patients were able to walk, indicative of a less severe phenotype. This proportion was considerably higher than estimates based on available case reports and case series (18, 61), suggesting that patients with a relatively less severe phenotype were overrepresented in this cohort. Although Remerand et al. reported on thyroid function tests, the peripheral phenotype had not been further detailed.

Hence, the establishment of a larger cohort was warranted, in order to obtain more robust phenotypic data covering both the neurological and peripheral phenotype. In an international multicenter effort, Groeneweg et al., described the phenotypic characteristics of up to 151 patients with MCT8 deficiency (15). Similar observations of key clinical neurological symptoms were found as in the cohort of Remerand et al., albeit with a much lower proportion of patient who had developed walking abilities (4 out of 77 patients). Also, this study was the first to systematically collect quantitative data on the motor and cognitive abilities of patients with MCT8 deficiency, as assessed with the Gross Motor Function Measure-88 and Bayley Scales of Infant Development III. These abilities plateaued at a median developmental age of well below 12 months, whereas the median age of evaluation was 6.4 years.

Next to neurodevelopmental symptoms, this study was the first to provide large-scale data on the peripheral phenotype (15). Observed hallmark features were elevated serum T3 concentrations (present in 95% of patients), hypothyroxinaemia (present in 89% and 90% of patients for serum free T4 and total T4 concentrations, respectively), with serum thyroid stimulating hormone (TSH) concentrations within the age-specific reference range in the majority (89%) of patients. Serum rT3 concentrations were low in 91% of patients, resulting in a high T3:rT3 ratio in all patients. No difference in serum T3 concentrations was observed in patients with severe *vs* less severe phenotype. In contrast, patients with a less severe phenotype showed significantly higher total T4 concentrations when compared to patients with a severe phenotype (although still below the reference interval). As a consequence, the T3:T4 ratio was lower in patients with a less severe phenotype. Importantly, of eight patients with T4-based neonatal screening data available, the majority of patients (88%) had total T4 concentrations below the 20th percentile (**Figure 3A**). None were identified through neonatal screening. Also, none would be identified as abnormal in TSH-based screening programs, which are commonly utilized (**Figure 3B**). Recent studies demonstrated that rT3 concentrations and T3/rT3 ratio might be suitable biomarkers in neonatal screening (63). As pregnancy and delivery are unremarkable in most cases (15) and treatment early in life can potentially improve the neurocognitive phenotype, early identification of patients with MCT8 deficiency through neonatal screening could be of large additive value.

As a consequence of the elevated serum T3 concentrations, clinical parameters and biochemical markers reflecting thyroid hormone action in peripheral tissues were altered. Being underweight was detected in 71% of patients and 84% of patients had hypotrophic musculature. Recurrent pulmonary infections were common, which might be related to the high incidence of impaired swallowing function (observed in 71% of patients) and increased susceptibility as a result of being

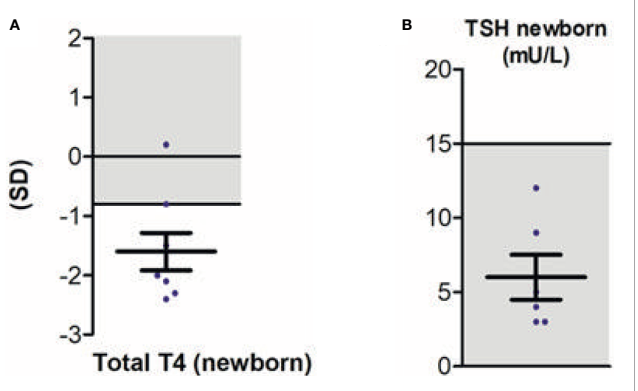

FIGURE 3 | Neonatal screening in patients with MCT8 deficiency; total T4 **(A)** and TSH **(B)**. Blue dots represent individual data points. Black bars indicate the mean ± SD. Grey areas indicate reference intervals. This figure was published in Disease characteristics of MCT8 deficiency: an international, retrospective, multicentre cohort study, Lancet Diabetes Endocrinol. 8(7), S Groeneweg et al., Figures 3D and s9I, 594-605, Copyright Elsevier (2020).

underweight. Moreover, signs of cardiovascular dysfunction were commonly observed, including premature atrial contractions (PACs), resting tachycardia and elevated systolic blood pressure (in 76%, 31% and 53% of patients, respectively). Serum sex hormone-binding globulin (SHBG) concentrations, considered to be a marker of thyroid hormone signalling in the liver, were elevated compared to its age-specific reference intervals in 88% of patients.

Whereas life span had previously been reported to appear relatively normal (14), systematic large-scale phenotyping showed a strongly decreased life expectancy in patients with MCT8 deficiency, with a median survival of 35 years (15). Proxies of the severity of both the peripheral and neurocognitive phenotype (being underweight early in life (1-3 years of age) and absence of head control before the age of 1.5 years, respectively) were strongly associated to increased mortality risk at a young age, with approximately 50% of severely affected patients dying in childhood. Importantly, sudden death was reported as a common cause of death. The observed high prevalence of cardiovascular abnormalities may point to a cardiac origin. Key characteristics such as cardiovascular function and nutritional status had been rarely documented in literature. These characteristics are potential key determinants of early death. Such detailed quantitative natural history data may serve as a control cohort in the evaluation of future therapeutic interventions, with placebo-controlled studies deemed not feasible due to the rarity of MCT8 deficiency.

DEVELOPMENT OF (POTENTIAL) THERAPIES

Currently, no therapeutic strategies are registered for MCT8 deficiency. The severity of this syndrome and its impact on quality of life of patients and their relatives, and the high mortality rate in infancy, warrant the need for adequate therapies. Since the description of the first patients with MCT8 deficiency, major research initiatives have been taken to design and develop therapies. This paragraph discusses the different treatment strategies that have been evaluated over time.

The ideal therapy should ameliorate or prevent the neurocognitive phenotype and should alleviate the peripheral thyrotoxicosis. It is generally accepted that putative beneficial effects on the neurocognitive phenotype are most prominent when treatment is commenced early in life, and are likely limited in older patients (analogous to congenital hypothyroidism). With symptoms of the peripheral phenotype linked to increased mortality risk at young age (15), therapies that can effectively and safely modulate the peripheral thyrotoxicosis should also be considered relevant, irrespective of their effects on neurocognitive outcomes.

Following the identification of the first patients with MCT8 deficiency and with hypothyroxinaemia being observed, patients were initially treated with oral T4 supplementation [(8, 9, 64–82), reviewed in (18)]. Upon treatment, the already elevated serum T3 concentrations further increased in a subset of patients, as a consequence of conversion of T4 to T3 in peripheral tissues. In line with this increase in serum T3 concentrations, body weight and other markers of thyrotoxicosis even further deteriorated. Hence, treatment with T4 monotherapy appears to aggravate the peripheral thyrotoxic phenotype. As T4 is not able to enter the human brain without functional MCT8, no improvement of neurocognitive symptoms was observed. Therefore, it is generally accepted that postnatal treatment with T4 monotherapy is not recommended in MCT8 deficiency. Following these observations, it was hypothesized that by combining T4 with propylthiouracil (PTU), which inhibits DIO1 in peripheral tissues, the aggravation of the thyrotoxicosis could be overcome. This strategy has been reported in five patients [(55, 83–86), reviewed in (18)] and resulted in a decrease in serum T3 concentration and improvement of biochemical markers and clinical symptoms of the peripheral thyrotoxicosis (decrease in serum SHBG concentrations, increase in body weight, decrease in heart rate). Following the experiences with T4 monotherapy, no alterations in the neurocognitive phenotype were observed upon treatment. Interestingly, neurocognitive improvement was observed upon intra-uterine instillation of T4 in one mother pregnant of a foetus with MCT8 deficiency, followed by T4 + PTU combination therapy after birth (87). It remains unclear why prenatal treatment had a beneficial effect on neurocognition, whereas this is not the case for postnatal treatment (9, 78). This can possibly be explained by the timing of the intervention or differential expression of alternative thyroid hormone transporters in the prenatal brain. However, as PTU carries a high risk of severe adverse reactions (e.g. agranulocytosis and liver failure), the pros and cons of treatment with a combination of T4 and PTU should be carefully balanced, in particular in vulnerable patients such as patients with MCT8 deficiency (88). Recent studies found that intranasal thyroid hormone administration, theoretically bypassing the BBB, did not result in alterations in cerebral thyroid hormone content, but rather aggravated the peripheral thyrotoxicosis in Mct8 KO mice (89). Hence, following the conclusion of the authors, intranasal thyroid hormone administration is likely not a valid treatment option in patients with MCT8 deficiency. The efficacy of this therapy has not been formally tested in patients.

With classic (anti-)thyroid drugs not being effective in MCT8 deficiency, alternative treatment options were explored. Research initiatives have been focussing on compounds called thyroid hormone analogues, which are able to cross the cell membrane independent of MCT8, with intracellular effects and degradation similar to T3 (90). In Mct8 KO mice, the thyroid hormone analogue diiodothyropropionic acid (DITPA) was able to effectively normalize serum TSH concentrations and Dio1 expression in the liver, indicative of a decrease of the peripheral thyrotoxic state (91, 92). However, as Mct8 KO mice do not fully resemble the human cerebral pathophysiology of MCT8 deficiency, these studies were less suited to address the effect of DITPA on the neurocognitive phenotype. Following these pre-clinical studies, four patients (median age 25 months, range 8.5 – 25 months) were treated with DITPA on compassionate use basis for a median time of 38.5 months (range 26 – 40 months) (85).

Upon treatment, serum T3 concentrations normalized and subsequent improvement of some markers of peripheral thyrotoxicosis, including body weight, was observed in some of the subjects. However, DITPA treatment did not result in improvements of the neurocognitive phenotypes in these patients, despite their relatively young age. After these reports, additional work showed that DITPA is able to cross the murine placenta independent of Mct8 (93). If similar observations are made for the human situation, this would classify DITPA as a potentially suitable compound for prenatal treatment of foetuses with MCT8 deficiency; this, however, remains to be elucidated. In zebrafish larvae lacking Mct8, DITPA was able to partially restore myelination six days after fertilization (94). Moreover, Lee et al. showed that DITPA restores myelination in oligodendrocytes derived from human embryonic stem cells, in which MCT8 was knocked down using short hairpin (sh)RNA technique (95). It should be noted that, despite its beneficial effects on the peripheral phenotype, DITPA is currently not commercially available.

Alongside DITPA, the therapeutic potential of the thyroid hormone analogue triiodothyroacetic acid (Triac) for MCT8 deficiency has extensively been studied. Triac is a naturally occurring thyroid hormone metabolite, which potently binds and activates the TRs and enters the cell independent of MCT8 (22). Following these observations, the efficacy of Triac was evaluated in Mct8/Oatp1c1 DKO mice. Importantly, a clear improvement in brain development was observed when mice were treated directly after birth (from postnatal day 1 to 12; dose 50 ng/g body weight), with complete restoration when high doses of Triac (400 ng/g body weight) were administered, as illustrated by normalization of cerebellar Purkinje cell dendritogenesis (calbindin immunoreactivity) and myelination (MBP immunoreactivity) (22). In line with these findings, Triac was able to completely rescue myelination in *mct8*$^{-/-}$ zebrafish larvae six days after fertilization, when brain damage has already occurred (94). An international phase II trial including 46 patients with a median age of 7.1 years demonstrated that Triac effectively reduces the high serum T3 concentrations and improved subsequent clinical and biochemical features of thyrotoxicosis, including body weight, heart rate, occurrence of PACs, SHBG and creatinine. In a subset of patients, treatment was extended up until 3 years and resulted in sustained beneficial effects on the peripheral phenotype. Except transient signs of increased thyrotoxicosis in a small subset of patients, no (severe) adverse events related to Triac were observed. Furthermore, a trend of improvement in neurodevelopment was observed in patients treated early in life (younger than four years at baseline) (96). Previous studies have demonstrated transplacental passage of Triac in women, also making Triac a suitable compound for prenatal treatment of foetuses with MCT8 deficiency (97).

In pursuit of the identification of thyromimetic molecules with a larger bioavailability in the brain, the thyroid hormone analogue sobetirome and its prodrug sob-AM2 have been studied in Mct8/Dio2 DKO mice (98). Although upon treatment serum T3 concentrations decreased, the effects of sobetirome and sob-AM2 on the peripheral phenotype and its safety profile remain unclear. In particular, upon sobetirome and sob-AM2 treatment, expression of T3-responsive genes did not alter in the liver and were increased in the heart, suggesting that these compounds may aggravate the peripheral thyrotoxic state. Cerebral *Hairless* expression levels were restored, indicative of a beneficial effect on cerebral thyroid hormone signalling. Additional evaluation of efficacy and safety is warranted before this drug can be used in a clinical setting.

Next to recovering thyroid hormone signalling *via* thyroid hormone analogues that enter the cell independent of MCT8, attempts to recover MCT8 function have been made. Gene therapy using adeno-associated virus 9 (AAV9) vector containing human MCT8 cDNA was able to increase cerebral thyroid hormone signalling in Mct8 KO mice (99). These effects were only observed after intravenous administration, but not after intracerebroventricular administration, which underlines the relevance of MCT8 at the BBB. Using a similar approach, Zada et al. showed that upregulation of the fusion protein Mct8-tagRFP completely rescued hypomyelination in *mct8*$^{-/-}$ zebrafish larvae (94). The effects of these different forms of gene therapy on the peripheral thyrotoxic phenotype as well as their safety profiles are to be explored. Hence, clinical evaluation of these promising therapies is not yet applicable.

In addition, different chemical chaperones have been exploited to improve trafficking and stability of mutant MCT8, thus potentially increasing MCT8-mediated thyroid hormone transport across the cell membrane. Depending on the cell model, different effects have been found. In overexpression models of the p.F501del mutation, treatment with phenylbutyrate and dimethylsulfoxide enhanced residual MCT8 transport capacity (100, 101). In fibroblasts containing this mutation, considered the most representative *ex vivo* model, these effects were not observed (25). The effects of such molecules on the thyrotoxic and neurocognitive phenotype are unclear, as none of these compounds have been tested in animal models for MCT8 deficiency. As this potential therapy is only applicable for a subset of mutations, its role in clinical praxis will likely be limited.

CONCLUSIONS AND FUTURE PERSPECTIVES

Since the first recognition of MCT8 deficiency, major discoveries have been made, helping to better understand this rare disorder. By utilizing different *in vitro, ex vivo* and *in vivo* models, it became clear that MCT8 particularly plays a crucial role at the BBB. Additional studies are warranted to fully understand the role of MCT8 within other parts of the brain. Results from recent deep-phenotyping re-emphasized the relevance of the peripheral phenotype in patients with MCT8 deficiency, as being underweight early in life as a proxy of the peripheral thyrotoxicosis, has been linked to increased mortality risk at a young age. These findings stress the importance of the thyrotoxic features as therapeutic target and thus the availability of treatment options that modulate the peripheral phenotype. The thyroid hormone analogue Triac, the only available safe treatment at this moment, is able to effectively and safely reduce the peripheral thyrotoxicosis in patients and might

improve the neurocognitive phenotype when treatment is initiated early in life.

Currently, a second international phase IIb trial studies the effect of Triac on neurocognitive outcomes in young patients (<30 months at baseline; NCT02396459). Another research initiative aims to treat women pregnant of a foetus with MCT8 deficiency with intra-uterine instillation of DITPA on compassionate use basis, in order to evaluate its effects on brain development when commenced early in life (NCT04143295). Aiming to gain a deeper understanding of the needs of patients and their parents and physicians, an international registry linked to the European Reference Network on Rare Endocrine Conditions centralizes knowledge on MCT8 deficiency (https://mct8registry.erasmusmc.nl/en/index.html). Together with other studies, these initiatives aim to provide additional insights in MCT8 deficiency and to decipher whether thyroid hormone analogues are able to modulate the neurocognitive phenotype when treatment is initiated early in life. With the availability of promising (prenatal) therapy, efforts should be made to detect MCT8 deficiency as early in life as possible. This goal could potentially be achieved by redefining neonatal screening programs. Given the rarity and complexity of MCT8 deficiency, international collaboration amongst researchers is warranted to attain these goals.

AUTHOR CONTRIBUTIONS

FSvG, NG, SG, and WEV reviewed available literature and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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Diagnosis and Management of Central Congenital Hypothyroidism

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Central congenital hypothyroidism (CH) is defined as thyroid hormone (TH) deficiency at birth due to insufficient stimulation by the pituitary of the thyroid gland. The incidence of central CH is currently estimated at around 1:13,000. Central CH may occur in isolation, but in the majority of cases (60%) it is part of combined pituitary hormone deficiencies (CPHD). In recent years several novel genetic causes of isolated central CH have been discovered (*IGSF1*, *TBL1X*, *IRS4*), and up to 90% of isolated central CH cases can be genetically explained. For CPHD the etiology usually remains unknown, although pituitary stalk interruption syndrome does seem to be the most common anatomic pituitary malformation associated with CPHD. Recent studies have shown that central CH is a more severe condition than previously thought, and that early detection and treatment leads to good neurodevelopmental outcome. However, in the neonatal period the clinical diagnosis is often missed despite hospital admission because of feeding problems, hypoglycemia and prolonged jaundice. This review provides an update on the etiology and prognosis of central CH, and a practical approach to diagnosis and management of this intriguing condition.

Keywords: central congenital hypothyroidism, isolated central congenital hypothyroidism, combined pituitary hormone deficiencies, etiology, diagnosis, management, pituitary stalk interruption syndrome

INTRODUCTION

Congenital hypothyroidism (CH) is defined as thyroid hormone (TH) deficiency at birth, either due to defective thyroid gland development or function (primary or thyroidal CH), or due to insufficient stimulation by the pituitary of an otherwise normal thyroid gland (central CH) (1). Central CH is often accompanied by other pituitary hormone deficiencies (combined pituitary hormone deficiency, CPHD), but can also be an isolated condition. Because TH deficiency early in life is harmful to brain growth and development, and difficult to recognize shortly after birth, newborn screening (NBS) programs for CH have been implemented in many countries worldwide since the 1970s. These programs enable early detection and treatment of CH, and successfully prevent brain damage and subsequent mental retardation (2).

The first NBS programs for CH were total thyroxine (T4)-based, combined with, or followed by thyrotropin (thyroid stimulating hormone, TSH) measurement (T4+TSH and T4-reflex TSH, respectively). Although the main objective of these programs was detection of primary CH, they also

detected central CH (2). In T4-based NBS, primary CH is suspected when T4 is low in combination with an elevated TSH concentration, and suspicion of central CH arises when T4 is low in combination with a normal TSH concentration. Unfortunately, fairly common but innocent thyroxine-binding globulin (TBG) deficiency also causes a low total T4 in combination with a normal TSH, resulting in a substantial number of false-positive referrals (3, 4). Therefore, many NBS programs switched to, or started with a TSH-based approach which only detects primary CH (5). In support of this, the general notion was that central CH is a rare and often mild condition, and that most neonates with central CH as part of CPHD are diagnosed clinically shortly after birth.

Nowadays only a few NBS programs aim at detection of both forms of CH: several state or regional programs in Italy, Japan, Spain and the USA, and the national programs of Israel and The Netherlands (2, 6–12). **Table 1** shows the characteristics of these programs. In Japan, the number of "false-positives" was reduced by measuring free T4 (FT4) and TSH. In The Netherlands a TBG measurement was added to the existing T4-reflex TSH approach (11, 13, 14) resulting in a three-step process in which T4 is measured in all newborns, and TSH and TBG in those with a T4 concentration belonging to the lowest 20% and 5%, respectively.

Since the start of NBS, the incidence of central CH in the Netherlands has steadily increased from 1:106,304 in the late 1970s/early 1980s to approximately 1:16,404 in the last two decades (15, 16). Recently, van Iersel et al. showed that between 2001 and 2011 29 children with probable central CH as part of CPHD were missed by the Dutch NBS program over a 10-year period. This would raise the Dutch incidence from 1:16,404 to around 1:13,000, consistent with data from Japan (7, 17).

During the past years the assumption that central CH is usually a mild condition has been refuted. Zwaveling-Soonawala et al. found that, based on initial FT4 concentration, more than half of all newborns with central CH have moderate to severe disease (18). The same applies to the notion that most neonates with central CH as part of CPHD are diagnosed clinically shortly after birth. In a recent study on clinical characteristics of Dutch central CH patients Naafs et al. reported that most neonates with central CH as part of CPHD were only diagnosed after notification of an abnormal NBS result, even though many patients were hospitalized in the first weeks of life for feeding problems, hypoglycemia or (prolonged) jaundice (19). This review provides an update on the etiology and prognosis of central CH, and a practical approach to diagnosis and management of this intriguing condition.

PHYSIOLOGY AND PATHOPHYSIOLOGY

Normal Hypothalamus-Pituitary-Thyroid Function

The thyroid hormones T4 (the prohormone) and triiodothyronine (T3; the active hormone) are produced and secreted by the thyroid gland in a molar ratio of about 11 to 1 (20). In serum, T4 and T3 are mostly bound to transporting proteins TBG, albumin and transthyretin; only a small part circulates as unbound free T4 and T3 (FT4 and FT3, respectively) (21).

TH enters target tissue cells by crossing the cell membrane facilitated by specific TH transporters. The intracellular amount of T3 depends on circulating T3, but a number of target tissues regulate their own optimal T3 content by enzymatically activating T4 into T3 by deiodinase types 1 and 2 (DIO1 and 2), or by converting T4 into the inactive TH reverse T3 (rT3) by deiodinase type 3 (DIO3); DIO3 also inactivates T3 into T2 (20). T3 exerts its action by binding to the thyroid hormone receptor alpha or beta (TRα or TRβ).

TH production is regulated by the hypothalamus-pituitary-thyroid (HPT)-axis, a classic endocrine negative feedback system. Depending on the amount of circulating TH the hypothalamus and pituitary adjust thyroid function by

TABLE 1 | Characteristics of NBS programs screening for both primary and central CH.

Country	NBS approach	Period after birth during which NBS is performed	T4, FT4 or T4/TBG ratio referral rules	Reference
Italy (regional program)	T4+TSH simultaneously	3-5 days of life	Referral if T4 <40 nmol/L	(6)
Japan (regional program)	FT4+TSH simultaneously	4-6 days of life	Referral if FT4 <0.5 ng/dL; second heel prick if FT4 <1.0 ng/dL, then referral if FT4 <1.0 ng/dL	(7)
Spain (regional program)	T4+TSH simultaneously	At 48 hours of life	Referral if T4 <6 μg/dL	(8)
USA (various regional/state programs)	Diverse approaches: T4+TSH simultaneously, T4-reflex TSH and TSH-reflex T4	Diverse periods ranging from first week to first month of life	Diverse approaches	(9)
USA (Northwest Regional NBS Program)	T4-reflex TSH	First NBS 1-2 days of life, second NBS 10-14 days of life	Referral if T4 <10th percentile	(4)
Israel (national program)	T4-reflex TSH	48-72 hours of life	Referral if T4 <10th percentile	(10)
The Netherlands (national program)	T4-reflex TSH-reflex TBG	4-7 days of life	Referral if T4 ≤-3 SD and TBG >40 nmol/L; if T4 <-1.6 SD, then TBG measurement; referral if T4/TBG ratio ≤17 in 1st and 2nd NBS result	(11, 12)

secreting more or less thyrotropin releasing hormone (TRH) and TSH, respectively, and thereby to maintain a stable serum TH/FT4 concentration (22). In healthy individuals TSH and FT4 concentrations show small variability while interindividual variability is large, as judged from the wide laboratory reference intervals. This suggests that each individual has their own specific HPT-axis set-point.

Central vs. Primary Hypothyroidism and Their Biochemical Characteristics

Hypothyroidism is defined as inadequate TH production causing TH deficiency at the target tissue level.

Primary hypothyroidism is caused by a defective thyroid gland and is characterized by a serum FT4 concentration below, and a TSH concentration above the reference interval. In subclinical (primary) hypothyroidism the serum FT4 concentration is still within the reference interval.

In central hypothyroidism the cause of the inadequate TH production is at the level of the hypothalamus or pituitary. In this scenario, decreased TH secretion is caused by quantitative or qualitative TSH deficiency (1). Biochemically, central hypothyroidism is defined as a FT4 concentration below the reference interval in combination with a normal, low, or slightly elevated TSH. The slightly elevated TSH concentrations observed in central hypothyroidism are partly explained by intact immunoactivity, but decreased bioactivity (23). Central hypothyroidism can be congenital or acquired (e.g., pituitary dysfunction after brain irradiation or trauma). While central CH may occur in isolation (TSH deficiency only), it is more often accompanied by other pituitary hormone deficiencies (combined pituitary hormone deficiency, CPHD (1).

In acquired central hypothyroidism, TH secretion can be reduced, while serum FT4 is still within the reference interval. This is explained by the abovementioned interindividual differences in HPT axis' set-point. This state is referred to as subclinical central hypothyroidism (24).

While diagnosing primary hypothyroidism is based on the finding of a clearly elevated TSH concentration, diagnosing central hypothyroidism relies on correct interpretation of FT4 concentration using age-specific reference intervals to recognize when FT4 is too low. However, the individual set-point may complicate correct interpretation. For example, while an FT4 concentration in the lower range of the reference interval may be normal for most individuals it does not necessarily exclude central hypothyroidism, especially in individuals with a set-point in the higher range. Yet, in many instances central hypothyroidism may be a rather straightforward diagnosis, when serum FT4 is clearly below the reference range, signs or symptoms of hypothyroidism are present and the medical history is suggestive for hypothalamic or pituitary damage or disease. This is often the case in acquired forms of central hypothyroidism, but also in congenital central hypothyroidism as part of CPHD (1).

Diagnosis is further complicated when clear signs or symptoms are absent and the medical history is uninformative, as can be the case with newborns with an abnormal NBS result suggestive for central CH; then a FT4 concentration around the lower limit of the reference interval may pose a diagnostic dilemma (by definition, 2.5% of healthy individuals have an FT4 below the reference interval).

In recent years knowledge about the etiology of central CH has increased significantly. With a combination of diagnostics, like basal and dynamic endocrine investigation, genetic testing and high-resolution magnetic resonance imaging (MRI) an increasing percentage of previously uncertain diagnoses can be confirmed.

In the next section, the various causes of isolated central CH and CH as part of CPHD are discussed in detail.

ETIOLOGY

Isolated Central CH

Currently, five genes related to isolated central CH have been identified. Molecular analyses of Mendelian forms in the 1980s and 1990s allowed recognition of pathogenic variants of the *TSHB* and *TRHR* genes, associated with TSH deficiency. Implementation of next-generation sequencing-based genetic testing in cohorts of central CH patients has significantly increased our knowledge of the etiology and clinical spectrum of isolated central CH. Recently, three other genes were implicated in isolated central CH: *IGSF1*, *TBL1X* and *IRS4* (18, 19, 25, 26). These relatively common forms of isolated central CH are X-linked, so most patients are boys. Cases of females with a mild central hypothyroidism phenotype carrying a mono-allelic pathogenic variant of these X-linked genes have been described. Isolated central CH is a monogenic disorder in all known cases. In a recent study, a high percentage of cases of isolated central CH could be explained by pathogenic variants in the five aforementioned genes (19). However, a number of cases remained unexplained, suggesting currently unrecognized pathogenic variants in other genes or perhaps a polygenic etiology. In patients with isolated central CH, structural hypothalamic-pituitary (HP) abnormalities have not been found. Severe neonatal health problems are rare. Older, seemingly asymptomatic patients have been detected through familial segregation analyses of probands with isolated central CH, stressing the rather mild character of some subtypes.

TSHB

TSHB (OMIM #188540) was the first gene associated with isolated central CH. It encodes the beta subunit of TSH, which belongs to the family of glycoprotein hormones. The alpha subunit of TSH (encoded by *GCA*) is common to the pituitary and placental glycoprotein hormones TSH, luteinizing hormone and follicle stimulating hormone (LH&FSH) and human chorionic gonadotropin (hCG), while the TSH beta subunit grants biological specificity to TSH. The first patients with isolated central CH due to *TSHB* pathogenic variants carried the homozygous c.145G>A p.(Gly49Arg) variant (NM_000549.4) (27). This variant affects the evolutionarily conserved CAGYC region (residues 47-51), which is important for normal folding and TSH-α/TSH-β dimerization. The phenotype, consisting of

quantitative TSH deficiency associated with mostly severe hypothyroidism, segregated in an autosomal recessive pattern. Later, patients were identified with different variants associated with qualitative TSH deficiencies characterized by measurable immunoreactive TSH without biological activity due to abolishment of signaling-specific domains but preserved TSH assay epitopes (28).

Given the substantial number of false-positives due to TBG deficiency in many T4-based NBS programs, it was stated that identification of heterozygous carriers of *TSHB* variants was preferable over T4-based NBS (29). Indeed, *TSHB* variants appear to be a very rare cause of isolated central CH (19), and the prevailing c.373del p.(Cys125Valfs*10) (NM_000549.4) variant was linked to a founder effect. However, the effectiveness of this approach has never been proven. Late diagnosed patients present with severe neurological deficits, while early treatment secures normal development (30). A meta-analysis of all recorded pre-treatment FT4 values underlines the severity of hypothyroidism due to biallelic *TSHB* variants (**Figure 1**).

TRHR

Not long after the discovery of pathogenic *TSHB* variants in isolated central CH, patients with biallelic pathogenic *TRHR* (OMIM #188545) variants were identified (32). *TRHR* encodes the TRH receptor, which is mainly expressed in thyrotrophs and lactotrophs. TRH is transported from the hypothalamus to the pituitary through the portal system to stimulate TSH and prolactin secretion. A logical assumption would be that beside *TSHB* and *TRHR* also the *TRH* (OMIM #613879) gene (encoding TRH) might be implicated in isolated central CH. Until now pathogenic variants of *TRH* have not been associated with central hypothyroidism in humans, but *Trh* knock-out mice have been generated (33).

Although a biallelic defective *TSHB* leads to severe hypothyroidism, this is not the case in defective *TRHR* (**Figure 1**). Different pathogenic variants underlie differences in TRH resistance (34, 35). Interestingly, even complete TRH resistance does not abolish TSHB and prolactin expression (35), an important insight into the molecular control of the HPT axis. The phenotype of moderate to mild central CH segregates in an autosomal recessive inheritance pattern, and may remain asymptomatic. However, treatment of asymptomatic patients is reported to improve their quality of life (QoL) (35). Heterozygous carriers of pathogenic *TRHR* variants with a mild effect on TRH resistance may have an elevated serum TSH; on the other hand, isolated TSH elevation instead of central hypothyroidism has been reported in homozygous carriers (34).

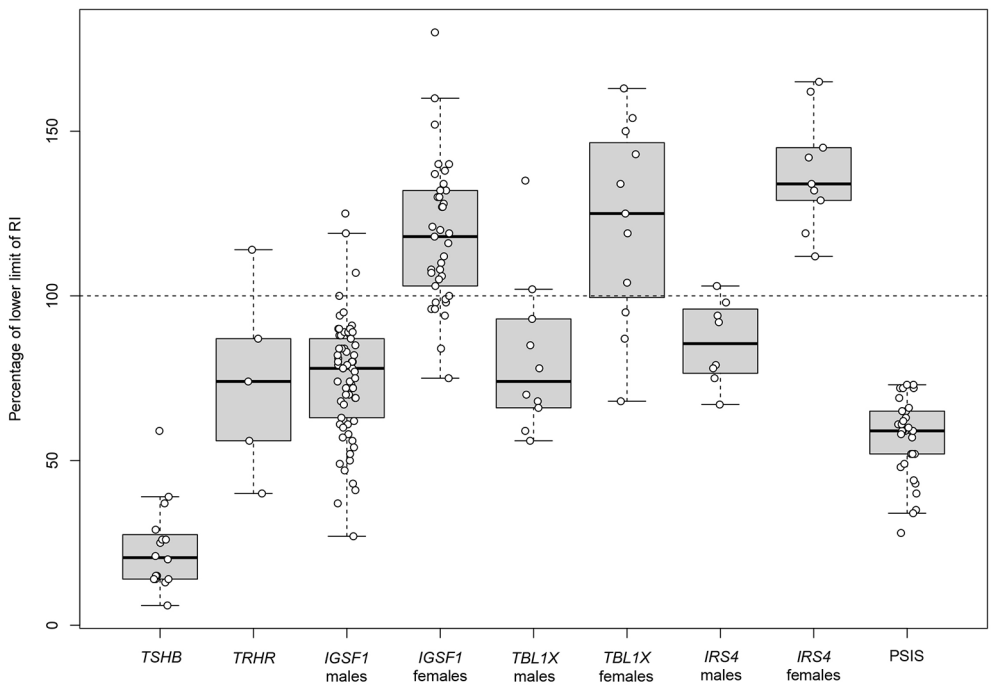

FIGURE 1 | Pre-treatment serum FT4 concentrations in patients with central congenital hypothyroidism. First pre-treatment serum FT4 concentrations in patients with isolated central CH and in patients with central CH as part of CPHD caused by PSIS. Data of isolated central CH were extracted from published studies/case reports (**Supplementary Table 1**), and expressed as percentage of the lower limit of given FT4 reference intervals. Data about central CH as part of PSIS were extracted from the study by Naafs et al. (19). FT4 values of PSIS patients measured at day 3-7 and day 12-16 after birth were compared to the lower limit of the plasma FT4 reference interval at these ages (20.5 pmol/L and 15.3 pmol/L, respectively) (31). Boxes represent median, 1st and 3rd quartile, and whiskers represent minimum and maximum. Individual data points are given as circles. CH, congenital hypothyroidism; CPHD, combined pituitary hormone deficiencies; FT4, free thyroxine; PSIS, pituitary stalk interruption syndrome; RI, reference interval.

IGSF1

The first patients with a recessive X-linked isolated central CH phenotype were reported in 2012 (36). Pathogenic variants in the immunoglobulin superfamily member 1 gene (*IGSF1*, OMIM #300137) were identified in 11 families. Until now, more than 40 pathogenic *IGSF1* variants have been described, and it is the most prevalent cause of moderate to mild isolated central CH in males and females (19).

In addition to central CH, IGSF1 deficiency in males leads to a heterogeneous phenotype, including macroorchidism, delayed pubertal testosterone rise (but a normal timing of testicular growth) and in some cases other pituitary hormone deficiencies, namely low serum prolactin and growth hormone (GH) deficiency (in childhood) (**Table 2**) (37, 38). Peculiarly, adult males exhibit increased growth hormone secretion, and as a result acromegaloid features (39). While IGSF1 deficiency segregates in a recessive X-linked pattern, females carrying a pathogenic variant present with varying FT4 concentrations, ranging from moderately reduced to normal (**Figure 1**). Other characteristics include delayed menarche, low prolactin and increased BMI. X-inactivation was studied in heterozygous female carriers to determine the cause of the observed female phenotypic heterogeneity; however, no correlation was established (37).

IGSF1 is expressed at high levels in testes and the pituitary, specifically in thyrotrophs, somatotrophs and lactotrophs (36). Its precise function within the context of the pituitary hormone axes and testicular function is still unknown (40). *Igsf1* knock-out mice have central hypothyroidism, decreased pituitary TRH receptor 1 mRNA expression and impaired responsiveness to TRH compared to healthy littermates (36, 41). This supports a role for impaired TRH action in the pathogenesis of the central CH. While mouse models have successfully recapitulated the IGSF1 deficiency central hypothyroidism phenotype (36, 41), they lack the testicular enlargement phenotype (42). Although macroorchidism may results from IGSF1 loss-of-function in testes (43), other *Igsf1* knock-out animal models need to be explored in order to further elucidate its underlying pathophysiological mechanism.

TBL1X

More recently the fourth molecular cause of isolated central CH was discovered. The first patients with a pathogenic variant in transducing-beta-like 1 gene (*TBL1X*; OMIM #300196) were identified in 2016 (44). An X-linked recessive inheritance pattern associated with an incomplete penetrance was observed. The phenotype consists of moderate to mild central hypothyroidism in hemizygous males, and mild central hypothyroidism to euthyroidism in heterozygous females (**Figure 1**). Again, X-inactivation studies could not explain phenotypic heterogeneity in females (44).

TBL1X mRNA is expressed in multiple tissues, including the pituitary and hypothalamus (44), and the TBL1X protein is an essential subunit of the nuclear receptor corepressors (CoRs) silencing mediator of retinoid and thyroid hormone receptors (SMRT) and nuclear receptor co-repressor1 (NCoR1). CoRs associate with unliganded thyroid hormone receptors (TRs) in order to repress transcription of genes that are positively regulated by T3. T3 binding results in the release of the corepressors and recruitment of coactivators leading to increased gene transcription. However, genes involved in the negative feedback regulation of the HPT-axis like *TSHB* and *TRH* are negatively regulated by T3 which means that their transcription is increased in the absence of T3. It was shown that the c.1145_1147del p.(Asn382del), c.1246A>T p.(Asn416Tyr) and c.1526A>G p.(Tyr509Cys) (NM_005647.3) variants resulted in reduced stimulation of *TSHB* and *TRH* promoters in a hypothalamic cell line (45, 46). Consequently, this may alter HPT-axis functioning to the point that the hypothalamus and pituitary are resistant to low TH levels, with a possible negative shift of the FT4 set-point.

Furthermore, some patients exhibit hearing thresholds lower than age-specific reference intervals, unrelated to pre-treatment serum FT4 levels (44, 47). It was suggested that due to TBL1X and subsequently NCoR-SMRT dysfunction there is differential expression of T3-regulated genes involved in ear development, such as pendrin (47). Similarly, the homologous gene *TBL1Y* is implicated in X-linked hearing loss (48).

An important question concerning the severity of the central hypothyroidism phenotype remains. It is not precisely known what the effect of *TBL1X* variants is on target tissue TH action. In light of this issue, patients with pathogenic *TBL1X* variants have normal TSH and FT4 responses to exogenous TRH administration, hinting at functional integrity of the pituitary.

TABLE 2 | Summary of key findings in the five genetic causes of isolated central congenital hypothyroidism.

Gene	Selected key findings	TRH test results
TSHB	Males and females (biallelic pathogenic variants): mostly severe hypothyroidism, TSH deficiency (quantitative or qualitative)	Severely reduced/absent TSH response, normal prolactin (PRL) response
TRHR	Males and females (biallelic pathogenic variants): moderate to mild hypothyroidism, elevated TSH Males and females (carriers): recurrent TSH elevation	Normal or absent TSH and PRL responses
IGSF1	Males (hemizygous pathogenic variant): moderate to mild hypothyroidism, macroorchidism, delayed pubertal testosterone rise, low prolactin, increased BMI and fat percentage, growth hormone deficiency (childhood), acromegaloid facies (adulthood) Females (heterozygous pathogenic variant): low-normal FT4 values to mild hypothyroidism, delayed menarche, low prolactin, increased BMI and fat percentage, acromegaloid facies (adulthood)	Normal or reduced TSH response, normal or reduced/absent PRL response
TBL1X	Males (hemizygous pathogenic variant): moderate to mild hypothyroidism, hearing deficits Females (heterozygous pathogenic variant): low-normal FT4 values to mild hypothyroidism	Normal TSH response, normal PRL response
IRS4	Males (hemizygous pathogenic variant): mild hypothyroidism Females (heterozygous pathogenic variant): low-normal FT4 values	Reduced TSH response, normal or slightly reduced PRL response

Also, patients that were identified at an older age have presented in seemingly asymptomatic states with normal intellectual development (44). A mouse model with a disturbed liver specific NCoR–TR binding showed upregulation of positive TH target genes in the presence of T3 (49). Accordingly, mice with global expression of this NCoR variant had biochemical features associated with central hypothyroidism, but normal growth and increased energy expenditure (50). Low TH and normal TSH concentrations in these mice may be an adaptive HPT-axis response to increased target tissue TH sensitivity. Deleterious *TBL1X* variants could have the same effect on transcription of positive TH target genes, but this has yet to be proven in animal studies.

Reported *TBL1X* variants mainly involve the WD40 domain (**Figure 2**), necessary for protein-protein and protein-chromatin interactions in the context of NCoR-SMRT action (44, 46, 47). In the first report on loss-of-function variants in *TBL1X*, families were identified with missense variants in the WD40 domain (44). Later, the phenotype of a boy with a *de novo* nonsense variant in the WD40 domain was described (47). The boy had central hypothyroidism and sensorineural hearing deficits like the previously identified patients, but also other signs that could be linked to NCoR-SMRT dysfunction, including attention deficit/hyperactivity disorder (ADHD), learning difficulties, fecal incontinence and Chiari malformation type I. More patients with premature termination codon variants need to be identified before its relation to these symptoms can be established. A phenotypic spectrum of TBL1X deficiency due to variable degrees of NCoR-SMRT dysfunction seems plausible.

IRS4

The most recently discovered genetic cause of isolated central CH was reported in 2018 (51). Five male probands from unrelated families with pathogenic variants in the insulin

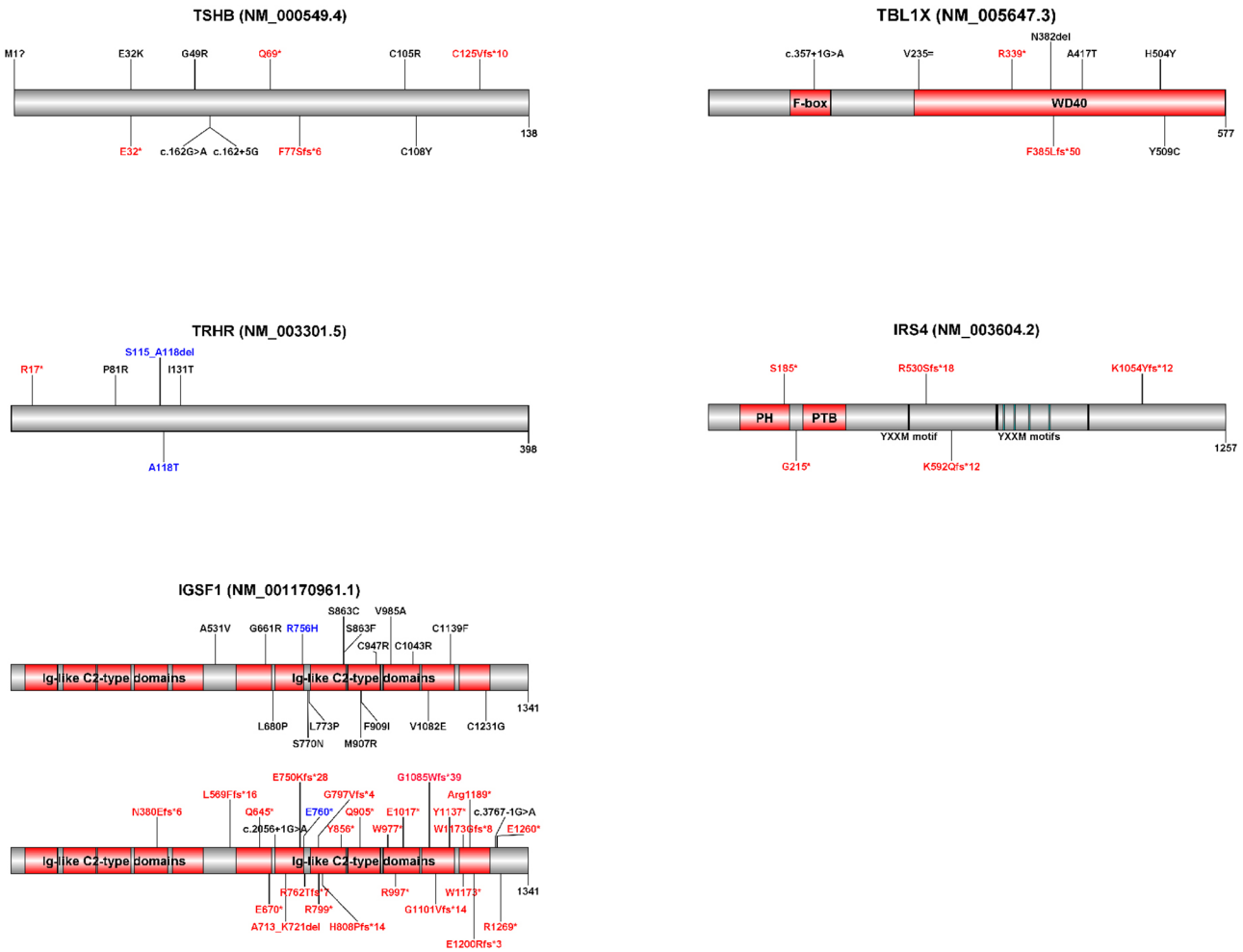

FIGURE 2 | Schematic representations of the five genes implicated in isolated central CH with known pathogenic variants. Variants displayed in red represent premature termination codon variants. Variants displayed in black represent missense, synonymous and in-frame indel variants. Variants displayed in blue were found on the same allele. Variant annotations may differ from published variants due to differences between transcripts (indicated in brackets). Diagrams were created with Domain Graph, version 2.0 (http://dog.biocuckoo.org/). Ig, immunoglobulin; PH, pleckstrin homology domain; PTB, phosphotyrosine binding domain.

receptor substrate-4 gene (*IRS4*) (OMIM #300904) had a phenotype consisting of solely X-linked central hypothyroidism and an abnormal TSH response to exogenous TRH. One patient had mild TSH elevation aside from low FT4. Females carrying a heterozygous pathogenic variant had low-normal to normal FT4 concentrations (**Figure 1**). All reported variants are premature termination codon variants. No other reports on the further phenotypic delineation have emerged since the first report, however additional patients have been identified (19).

IRS4 is expressed in hypothalamic nuclei, pituitary gland, ovaries, uterus, the thyroid gland and digestive tract among others (51, 52). *IRS4* encodes for the adaptor protein IRS4, which belongs to the family of IRS proteins. *IRS1* and *IRS2* have a widely distributed expression but limited shared amino acid sequence with *IRS4* (53). *IRS3* is a pseudogene in humans, and *IRS5* and *IRS6* have a more specific expression pattern, in liver and kidney, and skeletal muscle, respectively. Based on phylogeny studies, it was postulated that the amino acid sequence of IRS4 accelerated in early mammalian development, thus having distinct, but also overlapping functions compared with the other IRS proteins (53). IRS4 functions as an interface between hormone receptors with tyrosine or serine/threonine kinase activity (for example the insulin receptor, IGF1 receptor, FGF receptor 1, and BMP receptor 2), and signaling molecules with an SH2 domain (54, 55). Upon ligand activation of tyrosine or serine/threonine kinase receptors, intracellular sites are phosphorylated. IRS4 interacts with these phosphorylated sites, which results in phosphorylation of tyrosine or serine/threonine phosphorylation motifs. SH2 domain-containing proteins are then recruited and activated (54). Downstream signaling pathways of IRS4 include the MAPK cascade and the PI3K/AKT/mTOR pathway (56).

The pathophysiological mechanism of IRS4 pathogenic variants has not been elucidated yet. Several mouse models have failed to recapitulate the phenotype (51, 57, 58), possibly due to redundancy and overlapping functions of IRS proteins (53, 58–60). It was suggested that the central hypothyroidism phenotype is linked to leptin receptor (LEPR) dysfunction. Fasting signals are conveyed to hypothalamus through leptin/LEPR signaling, modulating *TRH* expression (61, 62). Since IRS4 is implicated in leptin/LEPR signaling (63), *IRS4* loss-of-function hypothetically diminishes *TRH* expression. Indeed, fasted subjects (an example of subjects with reduced leptin signaling) have similar TSH secretion patterns and TRH test results as patients with central hypothyroidism due to *IRS4* loss-of-function (51). However, patients with leptin deficiency and leptin resistance invariably present with central hypothyroidism, and have a different TSH secretion pattern in response to exogenous TRH than patients with *IRS4* loss-of-function. Moreover, patients with IRS4 dysfunction have blunted TSH responses to exogenous TRH, hinting at a defect at the level of the pituitary. The precise functions of IRS4 in the pituitary remain unclear. A pathophysiological mechanism of central hypothyroidism due to IRS4 dysfunction incorporating pituitary TRH resistance or defective TSH transcription/post-translational modification is conceivable.

Table 2 shows the key findings in the five genetic causes of isolated central CH. **Figure 2** shows schematic representations of the five genes implicated in isolated central CH with known pathogenic variants. **Supplementary Table 1** shows all reported pathogenic isolated central CH variants.

Central CH as Part of CPHD

Besides occurring in isolation central CH may be part of CPHD. During embryonic brain development complex cascades of signaling molecules and transcription factors influence midline brain structure formation, including HP development. The anterior pituitary lobe derives from oral ectoderm and consists of five specialized cell types, secreting six hormones: thyrotrophs producing TSH, somatotrophs producing GH, corticotrophs producing adrenocorticotropic hormone (ACTH), gonadotrophs producing LH and FSH, and lactotrophs producing prolactin. The posterior pituitary lobe derives from neural ectoderm and stores and secretes the hypothalamic produced hormones vasopressin and oxytocin. The anterior pituitary cell types receive input from hypothalamic hormones *via* the hypophyseal portal blood vessels in the pituitary stalk. The posterior pituitary has a neural connection with the hypothalamus by way of the hypothalamic-neurohypophyseal tract in the pituitary stalk (64).

CPHD is characterized by a very broad genetic and phenotypic heterogeneity with variable penetrance. In addition, CPHD not only seems to be of monogenic but also of di- and oligogenic origin. Defects in one or more genes involved in midline brain formation result in a variety of malformations. Genetic defects in factors involved in early embryonic brain development result in the most severe midline brain anomalies, while defects in transcription factors involved in the final steps of pituitary cellular differentiation cause milder CPHD without structural pituitary malformation (*POU1F1*, *PROP1*).

We divide CPHD into three main categories: 1) CPHD without pituitary malformation, 2) CPHD with isolated pituitary malformation, and 3) syndromic CPHD with pituitary and extra-pituitary malformations. The combination of pituitary hormone deficiencies and the severity are very variable. Overall GH deficiency is the most common endocrine deficit in CPHD, followed by central CH.

CPHD Without Pituitary Malformation

POU1F1

POU1F1 (previously known as *PIT1*) (OMIM #173110) was the first gene to be identified in CPHD patients. It is a pituitary-specific transcription factor expressed in the anterior pituitary, relatively late during pituitary development. It is responsible for differentiation of somatotroph, lactotroph and thyrotroph cells. In 1992 the first homozygous nonsense variant of *POU1F1* was identified in a CPHD patient from consanguineous parents (65).

Since this first description 38 pathogenic variants have been reported. Most are associated with an autosomal recessive inheritance pattern although a few autosomal dominant variants have been described. CPHD consists of TSH, GH and prolactin deficiency with normal gonadotroph and corticotroph axes. Patients often present at a very young age with growth retardation due to severe GH deficiency. Presentation of central CH is variable. Most patients have TSH deficiency early on, but it

may also occur later in childhood (66). Also cases with normal TSH have been reported. Neuroimaging usually shows a normal or hypoplastic anterior pituitary lobe, with a normal posterior lobe and pituitary stalk. *POU1F1* pathogenic variants are found in approximately 2.6% of non-familial CPHD cases, and in up to 21.6% in familial CPHD cases (67).

PROP1

In 1998 the first four families with CPHD due to homozygous and compound heterozygous *PROP1* (OMIM #601538) variants were described. Today, *PROP1* is the most frequent genetic cause of CPHD with a eutopic posterior pituitary (68). PROP1 is a transcription factor involved in differentiation of somatotroph, lactotroph, gonadotroph and thyrotroph lineages. CPHD consist of GH, prolactin and TSH deficiency, in addition to variable deficiency in LH&FSH and ACTH. Most affected individuals present with growth failure in infancy or early childhood due to severe GH deficiency. Central CH is usually mild and present later in infancy or childhood. ACTH deficiency occurs later on with a reported mean age at diagnosis of 25.3 years (range 7.4-67 years) (68). Most patients have a small or normal anterior pituitary with a normal pituitary stalk and posterior pituitary. Some cases of enlarged anterior pituitary gland during childhood have been described with subsequent involution over time. *PROP1* pathogenic variants are found in approximately 11% of CPHD patients with a frequency of up to 50% in familial cases and 7% in sporadic cases.

Isolated Pituitary Stalk Interruption Syndrome

The most common isolated pituitary malformation is referred to as pituitary stalk interruption syndrome (PSIS), and consists of a classic triad of interrupted or absent pituitary stalk, ectopic posterior pituitary and anterior pituitary hypoplasia or aplasia (**Figure 3**). It was first described in 1987 in a series of ten patients with "idiopathic pituitary dwarfism" showing an ectopic pituitary lobe and transection of the pituitary stalk upon magnetic resonance imaging (MRI) (69). Since then, there have been many reports on PSIS and a broad clinical, radiological and genetic heterogeneity has become apparent (70–72). The classic triad may not always be complete, but cardinal features include either interrupted/absent pituitary stalk or ectopic posterior pituitary. The position of the posterior pituitary varies and may be at the hypothalamic base along the pituitary stalk, or even in the normal position within the sella turcica. PSIS is considered a midline brain malformation and regarded to be at the mild end of the holoprosencephaly spectrum. PSIS most often occurs in isolation but it may also be associated with extra-pituitary malformations, mainly affecting brain, eye and craniofacial structures. The clinical phenotype of PSIS consists of anterior pituitary hormone deficiencies in variable combinations with normal posterior pituitary function. The normal posterior pituitary function reflects an intact neural hypothalamic-posterior pituitary connection while the hypothalamic-anterior pituitary connection, through the pituitary stalk, is disrupted.

Patients with PSIS may present immediately after birth with classic signs of hypopituitarism such as hypoglycemia, prolonged jaundice, undescended testes/micro-penis. Most often, however, patients present later on in childhood with poor growth. In a twenty-year cohort of 154 Dutch central CH patients detected by neonatal screening 39% (60/154) had isolated central CH and 61% (94/154) had CPHD. Of these 94 CPHD patients, 85% (80/94) had isolated PSIS and 15% (14/154) had syndromic PSIS (19). This makes isolated PSIS the most frequent diagnosis in central CH patients detected by the Dutch neonatal screening. In addition to central CH, 96% of the patients had GH deficiency, 86% ACTH deficiency and 74% LH&FSH deficiency. Posterior pituitary function was normal in all patients. The Dutch cohort consists of a specific subset of PSIS patients who all have central CH and therefore does not represent the whole spectrum of PSIS. Bar et al. reported on clinically detected PSIS patients including ten patients with a neonatal diagnosis and 47 patients diagnosed after presentation for growth retardation at a mean age of four years. Central CH was present in 80% of these patients (72). PSIS is also encountered in patients with isolated GH deficiency and this may be regarded the mildest phenotype of PSIS. However, in patients with an initial diagnosis of isolated GH deficiency, GH treatment frequently leads to a marked decrease in serum FT4 and thereby unmasks the diagnosis of central CH. In these patients with unmasked central CH after GH treatment, MRI usually reveals PSIS (17). Pituitary hormone deficiencies in PSIS are not always present at birth and may evolve throughout life, which is especially the case for ACTH deficiency. Therefore, long-term monitoring for development of additional anterior hormone deficiencies advised. In line with this all patients with apparently isolated GH deficiency should have close monitoring of FT4 levels under treatment and undergo pituitary MRI (73). See also the section on treatment and follow-up.

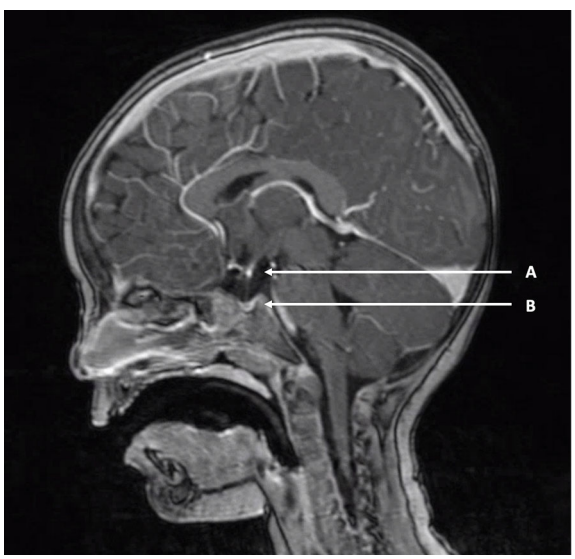

FIGURE 3 | Example of pituitary stalk interruption syndrome. T1-weighted MRI of a two-year-old boy with pituitary stalk interruption syndrome. **(A)** "Bright white spot" of ectopic posterior pituitary positioned at the hypothalamic base. **(B)** Hypoplastic anterior pituitary in the sella turcica. A thin pituitary stalk is visible between **(A, B)**.

Compared to isolated forms of central CH due to *IGSF1*, *TBL1X* and *IRS4* variants, FT4 concentrations in PSIS patients are usually lower and classify as moderate severe hypothyroidism (18). TSH concentrations in central CH due to PSIS are usually normal to slightly elevated. In the Dutch cohort of isolated PSIS patients the highest TSH measured was 12.9 mIUl/L (74). Mildly elevated TSH in PSIS is probably due to the absence of normal TRH stimulation leading to impaired TSH glycosylation and resulting in decreased bioactivity of circulating TSH. Since TSH values in isolated central CH cases are reported to be in the low-normal range, a mildly raised TSH concentration is suggestive of PSIS. Although the value of TRH-testing in the diagnosis of central CH is debated, in the Dutch cohort almost all PSIS patients had a delayed TSH rise (19). Ultimately MRI is the key to a definitive PSIS diagnosis. The posterior pituitary has a marked hyperintensity upon T1-weighted images making it recognizable as the "bright white spot" (**Figure 3**). While an ectopic position of the bright white spot is indicative of PSIS, absence of the bright white spot is found in up to 10 per cent of healthy individuals. In addition, the posterior pituitary hyperintensity may not be visible before the age of 2 months (75).

Monogenic causes of isolated PSIS are extremely rare and it has been suggested that, due to the low yield, genetic testing in non-familiar isolated PSIS cases is not indicated. Recent studies using next generation sequencing techniques have identified a few monogenic causes of isolated PSIS involving *CDON* (OMIM #608707), *GPR161* (OMIM #612250) and *GLI2* (OMIM #165230) genes (76–78). These genes are all involved in the Sonic Hedgehog signaling (SHH) pathway emphasizing the important role of this pathway in midline brain development. Rather than a monogenic etiology, there is growing evidence for a digenic or polygenic etiology for isolated PSIS (76, 79, 80). PSIS seems to result from a combination of defects in various genes in the Wnt, Notch and Sonic Hedgehog pathways involved in midline brain development (71, 81).

Syndromic CPHD

In syndromic forms of CPHD, pituitary insufficiency is accompanied by other cerebral and extra-cerebral abnormalities. Cerebral structures often include midline brain, eye, inner ear and craniofacial structures. Midline brain malformations may consist of holoprosencephaly, septo-optic dysplasia (SOD), absent corpus callosum, cerebellar malformations, Arnold Chiari malformation and also PSIS (syndromic PSIS). Cleft lip or palate and dental malformations such as single central incisor are examples of craniofacial malformations. Extra-cerebral structures that may be involved are heart, urinary tract, gastro-intestinal tract and axial skeleton.

Obvious birth defects, neurological and developmental problems will usually lead to an early diagnosis of syndromic CPHD. The yield of genetic testing is low with a genetic abnormality found in only 5-10% of sporadic cases. With an incidence of 1 in 10,000 live births SOD is one of the more common syndromic forms of CPHD consisting of two of the three features: optic nerve hypoplasia, midline forebrain abnormalities and pituitary hypoplasia. Hypopituitarism is reported in around two thirds of SOD patients with variable severity. *HESX1* (OMIM #601802), *SOX2* (OMIM #184429), *SOX3* (OMIM #313430) and *OTX2* (OMIM #600037) gene defects have been implicated in the etiology of SOD, although genetic defects are only found in up to 10% of SOD cases (64). Although SOD is one of the more common syndromic forms of CPHD, the frequency of central CH among patients with this condition is not known. It has been reported that approximately 62% to 80% of patients with SOD have hypopituitarism, with GH deficiency being the most common abnormality, but data on central CH are lacking (82, 83).

DIAGNOSIS

How Do Patients With Central CH Present?

As mentioned earlier, the incidence of central CH has increased about six-to eightfold in the past few decades. This probably reflects improved detection by NBS early in life and careful registration of affected children, rather than a true increase in incidence over time (2).

In countries that do not screen for central CH, affected children may be diagnosed clinically in the first weeks to months of life because of signs and symptoms of TH deficiency (see paragraph 4.2.2). When not clinically diagnosed early in life, these children may present later on with developmental delay (resulting from TH deficiency ± neonatal hypoglycemia), poor growth (GH deficiency) and delayed puberty (LH&FSH deficiency).

Since serum T4 concentrations in premature born infants are low these children are not screened for central CH, as this would result in a large number of false positive results. In the Dutch NBS program, for premature born children (gestational age ≤36 weeks and birth weight ≤2500 grams) only the TSH concentration is taken into account. Thereby primary CH is detected, but central CH is missed (11, 16).

In countries, states or regions with NBS for central CH many affected newborns are asymptomatic or only mildly symptomatic at presentation. Most children with central CH detected by NBS in the Netherlands have CPHD (around 60%) (19).Therefore, the benefit of early detection, diagnosis and treatment of central CH is prevention of possible neurological sequelae of TH deficiency or hypoglycemia due to GH deficiency and ACTH deficiency.

In order to allow early treatment of affected newborns and to avoid unnecessary treatment of false positives, *appropriate* and *rapid* diagnostics should be offered to all referred newborns.

Diagnostics in Case of Suspected Central CH

Figure 4 shows a proposed diagnostic algorithm to be used in newborns/neonates suspected of central CH. This algorithm is used in the Netherlands, and although it is designed for use after an abnormal NBS result suggestive for central CH – low total or free T4 in combination with normal TSH –, the algorithm can also be used when central CH (isolated or as part of CPHD) is suspected on clinical grounds early in life.

Medical History and Physical Examination

The medical history and physical examination should focus on signs and symptoms of TH deficiency, and deficiencies of pituitary hormones other than TSH. In the first weeks to months of life TH deficiency may cause feeding problems, drowsiness, (prolonged) jaundice, large tongue, umbilical hernia, and open large anterior fontanelle. Central CH as part of CPHD may also present with hypoglycemia (due to ACTH ± GH deficiency), sepsis-like illness (ACTH deficiency), undescended testes/micro-penis (boys; LH&FSH deficiency), elevated liver enzymes (ACTH deficiency and TSH deficiency), and midline defects associated with CPHD.

In addition, attention should be paid to the presence of conditions associated with transient low total and free T4 concentrations, like transient hypothyroxinemia of prematurity (THOP), non-thyroidal illness (NTI; also known as sick euthyroid syndrome), use of FT4 lowering medication and transient central CH due to maternal Graves' disease. The latter condition may be a reason for temporary levothyroxine (LT4) treatment (84–86).

Initial Diagnostics

In case of an abnormal NBS result, the initial step is confirmation of a low serum FT4 in combination with normal a TSH by (venous) blood collection. Optionally, TBG can be measured to test for TBG deficiency as cause of low NBS total T4 (2, 11). In the first two to three weeks of the neonatal period the lower limit of the FT4 reference interval lies considerably higher than later on; the same applies to the upper limit of the TSH reference interval (31, 87). This should be kept in mind when interpreting the measured FT4 and TSH concentrations. It may be necessary to perform additional diagnostics to confirm/rule out THOP, NTI and maternal Graves' disease (**Figure 4**).

Diagnostics and Interpretation

Signs and symptoms of TH deficiency or of pituitary hormone deficiencies other than TSH are highly suggestive of the diagnoses isolated central CH or central CH as part of CPHD. The same applies to persistently low serum FT4 concentrations with normal TSH in the absence of aforementioned transient conditions. Additional support for the diagnosis central CH can be obtained by diagnosing one or more other pituitary deficiencies, finding variants in genes causing isolated central CH, or finding structural HP abnormalities associated with CPHD, like PSIS.

Management of children diagnosed with CPHD differs significantly from those with isolated central CH. Not only with respect to additional risks, but also regarding supplementary diagnostics and treatment. For instance, ACTH ± GH deficiency may cause hypoglycemia and warrant glucose monitoring. In case of ACTH deficiency, it is important to first start hydrocortisone treatment should be started before LT4, since LT4 treatment may provoke a potentially life-threatening adrenal crisis in patients with untreated ACTH deficiency. It is therefore important to test for ACTH deficiency early on (1, 88).

In our experience, this can be done by monitoring for hypoglycemia and by performing a low dose (LD, 1 μg) ACTH test (89). This may require a short hospital admission. If supplemented by (rapid) genetic testing for gene variants causing isolated central CH (which can be omitted if there is convincing evidence of additional pituitary hormone deficiencies, for instance a clearly abnormal LD ACTH test [that was technically well-executed]), there are three possible outcomes/scenarios:

A) abnormal LD ACTH test result;
B) normal LD ACTH test result but no pathogenic gene variant found;
C) normal LD ACTH test result and pathogenic variant in *TSHB*, *TRHR*, *IGSF1*, *TBL1X* or *IRS4*.

MANAGEMENT

An abnormal LD ACTH test (scenario A) points to central CH as part of CPHD. In that case LT4 treatment should be preceded by hydrocortisone supplementation. In case of a borderline LD ACTH test result, consider prescribing hydrocortisone only during illness, or other forms of severe stress. LD ACTH testing is an error-prone procedure; requiring dilution of synthetic ACTH and my lead to a false abnormal test result. When doubting the reliability of the LD ACTH test result, consider rapid genetic testing for causes of isolated central CH. The finding of a pathogenic variant in one of the five isolated central CH genes will prevent unnecessary hydrocortisone treatment and additional diagnostics.

When the LD ACTH test result is normal, LT4 treatment can be started safely. Finding a pathogenic variant in *TSHB*, *TRHR*, *IGSF1*, *TBL1X* or *IRS4* (scenario C), confirms the diagnosis isolated central CH. Parents can be counseled on possible associated (future) health problems, like a small chance of partial GH deficiency and later on GH excess, delayed puberty and macroorchidism in IGSF1 deficiency syndrome, or hearing loss due to *TBL1X* loss-of-function (37, 44).

In scenario B – a normal LD ACTH test, but no pathogenic variant found -, the diagnosis can be either central CH as part of CPHD or (genetically unexplained) isolated central CH. In this scenario LT4 treatment can be started but as long as CPHD is not ruled out children should be followed-up as possible CPHD (scenario A and B). The diagnosis CPHD can be (further) substantiated by performing an MRI of the HP region and finding PSIS. MRI can be delayed if anesthesia is undesirable. A TRH test may be helpful in differentiating between central CH as part of CPHD and isolated central CH (90). A delayed TSH rise suggests central CH as part of CPHD, a normal rise but low peak value suggests isolated central CH (19, 90). A TRH test, however, should be performed before the start of LT4 treatment. Since ACTH deficiency may develop later on the importance of periodic testing for central adrenal insufficiency cannot be stressed enough.

In accordance with the recently updated CH consensus guidelines, treatment of central CH consists of once daily administration of LT4 (orally; tablets or liquid preparation) (91).

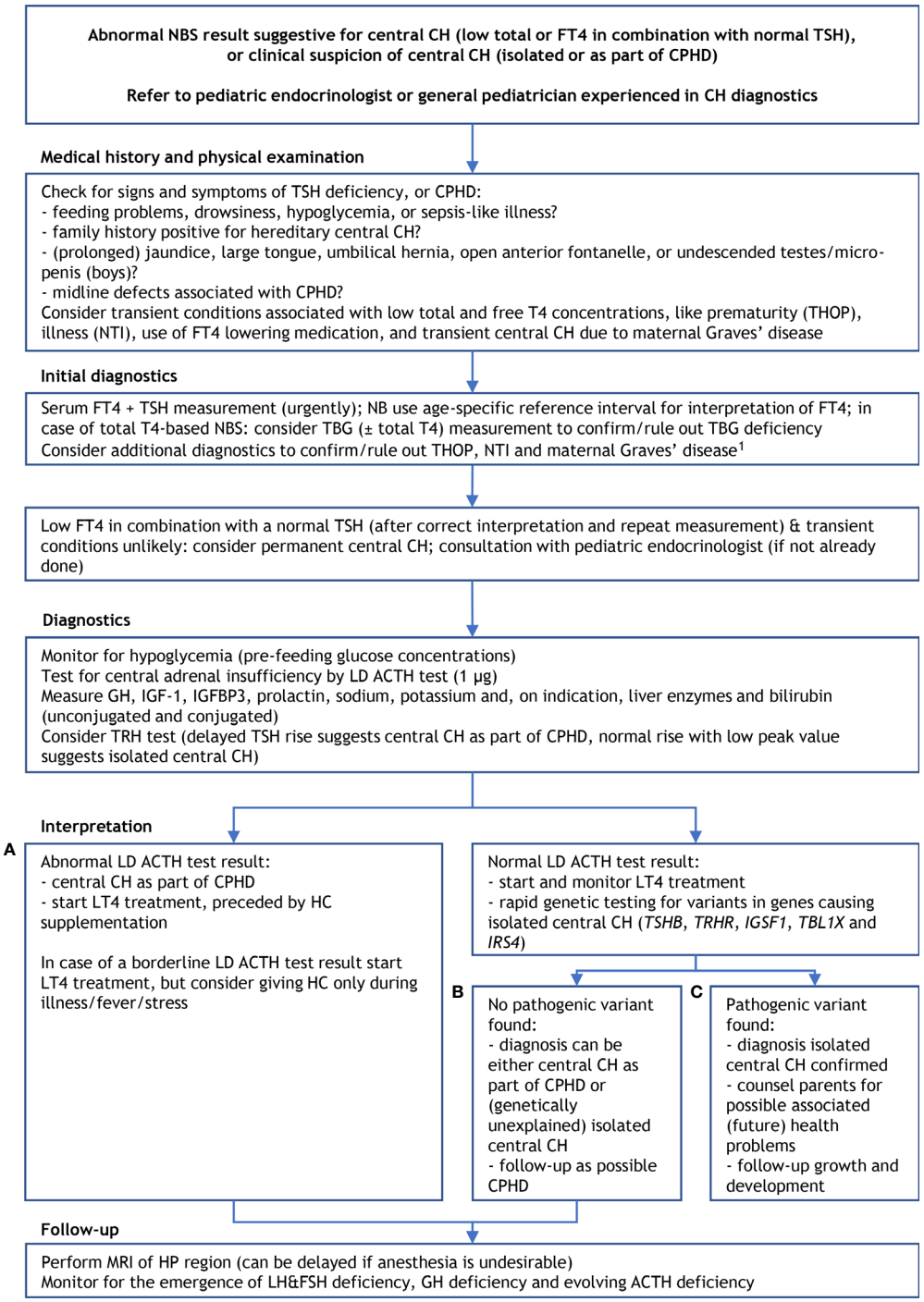

FIGURE 4 | Proposed diagnostics after an abnormal newborn screening result suggestive for central congenital hypothyroidism. [1] suspicion of THOP: re-measure FT4 and TSH at term age, suspicion of NTI: measure (F)T3 ± rT3, re-measure FT4 and TSH at recovery; suspicion of transient central CH due to maternal Graves' disease: measure TSHRAb and maternal thyroid function, and start thyroxine treatment when the newborn's FT4 is too low. ACTH, adrenocorticotropic hormone; CH, congenital hypothyroidism; CPHD, combined pituitary hormone deficiency; FT4, free thyroxine; FSH, follicle stimulating hormone; GH, growth hormone; HC, hydrocortisone; HP, hypothalamic-pituitary; IGF-1, insulin-like growth factor 1; IGFBP3, insulin-like growth factor binding protein 3; LD, low dose; LH, luteinizing hormone; LT4, levothyroxine; MRI, magnetic resonance imaging; NBS, newborn screening; NTI, non-thyroidal illness; rT3, reverse triiodothyronine; T4, thyroxine; TBG, thyroxine binding globulin; TH, thyroid hormone; THOP, transient hypothyroxinemia of prematurity; TRH, thyrotropin releasing hormone; TSH, thyroid stimulating hormone; TSHRAb, TSH receptor antibodies. **(A–C)** refer to three scenarios of different outcomes of the work-up of central CH (see the *Management* section).

In severe central CH (serum FT4 before treatment <5 pmol/L), the LT4 starting dose should be at least 10 µg/kg per day, in order to rapidly bring FT4 within the age-specific reference interval. To reduce the risk of overtreatment, a lower starting dose (5-10 µg/kg per day) can be used in milder forms. The (biochemical) long-term aim of LT4 treatment is to bring and keep serum FT4 in the *upper half* of the age-specific reference interval. Although randomized clinical trials testing this approach in children are lacking, data from adult studies support this approach (92, 93). The biggest difference between treatment of primary and central CH is in treatment monitoring, with serum FT4 (instead of TSH) being the most important parameter. If TSH prior to treatment was low, subsequent TSH measurements can be omitted. With regard to treatment monitoring, clinical and biochemical follow-up is similar to primary CH. The first evaluation should be scheduled one to two weeks after the start of treatment. Subsequent evaluations should take place every two weeks until normalization of serum FT4. Thereafter, the frequency can be lowered to once every one to three months until the age of 12 months, every two to four months during the second and third years of life, and every three to six months thereafter. Blood collection for serum FT4 measurement should be performed before, or at least four hours after the last (daily) LT4 administration (91). When under- or overtreatment is suspected, measurement of TSH, and free or total T3 may be helpful. Undertreatment should be considered when FT4 is around the lower limit of the reference interval, particularly if TSH >1.0 mIU/L. Overtreatment should be considered when FT4 is around or above the upper limit of the reference interval, and accompanied by clinical signs of thyrotoxicosis or a high (F)T3 concentration (1).

As already mentioned, in a study on the clinical characteristics of 92 Dutch children with central CH early detected by neonatal screening (57 as part of CPHD, 35 isolated disease), Naafs et al. found that 86% of the patients with CPHD also had ACTH deficiency, and that 96% and 74% had GH and LH&FSH deficiency, respectively (19). Most cases of ACTH deficiency (35 of 49) were diagnosed in the neonatal period, but nine cases between the ages of two months and one year, and the remaining five cases between the ages of one and 14 years. GH deficiency was diagnosed at a mean age of 1.3 years (range age 21 days to 9.2 years), and LH&FSH deficiency both in the first months of life and around the age of 12.8 years.

The implications of these findings are that all children diagnosed with central CH as part of CPHD, and all children without a genetically confirmed diagnosis of isolated central CH should be followed-up and monitored for the emergence of LH&FSH deficiency, GH deficiency and, evolving ACTH deficiency.

PROGNOSIS

Since the start of NBS for CH the neurodevelopmental prognosis of early detected and treated children with primary CH has improved dramatically (91). In 1980, Klein reported a mean IQ of 76 observed in over 800 patients with primary CH clinically diagnosed before the era of NBS (94). More recently, in a systematic literature search and review, Grosse et al. identified four population-based studies conducted in high income countries and found a somewhat higher mean IQ of 85 among children with clinically diagnosed primary CH prior to the introduction of NBS; 8-28% of these children were classified as having intellectual disability (defined as an IQ <70) (95).

In contrast, the latest long-term follow-up studies in patients with primary CH show that when LT4 treatment is started before the (mean) age of 10 days with a starting dose of at least 10 µg/kg per day, affected children have a neurodevelopmental outcome similar to that of unaffected siblings in the second decade of life (91, 96, 97). Although randomized clinical trials have not been conducted, the results of these studies indicate that early detection and adequate LT4 treatment of newborns with primary CH prevents brain damage and developmental delay.

Comparable outcome data of children with central CH are scarce. In a recent systematic review with meta-analysis of individual patient data, Naafs et al. identified six studies of reasonable quality on neurodevelopmental outcome in patients treated for central CH from which data of only 30 patients (27 with central CH as part of CPHD) was sufficient for analysis. While mean full scale intelligence quotient (FSIQ) was normal in these 30 patients (97; 95% confidence interval (CI) 88-105), 27% had a FSIQ below 85 (≥1 SD below norm score), and 10% below 70 (≥2 SD below norm score). Since in half of the studies the age at start of treatment was not available reliable conclusions could not be drawn (98). Subsequently, Naafs et al. performed a study on cognitive and motor outcome in a large cohort of Dutch patients with early-detected central CH (26). In this cross-sectional study, FSIQ was measured in 52 patients with CPHD and 35 patients with isolated central CH born between 1995 and 2015, with 52 unaffected siblings as controls. Secondary outcomes were intelligence tests' subscales and motor function. Mean FSIQ was 90.7 (95% CI 86.4-95.0) in CPHD patients and 98.2 (95% CI 93.0-103.5) in isolated central CH patients. CPHD patients had lower FSIQs than siblings (mean difference -7.9 points, 95% CI -13.4 to -2.5), but FSIQs of isolated central CH patients and siblings were similar. Processing speed was lower in both patient groups than in siblings; motor difficulties occurred significantly more often in patients (33%) versus siblings (5%; p=0.004).

These data suggest that early detection and TH treatment of newborns with isolated central CH results in a normal IQ later in life, like in patients with early detected and treated primary CH. The approximately 0.8 SD lower IQ in patients with CPHD may be explained by ACTH and GH deficiency (present in 88% and 96% of patients, respectively), resulting in neonatal hypoglycemia (documented in 55% of patients; mean lowest glucose level 1.2 ± 0.8 mmol/L). Just like in primary CH, the lower processing speed and the more frequent occurrence of motor difficulties in isolated central CH and in CPDH patients may be related to pre- and early postnatal TH deficiency (26).

Naafs et al. also studied health-related QoL in the same cohort of patients with early-detected central CH and their unaffected siblings (99). Patients ≥8 years old filled in self-reports, and parents of patients aged three to 18 years old filled in parent-reports of the Pediatric Quality of Life inventory (PedsQL™) and

the PedsQL Multidimensional Fatigue Scale. Patients with isolated central CH (n=35) and siblings showed similar scores on all subscales, both in the self-reports and parent-reports. Self-reported sores of CPHD patients (n=53) were also similar to those of siblings. However, parent-reported total HRQoL and fatigue scores of CPHD patients were significantly lower than those of siblings. This indicates a perceived difference in perception between patients and their parents.

Despite these positive and promising results, the remaining question is whether early detection and treatment of children with central CH really improves their neurodevelopmental outcome and, if so, how much. Although a few studies not included in the systematic review by Naafs et al. suggest that it does (100, 101), this question can only be answered by conducting neurodevelopmental outcome studies in patients with late-detected central CH. Since isolated CH may not be clinically detected at all, such studies may have to focus on patients with CPHD due to PSIS, which was present in 88% of the patients evaluated by Naafs et al. (19) Given the rarity of this disorder this can only be achieved by international collaboration for instance in the form of an international patient registry.

CONCLUSIONS

Based on data from T4- and FT4-based NBS programs that detect central CH, its incidence is currently estimated at around 1:13,000. Approximately 60% of affected children have central CH as part of CPHD, the others have isolated central CH (≈40%). Most CPHD cases are due to isolated PSIS, a condition of which the etiopathogenesis is still not well understood. In contrast, up to 90% of cases of isolated central CH can be explained by pathogenic variants in *TSHB*, *TRHR*, *IGSF1*, *TBL1X* or *IRS4*.

In the last few years, it has been shown that central CH is a more severe condition than was previously thought, and that in the neonatal period the clinical diagnosis is often missed despite hospital admission because of feeding problems, hypoglycemia and prolonged jaundice. However, with early detection by NBS quickly followed by the right diagnostics and treatment children with central CH, both isolated and CPHD, have an excellent neurodevelopmental prognosis, comparable to unaffected siblings.

AUTHOR CONTRIBUTIONS

NZ-S and ASPvT drafted the manuscript. PL and JCN retrieved data for **Figure 1** and **Supplemental Table 2**. All authors contributed to the article and approved the submitted version. ASPvT supervised the whole process.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2021.686317/full#supplementary-material

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Thyroid Gene Mutations in Pregnant and Breastfeeding Women Diagnosed With Transient Congenital Hypothyroidism: Implications for the Offspring's Health

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Fetus and infants require appropriate thyroid hormone levels and iodine during pregnancy and lactation. Nature endorses the mother to supply thyroid hormones to the fetus and iodine to the lactating infant. Genetic variations on thyroid proteins that cause dyshormonogenic congenital hypothyroidism could in pregnant and breastfeeding women impair the delivery of thyroid hormones and iodine to the offspring. The review discusses maternal genetic variations in thyroid proteins that, in the context of pregnancy and/or breastfeeding, could trigger thyroid hormone deficiency or iodide transport defect that will affect the proper development of the offspring.

Keywords: offspring, pregnancy, breastfeeding, iodine, thyroid hormones, congenital hypothyroid women, genetic counseling

INTRODUCTION

Pregnancy and lactation are challenging physiological periods due to the mother supplies thyroid hormones and iodine to the fetus and infant, respectively. Given that, there is an increase on iodine and thyroid hormone requirements, a proper iodine status and thyroid hormone levels in pregnant and breastfeeding women must be guaranteed (1). Unfortunately, iodine insufficiency and thyroid hormone (TH) deficiency are frequent in pregnancy and breastfeeding (2). The statistics showed that 2.5% of pregnant and breastfeeding women consume insufficient iodine in their diet (3) and the prevalence of hypothyroidism is about 2%–3% in pregnant women (4). The progeny gestated and/or lactated under iodine deficiency or thyroid hormone deficiency, like hypothyroidism or hypothyroxinemia, is at higher risk of suffering irreversible cognitive impairment like attentional deficit, low intelligent quotient, and intellectual disability (5). Women carrying thyroid gene variations could be more sensitive to suffer thyroid hormone deficiency during pregnancy and/or iodide transport defect (ITD) during lactation.

Special attention are those women that were diagnosed at birth with transient congenital hypothyroidism (TCH). TCH is a temporary deficiency of thyroid hormones identified after birth (6). TCH has several different causes like endemic iodine deficiency, iodine excess, maternal antithyroid medication, maternal antibodies, and genetics. This review will focus on thyroid gene variations that will risk the pregnant or lactating women to suffer thyroid hormone deficiency or ITD during pregnancy and/or lactation (7, 8). We will review these thyroid gene variations reported in the literature that caused dyshormonogenic TCH. The point of this review is to provide awareness to scientists and physicians to prevent thyroid hormone deficiency and ITD during pregnancy and lactation in those women diagnosed with dyshormonogenic TCH. These maternal genetic mutations on thyroid proteins could irreversibly damage the appropriate development of the offspring.

FUNCTIONAL MATERNAL THYROID PROTEINS ARE NEEDED FOR FETUS DEVELOPMENT

In the first 5 months of pregnancy, the maternal thyroid gland will be challenged to supply THs to the fetus, especially T_4 (9, 10). Thus, the mother will synthesize THs for her own needs and the fetus (**Figure 1**) (11, 12). This new scenery causes physiological stress to the maternal thyroid gland. To overcome this challenge, the pregnant woman should increase her daily iodine intake to 250 µg/day. The thyroid-stimulating hormone (TSH) and the human chorionic gonadotropin (hCG) will stimulate the maternal thyroid gland to produce T_4 and T_3 during the first trimester of pregnancy (13, 14). The hCG shares structural features with TSH (15). hCG and TSH will increase iodine uptake and thyroid hormone synthesis by binding to the TSH receptor (16). TSH and hCG will stimulate maternal thyroid gland growth and the expression of thyroid proteins like Na^+/I^- symporter (NIS), pendrin (PDS), thyroglobulin (TG), thyroid peroxidase (TPO), dual oxidase 2 (DUOX2), and dual oxidase maturation factor 2 (DUOXA2) (17). The expression of NIS at the basolateral membrane of the thyrocyte will increase the uptake of iodide from the blood into the thyrocyte. Iodide will diffuse through the cytoplasm to the apical side of the thyrocyte, where transporters like PDS, anoctamin-1 (ANO1), and SLA26A7 will transport it to the colloid (18). The thyrocytes also express the cystic fibrosis transmembrane conductance regulator (CFTR) (18). CFTR transports chloride, and more studies are required to evaluate whether CFTR transports iodide. At the colloid, iodide will be oxidized and incorporated in the tyrosyl residues of TG by TPO (18). The organification of TG requires

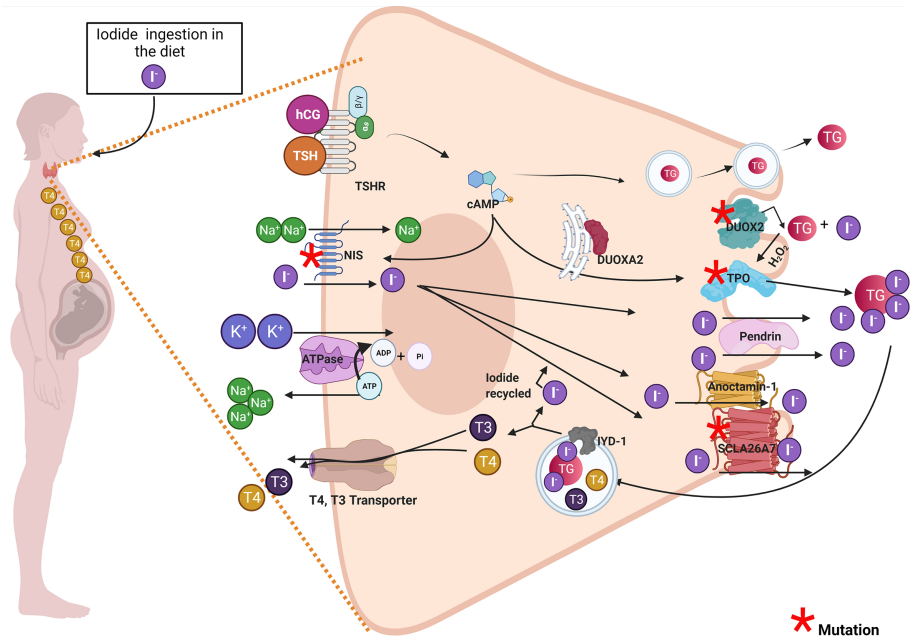

FIGURE 1 | Pregnant woman diagnosed with transient congenital hypothyroidism due to mutations in thyroid proteins could develop thyroid hormone deficiency and impaired the offspring's development. The drawing shows a thyroid cell of a pregnant woman and the principal thyroid proteins involved in thyroid hormonogenesis. Proteins with red asterisk have been associated in the literature with thyroid dyshormonogenesis in transient congenital hypothyroid patients. Among these proteins are DUOX2, DUOXA2, DUOX1, DUOXA1, TPO, NIS, and SLCA27 (see red asterisk). The maternal thyroid gland must synthesize thyroid hormones for mother and the fetus. The high demand for thyroid hormone synthesis in pregnancy could stress the thyroid gland especially in women carrying genetic variations on thyroid proteins and that were diagnosed with transient congenital hypothyroidism (TCH). These women diagnosed with TCH (carrying monogenic mutations in DUOX2 or DUOXA2 or digenic mutations in DUOX2 and DUOXA1 or mutations in TPO or NIS) that were euthyroid before pregnancy could develop thyroid hormone deficiency like hypothyroidism or hypothyroxinemia during pregnancy. Both hypothyroxinemia and hypothyroidism are thyroid hormone deficiency conditions that risk the proper fetus development and have deleterious consequences in the offspring.

hydrogen peroxide (H_2O_2) which is supplied by dual oxidase system (DUOX). It has been described that two DUOX enzymes (DUOX1 and DUOX2) and their respective maturation factors (DUOXA1 and DUOXA2). These maturations factors reside at the endoplasmic reticulum, and they are essential for DUOX function (**Figure 1**). hCG and TSH will stimulate the endocytosis of iodinated TG. In the endo-lysosomes, TG will be digested by proteolytic enzymes like lysosomal dipeptidase, glutamate carboxypeptidase, and cathepsins B, K, L, and S releasing T_3 and T_4. THs will efflux the thyroid cell to the blood stream mainly by transporters like monocarboxylate transporter (MCT8) (19). It has been reported that certain genetic variations in thyroid proteins affects the function of the thyroid gland (20–22). There are mutations in thyroid genes that cause thyroid dyshormonogenesis, which is responsible for 10%–15% of congenital hypothyroidism (CH) (23). Newborns diagnosed with CH must be treated immediately with T_4 to avoid neurological damage (23). Moreover, the etiology of CH should be determined by measurement of serum T_3, T_4, TSH, and TG and thyroid ultrasonography, thyroid scintigraphy, and perchlorate discharge test should be performed (23, 24). These biochemical and clinical analyses will reveal if the infant suffers transient congenital hypothyroidism (TCH) or permanent congenital hypothyroidism (PCH) (24). Those patients that have PCH will be kept for life with T_4 treatment. Instead, thyroid function will be reassessed to those patients diagnosed with TCH at 3 years of age. If their thyroid function has been normalized, the T_4 treatment will stop. This situation occurs for patients with TCH given that by progressing in age they require less thyroid hormone production related to their weight and the thyroid gland will be able to overcome the demands (23–26). *Moreno et al.* emphasize that it is crucial for those women that have been diagnosed with TCH due to monoallelic or biallelic mutations on thyroid proteins to be followed up during pregnancy for the risk of suffering hypothyroidism in this period and therefore will affect the appropriate development of the fetus (23, 27). Thyroid hormone synthesis can be impaired in pregnant women diagnosed at birth with TCH. Even though they were euthyroid before pregnancy, they could fall back in thyroid hormone deficiency due to expressing variants of thyroid protein that are in some degree functionally impaired (28). The thyroid gland of these women during pregnancy could be at risk of dyshormonogenesis endangering the proper development of the fetus. The recommendation of the Endo-European reference network (ERN) that patients with TCH should receive genetic testing to identify mutations causing thyroid dyshormonogenesis is of importance (23, 24). In the next section, the mutations in thyroid proteins that have been described to cause TCH will be discussed.

VARIATIONS IN MATERNAL THYROID GENES THAT COULD IMPAIR THE DEVELOPMENT OF THE FETUS

It has been described in the literature that variations on thyroid proteins like NIS, DUOX2, DUOXA2, TPO and Tg are responsible for TCH. In this section we briefly discuss the relevance of of these proteins variations in TCH women when they reached pregnancy. The NIS mutants could induce ITD causing TCH or PCH (29). However, the severity and the onset of the disease phenotype will depend on the type of NIS mutation, if it is monoallelic or biallelic, the period of life, and iodide ingestion (30, 31). Therefore, physicians should prevent thyroid hormone deficiency in pregnant women diagnosed with TCH carrying NIS mutations (**Figure 1**). There are other transporters expressed in the thyroid cells like PDS, ANO-1, SLC26A7, and CFTR (32–34). PDS, ANO-1, and SLC26A7 are localized at the apical side of the thyrocyte, and their physiological role have been associated with iodide efflux into the colloid (35). Mutations on *SLC26A4* that encodes PDS has been associated with Pendred syndrome, characterized by congenital bilateral sensorineural hearing loss and in childhood can appear diffuse or multinodular goiter (36). Most of the patients with Pendred syndrome are euthyroid; however, their thyroid function can be impaired if they are exposed to low iodine intake (37). ANO-1 and CFTR variants have not been associated with TCH. A study performed in Saudi Arabia, by using whole-exome sequencing (WES) described mutations in SLC26A7 that could be responsible in part for CH (38). Another thyroid protein variants that have been reported to be responsible for TCH are monoallelic and biallelic mutations of DUOX2 (27, 39, 40). DUOX2 is a transmembrane protein localized at the apical side of the cell, and it belongs to (NADPH)-oxidase family whose function is involved in H_2O_2 generation (**Figure 1**). H_2O_2 is required for thyroid hormone synthesis specifically for iodide organification in tyrosyl residues of TG by TPO (**Figure 1**) (35). The prevalence of *DUOX2* mutations is highly variable in the population. From 2019, approximately 105 *DUOX2* mutations are described. Some of them were in-frame deletions, missense, nonsense, splice site, and frameshift mutations (41, 42). Moreover, mutations in the gene *DUOXA2* were also reported in TCH patients (43). DUOXA2 is an endoplasmic reticulum transmembrane protein that helps DUOX2 mature through the ER and translocate to the plasma membrane (44). Zamproni et al. found a biallelic mutation in the *DUOXA2* gene that leads to a partial iodide organification defect (45) and Liu et al. found a monoallelic missense mutation in DUOXA2 that causes mild TCH (46). Besides, DUOX2 and DUOXA2 thyrocytes also express DUOX1 and DUOXA1. DUOX1 has 83% protein sequence homology with DUOX2; however, its expression is lesser than DUOX2 and its function in the thyroid gland is not clear yet as DUOX1 mutations do not cause CH (47). Aycan et al. described two patients with severe CH that have biallelic mutations in DUOX2 and DUOX1, suggesting that DUOX1 can replace the function of DUOX2 when this enzyme is not available (48). DUOXA1 in similar fashion as DUOXA2 helps DUOX1 to mature through the ER and mutations in DUOXA1 has not been associated yet with CH (47). The possible implication of DUOX1 and DUOXA1 in CH is under intense debate, as the related mutations were reported to be always associated with DUOX2 or DUOXA2 mutants which are the principal proteins involved in the thyroid H_2O_2-generating system. Interestingly, Wang et al.

sequence 16 genes related with CH by next-generation sequence (NGS) in 377 CH patients and found patients with biallelic mutations in DUOX2 and DUOXA1, suggesting that DUOX1 and DUOXA1 could also play a role in thyroid hormone synthesis (49). TPO is responsible for iodide organification and the coupling of tyrosyl residues, essential steps in thyroid hormone synthesis (50). Thus, the expression of TPO variants will severely affect thyroid hormone synthesis (37). In fact, it has been reported that TPO biallelic mutations caused PCH (51). Another study, performed in 243 Russian patients also showed an increased number of variants identified in thyroid dyshormonogenesis-associated genes mostly of the variants found in the *TPO* gene (52). It has been shown that certain patients with monoallelic mutations in *TPO* could develop PCH (53, 54), mild hypothyroidism (55), and TCH (56). It has been reported that there are 229 mutations in *TG*, the precursor of thyroid hormones (57). Some of them occur as biallelic or monoallelic and some are found inherited as monogenic or polygenic (57). A targeted NGS study performed in 19 patients mostly from France with CH due to dyshormonogenesis showed that TG was a site with the higher number of identified mutations followed by DUOXA2 (58). Interestingly, mutations in *TG* cause euthyroidism to mild or severe CH (57). Variants in iodotyrosine deiodinase (IYD) cause dyshormonogenic PCH, and there are no monoallelic mutations associated to TCH (57). Molecular biology techniques, like NGS and WES will help to understand the genetic component of CH. These molecular techniques like NGS and WES will provide data to fill the complex analysis of gene panels aiming to understand the mechanisms underlying disease development (59). Genetic counseling is recommended for women previously diagnosed with TCH to prevent thyroid hormone deficiency during gestation, and it is highly recommendable to follow thyroid function during pregnancy.

PROPER IODIDE NUTRITION IS AN ESSENTIAL REQUIREMENT FOR INFANT THYROID HORMONE SYNTHESIS

Neonates and lactating infants require thyroid hormones for proper physical and neurological development. Even though there are traces of maternal thyroid hormones in the milk, the main source of these hormones for the infant derives from their own thyroid gland (60, 61). Thus, neonates and infants are required to ingest iodine for thyroid hormone synthesis (25). The iodine demand for the newborn is higher than an adult if we ponder their weight (62). Newborns, until 5 months of age need 15 μg/kg/day of iodine, and infants are required 90 μg/day of iodine (63). The newborn thyroid gland has a small iodine reserve close to 100 μg. Therefore, by considering their high demand for this micronutrient, the infants should have a daily intake of iodine (64). Breast milk is an excellent source of iodine; however, its content can vary from 5.4 to 2.170 μg/L (26, 65). Physiologically, iodine content in breast milk will depend mainly on the mother's iodine consumption being influenced by iodization programs (25, 40). According to the WHO/UNICEF and the International Council for Control of Iodine Deficiency Disorders (ICCIDD), the recommended iodine intake for lactating women is 250 μg/day to ensure 100–150 μg of iodine/dl in the breast milk (26). Iodine is accumulated into the mother's milk in the form of iodide, and this transport is mediated by NIS that is expressed in the mammary gland physiologically only during lactation (**Figure 2**) (67). NIS transports iodide against its electrochemical gradient from the blood into lactocytes, and its expression is regulated by oxytocin and prolactin (67). Other transporters like CFTR and ANO-1, that efflux iodide at the thyrocyte, are also expressed in the mammary gland (68, 69). Prolactin also regulates the expression of PDS, an apical thyroid iodide transporter, in lactocytes during lactation (70). The role of these transporters in iodide transport in the mammary gland must yet be elucidated. Iodide inside the lactocytes is oxidized and incorporated into casein by a lactoperoxidase (LPO), forming iodocasein molecules (**Figure 2**). These iodocasein molecules are aggregated inside micelles and will be secreted into breast milk (**Figure 2**) (71). The concentration of iodide in breast milk depends on environmental and genetic factors. Women living in regions with insufficient iodine intake are at risk of iodine deficiency during lactation (72). Among the environmental factors are the mother's diet and the ingestion of thiocyanate and perchlorate (73–75). Perchlorate and thiocyanate are NIS inhibitors of iodide transport (29, 76, 77). Perchlorate is a competitive inhibitor of iodide; Llorente-Esteban et al. showed that the tiny perchlorate addition increases NIS K_m for iodide impairing thyroid hormone synthesis (78). Thiocyanate is a potent NIS inhibitor, and it has been described as a noncompetitive inhibitor (76, 79, 80). Therefore, the presence of these molecules in the mother's diet will reduce the iodide uptake in lactocytes, diminish the concentration of iodide in breast milk, and reduce thyroid hormone synthesis in the infants affecting their development (81). Concerning genetic factors, particular attention should be taken with congenital hypothyroid women because they can carry NIS or LPO variants that impair the accumulation of iodide in breast milk during lactation. In the next section, NIS variants will be discussed.

NIS VARIANTS THAT AFFECT IODIDE CONCENTRATION IN BREAST MILK

NIS is the principal molecule responsible for iodide uptake in the mammary gland playing a pivotal role in iodide concentration in breast milk (67, 82) (**Figure 2**). Eighteen NIS mutations have been reported in the *Slc5a5*, the gene that encodes for *NIS* (30, 83, 84). The type of mutations in *Slc5a5* includes nonsense, alternative splicing, frameshift, deletion, and missense, and they are responsible for causing ITD (20). ITD occurs as autosomal recessive disorder that results in CH (29). It has been reported that the severity and the onset of the CH varies and depends on the type of NIS mutation and iodide ingestion (30, 31). An important issue to consider is the situation of

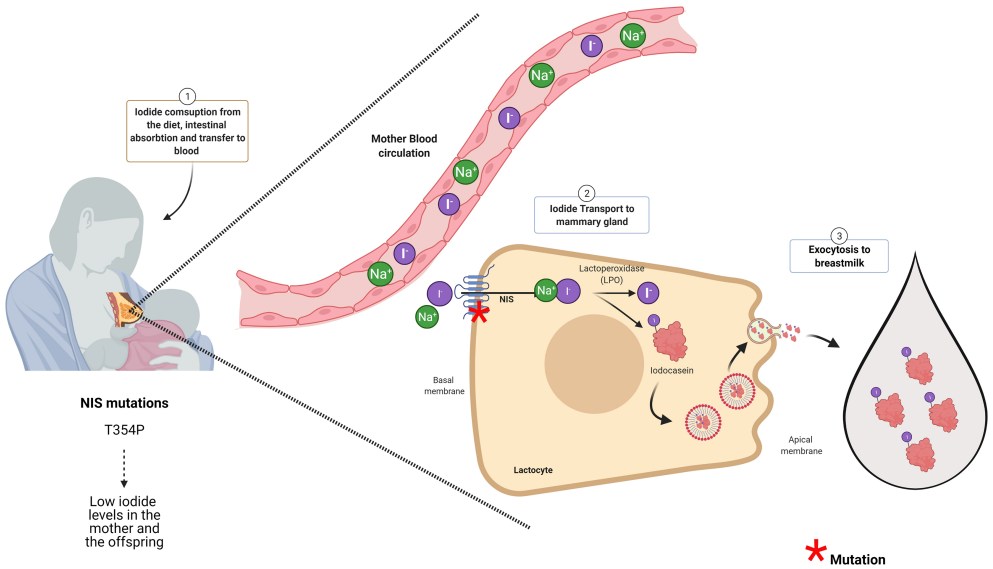

FIGURE 2 | NIS and LPO are essential proteins for iodide accumulation in breast milk. The infant synthesizes its own thyroid hormones. For that, the mother should consume enough iodine to supply her baby with this micronutrient through the breast milk. The Na^+/I^- symporter (NIS) at the intestine will transport iodide into the blood NIS expressed at the lactocytes will transport iodide from the blood to inside these cells. NIS in the lactocytes is located at the basal membrane. The iodide inside the lactocyte is incorporated into casein by the action of lactoperoxidase (LPO). Iodocasein molecules will be aggregate inside micelles. The micelles will fuse with the apical membrane to release their content by exocytosis to breast milk. Woman diagnosed with congenital hypothyroidism (CH) carrying NIS mutation (like T354P) accumulated low iodide in the milk even though she was under levothyroxine (LT_4) treatment. Thus, women carrying NIS mutants that caused congenital hypothyroidism besides T_4 treatment should receive iodide supplementation (66). Mutations in proteins with red asterisk have been associated in the literature with low iodide in breast milk as it is for NIS (see red asterisk). Even though, LPO is an essential enzyme for iodide presence in the milk, there are no reports in the literature indicating that mutations in LPO affects iodide content in breast milk.

breastfeeding women diagnosed with CH bearing NIS mutation and treated with levothyroxine (LT_4) (84). Even though LT_4 will protect the mother from suffering hypothyroidism, their lactating offspring will not receive enough iodide, given that NIS is the only iodide transporter described in the mammary gland so far. PDS is also expressed at the mammary gland, its expression is stimulated by prolactin; however, PDS mutations affecting iodine content in breast milk has not been described (85). Regarding the NIS variants, there is only one report by Mizokami et al., showing ITD at the mammary gland during lactation (66). Mizokami et al. reported the case of a woman diagnosed with CH that received LT_4 treatment (150 µg/day of LT_4) and had low levels of iodide in her breast milk (54 µg/L) (66). Iodine supplementation was given to the mother to reach the minimum level of 90 µg/L of iodide in milk. This amount was accepted as sufficient to fulfill the newborn iodine necessities (66). The breastfeeding woman carried a missense variation in the *slc5a5* gene previously described by Fujiwara et al. This variant has a cytosine instead of adenosine in codon 354, leading to an exchange of threonine for proline (T354P) in NIS protein (86). NIS T354 mutant has lost function and is highly prevalent in the Japanese population (87). Regarding LPO, an important enzyme for iodide accumulation in breast milk, there are no specific mutations described yet in this protein that affects iodide content in breast milk.

DISCUSSION AND CONCLUDING REMARKS

It is well established that patients born with PCH should be immediately treated with LT_4 for life. However, those patients with TCH will stop LT_4 treatment at a certain age when their thyroid function normalizes and present normal TSH and fT_4, in fact most TCH patients are euthyroid at adulthood. Besides that, genetic counseling is highly recommended especially for women. Genetic counseling will allow to identify the mutations that are responsible for thyroid dyshormonogenesis. As we discussed in this review, proper maternal thyroid function in pregnancy is essential for appropriate fetus development. The requirement for thyroid function during pregnancy is higher in pregnant women than for nonpregnant women. Pregnancy requires increasing iodine consumption and the demand for functional thyroid proteins. The identification of mutations in thyroid proteins will help physicians to protect the women during pregnancy to suffer hypothyroidism or hypothyroxinemia. Women carrying genetic mutations on *DUOX*2, *DUOXA2*, TPO, or *NIS* gene, they must receive prophylactic LT_4 treatment during pregnancy due to the higher probability to develop thyroid hormone deficiency during this period. The relevance of NIS mutants surpasses pregnancy, given they are also significant for infant development during breastfeeding. If the CH woman carries

mutations of NIS protein, besides T_4 treatment, she must receive prophylactic iodine supplementation during breastfeeding. Given that T_4 treatment will only rescue the mother from hypothyroidism and not his/her lactating infant for thyroid hormone deficiency, medical doctors should care to supply the mother with enough iodide. One approach was described by Mizokami et al. The authors administered iodide to the lactating woman that carries NIS T354P mutation and follows the measurement of breast milk iodine concentration (BMIC) (66). This action restored the normal levels of iodide and protected the neonate from suffering the health consequences of hypothyroidism (66, 85). There are certain NIS variants that cause severe CH, and in these cases, mother supplementation with iodide will not rescue the lactating infant. In these cases, it will be highly recommendable to directly supplement the baby by giving him/her iodine through baby formula (63). Baby formula should have 10–60 μg of iodine/100 kcal or 5–75 μg of iodine/100 kcal according to the regulations of the Commission of European communities (88) and the US Food and Drug Administration (FDA), respectively (89).

Genetic counseling is essential for CH women to determine if they will need iodide supplementation during breastfeeding to ensure the proper development of their infant. LPO mutants had not yet been described in the literature, and they have not even been related to low iodide levels in breast milk. Based on the physiological relevance of iodide for the infant's proper development, we highly recommend measuring its content in breast milk.

CONCLUSIONS

The concluding remark of this mini review is to highly recommend women that have been diagnosed with dyshormonogenic CH to counsel for genetic study before pregnancy, aiming to determine which thyroid gene/s is or are mutated. By using this strategy, medical doctors could design the prophylactic treatment by giving the patient LT_4 and/or iodide. This information will protect the offspring during gestation and breastfeeding from cognitive impairment. The mother is the only source of thyroid hormones at the early pregnancy and the source of iodide during breastfeeding. Women diagnosed with TCH who bear mutations in *DUOX2*, *DUOXA2*, *TPO*, *TG*, *SLC26A7*, or *NIS* genes and are euthyroid before pregnancy should be prophylactic treated with LT_4 during pregnancy, aiming to have appropriate thyroid hormone level for the development of the fetus. Although women who carry mutations for NIS are treated with LT_4 during lactation, they must increase the consumption of iodide, and BMCI should be monitored. The fact that there is only a single report in the literature associating that a lactating woman carrying a NIS mutant should receive iodine supplementation during lactation to protect the development of the lactating infant, increases the alarm for gynecologist and endocrinologist to evaluate for NIS variations in women diagnosed with TID. Moreover, it is necessary to encourage NGS and WES analyses in patients diagnosed with TCH due to dyshormonogenesis. These techniques will unveil the complexity of thyroid genes that are required for the proper function of thyroid gland for the human health protection.

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MO and CR worked: All authors have made a substantial, direct, and intellectual contribution to the work and approved it for publication. All authors have read and agreed to the published version of the manuscript. All authors contributed to the article and approved the submitted version.

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