Title: Congenital hypothyroidism: update and perspectives

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Abstract

Congenital hypothyroidism (CH) may be primary, due to a defect affecting the thyroid gland itself, or central, due to impaired thyroid stimulating hormone (TSH)-mediated stimulation of the thyroid gland as a result of hypothalamic or pituitary pathology. Primary CH is the most common neonatal endocrine disorder, traditionally subdivided into thyroid dysgenesis (TD), referring to a spectrum of thyroid developmental abnormalities, and dyshormonogenesis, where a defective molecular pathway for thyroid hormonogenesis results in failure of hormone production by a structurally intact gland. Delayed treatment of neonatal hypothyroidism may result in profound neurodevelopmental delay, therefore CH is screened for in developed countries to facilitate prompt diagnosis.

Central Congenital Hypothyroidism (CCH) is a rarer entity which may occur in isolation, or (more frequently) in association with additional pituitary hormone deficits. CCH is most commonly defined biochemically by failure of appropriate TSH elevation despite subnormal thyroid hormone levels and will therefore evade diagnosis in primary, TSH-based CH screening programs. This review will discuss recent genetic aetiological advances in CH and summarize epidemiological data and clinical diagnostic challenges, focussing on primary CH and isolated CCH.

Introduction

Primary congenital hypothyroidism (CH) occurs due to defective thyroid gland
development or hormone biosynthetic function, and is traditionally sub-classified as thyroid dysgenesis (TD) or dyshormonogenesis. TD refers to a spectrum of aberrant thyroid gland development, most commonly involving thyroid ectopy, an abnormally situated and mostly small thyroid gland. Complete absence of the thyroid gland (athyreosis) affects 20-30% of TD cases and a small minority exhibit a normally-located but hypoplastic thyroid (1). Dyshormonogenesis refers to failure of thyroid hormone production by a normally located, sometimes goitrous thyroid gland in which the molecular pathway for thyroid hormone biosynthesis is disrupted (2). Historically, 75-85% of CH cases have been attributed to TD with the remainder occurring due to dyshormonogenesis. However, studies undertaken more recently using lower screening TSH diagnostic cut-offs have reported a doubling in the incidence of CH largely due to increased diagnosis of cases with gland in situ (GIS) (3). TD is generally considered to be a sporadic disease for which the underlying aetiology is usually not clear. Genetic causes involve genes mediating thyroid differentiation, migration and growth, however, less than 5% TD cases are attributable to a mutation in a known TD-associated gene. In contrast, the majority of individuals with dyshormonogenesis harbour mutations in genes encoding known components of the thyroid hormone biosynthesis machinery (reviewed in 2, 4).

Central CH (CCH) occurs when defective stimulation of a normal thyroid gland by thyroid stimulating hormone (TSH) results in inadequate thyroid hormone biosynthesis. Hypothalamic or pituitary pathology causes a qualitative or quantitative deficit in TSH synthesis or secretion, and in the majority of cases, the molecular basis has remained unresolved (5). Although CCH most commonly manifests with normal or subnormal TSH levels despite subnormal thyroid hormone concentrations, mildly
elevated serum levels of immunoreactive TSH with impaired bioactivity may be
detected if the defect is predominantly hypothalamic (maximum 12.9mIU/l in one
study), with the potential for misdiagnosis as subclinical or mild primary
hypothyroidism (6). Although assumed to be rare, CCH may be more common than
previously appreciated with an incidence of up to 1:16,400 to 1:21,000 in the
Netherlands (7, 8). Most individuals with CCH exhibit additional pituitary hormone
deficits, but isolated TSH deficiency occurs with an estimated incidence of around
1:65,000, often as a result of defects in genes controlling the TSH biosynthetic
pathway (5, 9).

Adequate circulating thyroid hormone levels are a prerequisite for normal childhood
growth and neurodevelopment, therefore prompt detection and treatment of CH is an
important public health concern. Although most industrialized countries operate
neonatal screening programmes for CH, most employ a primary TSH-based
methodology which will detect primary CH but not CCH. This review will focus on
primary CH and isolated CCH, incorporating discussion of genetic aetiological
advances as well as summarizing recent epidemiological data and clinical diagnostic
challenges for these conditions.

**Primary Congenital Hypothyroidism**

**Epidemiology**

Prior to the development of CH screening, the incidence of primary CH was estimated
at 1:7,000 (10, 11), however, once screening was introduced, the actual incidence was
found to be almost double these original estimates at 1:3,000-4,000 (12). Over the last
two decades, the detected incidence of CH has doubled again, largely due to increased
diagnosis of cases with gland-in-situ (GIS), with a lesser contribution from thyroid
ectopy or cases with a structurally abnormal gland in situ. The number of children
with athyreosis seems unchanged (3).

The contributing factors underlying this altered incidence are complex (13, 3, 14, 15,
16). Some argue that the increase in CH cases is due to a generalized lowering of
newborn screening cutpoints that has arisen with assay changes and reports of missed
cases of CH. However, the prevalence of CH has also increased in programmes where
the cutpoints have not been altered (14, 15). Additional influences include changes in
ethnicity and demographics of the populations screened (14, 16, 17) and dietary
iodine insufficiency may also be contributing to the number of children with slightly
higher TSH concentrations. (18). The extent to which iodine deficiency interacts with
variants in the genes mediating thyroidal iodine metabolism to provoke CH remains
unclear.

In addition to the overall increased incidence of CH, there is also an increase in the
proportion of children with transient hypothyroidism. These children meet the
biochemical criteria for CH treatment in infancy but no longer require levothyroxine
when retested around three years of age. Transient CH may reflect mild
dyshormonogenesis with failure of adequate thyroxine (T4) production to meet the
increased requirements in the first months of life, but sufficient hormone biosynthesis
for later childhood years. The risk of subclinical or overt hypothyroidism in later life
and potential for hypothyroidism in pregnancy when there is a further increased
physiological demand for levothyroxine has not yet been fully evaluated in such
patients.

**Genetic advances in Primary CH**

**Monogenic causes of Thyroid Dysgenesis**

In more than 95% cases, the aetiology for thyroid dysgenesis cannot be identified, however, genetic ascertainment in CH cases has established five key monogenic causes of thyroid dysgenesis: loss-of-function mutations in *TSHR* (TSH receptor), *NKX2-1* (NK2 homeobox 1, previously known as TTF1), *PAX8* (Paired box 8), *FOXE1* (Forkhead box E1, previously known as TTF2) and most recently, *GLIS3* (GLIS family zinc finger 3). *PAX8, NKX2-1* and *FOXE1*, together with *HHEX* (Haemopoetically expressed homeobox) encode an indispensable quartet of transcription factors which define early thyroid development in both humans and mice. Although these transcription factors act in concert to mediate organogenesis only in epithelial thyroid follicular cells, they also have individual developmental roles in extra-thyroidal tissues (19). TSHR is the thyroidal G-protein coupled receptor for TSH (20), and GLIS3, plays a role in both transcriptional repression and activation in multiple organs from early in embryogenesis (21).

**Key Transcription factor mutations in TD**

Monogenic loss-of-function mutations in *NKX2-1*, *PAX8* and *FOXE1* are rare but well-established causes of TD, and deficiencies in these genes underlie distinct syndromes reflecting their additional, extrathyroidal expression patterns (Table 1),
however, expressivity and phenotypic penetrance may be highly variable, even within
the same family (1, 22).

**NKX2-1**

Heterozygous loss-of-function *NKX2-1* mutations are the most frequent transcription
factor mutation in CH. *NKX2-1* plays a major role in regulation of key genes
involved in thyroid differentiation (including *TSHR, TG* and *TPO*), and *Nkx2-1* null
mice also exhibit a visible embryonic thyroid bud which disappears around E10.5-11,
suggesting that *Nkx2-1* may play a role in thyroid precursor cell survival (23, 24).
Additionally, *Nkx2-1* is required for maintenance of the normal architecture and
function of differentiated thyroid (25) and has extra-thyroidal roles, being required for
pulmonary surfactant production and contributing to development of the ventral
forebrain, and hypothalamic neurons. Consequently, *NKX2-1* mutations result in
cerebral and pulmonary phenotypes in addition to thyroid dysfunction, with a
complete triad constituting ‘brain-lung-thyroid’ syndrome. Figure 1A summarizes the
distribution of phenotypes in cases with *NKX2-1* mutations. Neurological
manifestations typically include a benign hereditary chorea but may comprise
hypotonia, ataxia and developmental delay. Hypothyroidism is usually mild or
subclinical with normal thyroid morphology although gland hypoplasia, hemiagenesis
or athyreosis are also described (26). Pulmonary compromise includes infant
respiratory distress syndrome (IRDS), recurrent infections and airways
hypersensitivity and carries an associated mortality of up to 16% (22, 27).

*NKX2-1* mutations may exhibit autosomal dominant inheritance with variable
expressivity and penetrance, but frequently occur de novo, which, if confirmed, may reassure parents that the risk to future offspring is minimal (22). Most mutations are thought to result in a phenotype due to haploinsufficiency, but a minority of variants with dominant negative effects have also been described (22, 28). Deletions involving NKX2-1 are common and should be specifically excluded in brain-lung-thyroid syndrome; moreover, deletions proximal to NKX2-1 have also been implicated, suggesting the presence of an upstream enhancer in this region (27).

PAX8

PAX8 is also crucial for expression of the genes involved in thyroid differentiation and hormone biosynthesis including TG, TPO and SLC5A5 (19, 29, 30). Additionally, the disappearance of the thyroid cell precursors in Pax8 null mice at around E11–11.5, attests to a likely role for PAX8 in thyroid precursor cell survival (31). Indeed, Pax8 also controls thyroid cell survival in adults, and is involved in the maintenance of adult thyroid follicular cell differentiation (32).

Twenty-nine heterozygous loss-of-function PAX8 mutations defined as ‘disease causing’ are reported in HGMD (Human Gene Mutation Database (HGMD®) Professional 2018.1), the majority comprising substitutions affecting the DNA binding domain. Inheritance is autosomal dominant with variable expressivity and penetrance and both dominant negative effects and haploinsufficiency may mediate disease phenotype (33, 34). Affected patients predominantly exhibit thyroid hypoplasia, however, GIS, ectopy and athyreosis may also occur (Figure 1B) (35). Despite CH in most affected patients, thyroid dysfunction may also be transient or subclinical and manifest in later childhood or adulthood (36). PAX8 is also expressed
in the nephrogenic mesenchyme and rarely, mutations have been associated with urogenital tract abnormalities (36).

FOXE1

FOXE1 is a transcription factor in the forkhead domain family which is expressed in the developing oropharynx, esophagus, choanae and hair follicles. Foxe1 null mice develop a rudimentary thyroid bud which either remains in an ectopic site at the base of the tongue, or disappears, supporting a role for FOXE1 in thyroid migration as well as differentiation and survival of the developing thyroid gland. Additionally, FOXE1 has a role in maintenance of the mature, differentiated thyroid, for example enabling expression of genes involved in thyroid hormone biosynthesis. (37, 38).

At least seven biallelic CH-associated FOXE1 point mutations have now been reported and affected patients typically exhibit athyreosis or severe thyroid hypoplasia, in association with cleft palate and spiky hair (Bamforth-Lazarus syndrome). Choanal atresia and bifid epiglottis may also occur, reflecting the expression of FOXE1 in epiglottis, palate, oesophagus, definitive choanae and hair follicles (39). Inheritance of FOXE1 mutations is autosomal recessive and all described mutations cluster in the forkhead DNA binding domain. All except one impair both DNA binding and transcriptional activity of FOXE1 resulting in loss of function. A single gain of function mutation in the same region results in enhanced activity of TG and TPO promoters, but the associated clinical phenotype is indistinguishable from that associated with loss-of-function mutations (40).

GLIS3
GLIS3 is a member of the GLI-similar 1-3 (GLIS1-3) subfamily of Krüppel-like zinc finger protein transcription factors which play a key regulatory role in embryogenesis. GLIS3 may act as a transcriptional activator or repressor and is particularly important in the regulation of pancreatic β cell generation and maturation, insulin gene expression, thyroid hormone biosynthesis, spermatogenesis, and the maintenance of normal kidney function. Biallelic loss-of-function mutations in GLIS3 have now been described in children from 14 different families and are robustly associated with CH in all but one case as part of a variably penetrant multisystem phenotype consistently including permanent neonatal diabetes. (Table 1, reviewed in 21). Inheritance is autosomal recessive. Thyroid morphology ranges from apparently normal to hypoplasia or gland athyreosis, however histology from an ultrasonographically normal gland demonstrated abnormal architecture with a paucity of colloid and extensive fibrosis. Significant TSH resistance frequently compounds management of children harbouring GLIS3 mutations, perhaps explained by its actions downstream of TSH and the TSHR, since GLIS3 is indispensable for TSH/TSHR-mediated proliferation of thyroid follicular cells and biosynthesis of thyroid hormone (41, 21). Additionally, in some cases, elevated TSH and thyroglobulin levels appear to be resistant to treatment with conventional doses of levothyroxine therapy despite normalization of free T4 (21).

**TSHR**

TSHR mutations result in TSH resistance, for which the associated phenotype is dependent on both the deleteriousness of the mutation, and the number of mutated TSHR alleles, since inheritance may be either dominant or recessive (1). Complete TSH resistance manifests as severe biochemical CH with orthotopic gland hypoplasia,
however, at the milder end of the spectrum, isolated hyperthyrotropinaemia may be associated with preserved thyroid hormone biosynthesis from a normal-sized thyroid gland. Early thyroid development, and thyroglobulin (TG) synthesis at the onset of folliculogenesis is TSH independent. Therefore, even severe TSHR mutations cause only apparent athyreosis, i.e. marked thyroid hypoplasia which is undetectable on imaging but associated with a measurable serum thyroglobulin (TG) confirming the presence of some residual thyroid tissue (20). TSH plays a role in thyroid growth from the third trimester and subsequently stimulates thyroid hormone synthesis and release.

Around 100 likely inactivating TSHR mutations have been reported, the incidence of which depends on the clinical characteristics and ethnicity of the CH population screened. Heterozygous TSHR mutations causing partial TSH resistance, are most frequently described, with incidences of 11-29% in Italian studies of non-autoimmune hyperthyrotropinaemia (42). The contribution of TSHR mutations to orthotopic thyroid hypoplasia is more difficult to estimate, since most studies involving such cases include additional thyroid morphologies, but in a consanguineous Pakistani and Turkish cohort, TSHR mutations were the most common genetic cause of non goitrous CH, affecting 5% families (43). Founder mutations also operate, e.g. TSHR p.R450H in individuals from East Asia (1).

TSHR mutations occur throughout the protein, with nonsense and frameshift mutations generally decreasing levels of TSHR expressed at the plasma membrane. Most point mutations result in decreased signal transduction by the receptor, but may also affect ligand binding, depending on their location. A dominant negative
mechanism may contribute to phenotypic severity in some cases, e.g. in association
with TSHR p.C41S, which entraps wild-type receptor intracellularly by forming
oligomers (reviewed in 42).

In complete TSH resistance with subnormal FT4 levels, levothyroxine replacement is
unequivocally required but the need for treatment in partial TSH resistance remains
controversial since, in this context, elevated TSH levels are sufficient to maintain
normal circulating thyroid hormone concentrations (42). Additionally, exogenous T3
administration elicits normal fractional changes in serum TSH and peripheral
biomarkers of thyroid hormone action, signifying normal sensitivity of pituitary
thyrotrhops and peripheral tissues to thyroid hormone (44, 45). The mechanisms
permitting continued secretion of high levels of TSH with T4 levels within the normal
range are unclear, but this biochemical signature may represent a resetting of the
threshold for TSH suppression by circulating thyroid hormones. Accordingly,
supraphysiological FT4 concentrations are required to achieve a TSH level in the
normal range (44, 45). The molecular mechanisms supporting an altered TSH set
point have not yet been elucidated, and may include altered expression of genes
involved in set point regulation at different points in the hypothalamic-pituitary-
thyroid axis, or genes mediating thyroid hormone metabolism (20).

Since peripheral sensitivity to thyroid hormone is preserved, the doses of
levothyroxine required to normalize TSH in partial TSH resistance may provoke
thyrotoxic symptoms, and anecdotal reports of normal growth, development and
pituitary size in untreated cases despite chronically raised TSH support the argument
that levothyroxine treatment may be unnecessary (44, 45). Two recent studies from
Israel and Italy have addressed this question in patients with TSHR mutations; in the Italian cohort, the effect of thyroid hormone replacement on developmental parameters and biomarkers of thyroid hormone action was also assessed. Both studies concluded that biochemical features associated with heterozygous TSHR mutations (elevated TSH, normal circulating thyroid hormone concentration) were generally stable, representing a compensated state which may not require thyroid hormone replacement. However, in patients with either heterozygous mutations and additional risk factors for thyroid dysfunction, or biallelic mutations, compensation of thyroid hormone biosynthesis by elevated TSH levels may be incomplete and progress, resulting in deteriorating thyroid hormone levels and a need for levothyroxine treatment (46, 47). Larger studies, including monoallelic TSHR mutations associated with a broader spectrum of TSH levels are still required to guide management definitively. Meanwhile, levothyroxine replacement therapy in partial TSH resistance should be considered on a case by case basis and is not indicated for the treatment of elevated TSH alone although in some affected individuals, signs and symptoms of hypothyroidism, or declining thyroid hormone levels may justify intervention.

**Additional genes associated with TD**

**NKX2-5**

NKX2-5 belongs to the NK-2 family of homeodomain-containing transcription factors and was initially an attractive candidate gene for TD, having been shown to play a role in murine thyroid development. Nkx2-5 is expressed during early murine thyroid morphogenesis and murine Nkx2-5 null embryos exhibit thyroid bud
hypoplasia. However, despite an initial report of four patients with heterozygous loss-of-function NKX2-5 mutations and thyroid ectopy or athyreosis, the role of NKX2-5 in TD remains ambiguous. Inheritance in these families was autosomal dominant but mutation penetrance was highly variable (carrier parents frequently had normal thyroid morphology and biochemistry) and pathogenic mutations may also occur either in healthy populations (p.R25C, MAF >1%) or in association with isolated congenital heart disease (48, 49). Although NKX2-5 variants may contribute to TD risk, other factors/genes are likely to play a significant role modulating penetrance and expressivity.

**JAG1**

Jagged1 (JAG1) is a Notch receptor ligand expressed in thyroid, which may play a role in thyroid specification in zebrafish, as well as in differentiation and maintenance of thyroid precursor cells (50). JAG1 plays a role in zebrafish thyroid development and variably penetrant, human heterozygous loss-of-function JAG1 mutations are associated with Alagille syndrome in which congenital heart disease (CHD) associates with variable hepatic, eye and skeletal defects together with dysmorphic facies. Evaluation of thyroid function in 21 cases with Alagille syndrome revealed mild, non-autoimmune hypothyroidism in six individuals. Additionally, 4% cases in a CH cohort without associated Alagille syndrome identified heterozygous JAG1 mutations (with associated CHD in two patients). Rare JAG1 variants have also been detected in an unrelated CH cohort substantiating the notion that JAG1 may contribute to the pathogenesis of CH. (51, 52).

**CDCA8 (BOREALIN)**
CDCA8 is a member of the Chromosomal Passenger Complex with roles in the processes of chromosome segregation and cytokinesis. It is expressed in human thyroid tissue during embryonic development and is the most recently-identified genetic cause of TD with mono- and biallelic loss-of-function mutations reported in three unrelated families. A homozygous CDCA8 mutation (p.S148F) in two siblings was associated either with CH and thyroid ectopy, or euthyroidism with thyroid hemiagenesis. In two more families, heterozygous CDCA8 mutations were associated with CH and either thyroid ectopy or athyreosis. Euthyroid heterozygous parents exhibited variable thyroid structural abnormalities (asymmetry, nodules) and one developed papillary thyroid cancer. Expression of mutant CDCA8 in a thyroid cell line resulted in altered cellular migration and adhesion by decreasing the expression of genes implicated in focal adhesion (53).

Netrin 1 (NTN1)

The close proximity of the developing thyroid to cardiac mesenchyme and vasculature, and the increased frequency of cardiovascular malformations in patients with TD, have led to the suggestion that non-cell autonomous mesenchyme derived factors may play a role in thyroid development. Zebrafish have proved a useful tool in investigating the mechanisms involved and Netrin 1-deficient zebrafish embryos demonstrate defective aortic arch artery formation in addition to abnormal thyroid morphogenesis. Since ntn1a, the zebrafish paralog of human NTN1, is expressed in pharyngeal arch mesenchyme but not in thyroid tissue, it is likely that the thyroid fails to develop due to lack of guidance cues from dysplastic vasculature. A single patient with VSD and thyroid ectopy was reported in whom a heterozygous deletion involving part of NTN1 was detected in addition to a 47, XYY karyotype and an
atypical 22q11 deletion. However, the extent to which \textit{NTNI} mutations contribute to shared thyroid and cardiac congenital defects in the population remains unclear (54).

** Syndromes which may be associated with CH. **

Risk of CH may be increased in the context of several syndromes with an underlying genetic basis and predominantly extrathyroidal associated abnormalities. Candidate genes involved include \textit{SALL1} (Townes-Brocks syndrome), \textit{TBX1} (di George syndrome), \textit{URB1} (Johanson-Blizzard syndrome), \textit{DYSR1A} (Trisomy 21), \textit{ELN} (Williams-Beuren syndrome), \textit{KMT2D/MLL2}, \textit{KDM6A} (Kabuki syndrome) and \textit{KAT6B} (Ohdo syndrome, Genitopatellar syndrome) (4). The underlying mechanism for CH in these conditions is generally unclear, and the genes involved usually demonstrate ubiquitous expression, including expression in thyroid. \textit{TBX1} is a non-cell autonomous factor likely derived from cardiac mesenchyme, required for thyroid development (38).

** Genetic Causes of Dyshormonogenesis **

Unlike thyroid dysgenesis, the majority of dyshormonogenesis has an identifiable genetic basis. Thyroid hormone biosynthesis at the apical surface of polarized thyroid follicular cells requires an intact synthesis pathway comprising transporter molecules, enzymes, thyroglobulin (TG) and adequate iodide substrate (Figure 2). Genetic causes of dyshormonogenesis comprise loss of function mutations in genes encoding components of the thyroid hormone biosynthetic machinery resulting in inadequate thyroid hormone synthesis with or without compensatory goitre. Mutations may
involve TG, TPO, SLC26A4 (Pendrin), SLC5A5 (NIS), DUOX2, DUOXA2 or IYD.

Inheritance patterns and associated features are dependent on the site of the synthesis defect and summarized in Table 2 (2, 1).

The frequencies of dyshormonogenesis-associated mutations are heavily influenced by ethnicity, including the presence of founder mutations, and selection criteria for the study population. TG mutations are a common cause of dyshormonogenesis with an estimated frequency of at least 1:100,000 births (55). TPO defects represent the commonest cause of total iodide organification defect (TIOD, 56), and frequently underlie dyshormonogenesis in European and Pakistani cases (57) but may occur less frequently in East Asian individuals (58, 59). DUOX2 mutations are now frequently reported, especially in East Asian individuals, with a monoallelic frequency of up to 1:13,501 in Korean cases (58, 59) and also account for 37% CH due to partial iodide organification defect (PIOD) in an Italian series (60). Some pathogenic DUOX2 mutations have a minor allele frequency of >1% in certain populations (e.g. p.Q570L in South Asians) suggesting that they may be an even more frequent contributor to CH than previously recognized (60). Mutations in SLC5A5, and IYD seem rare, with IYD mutations only identified in five families (55) and DUOXA2 mutations are also uncommon although probably occur most frequently in East Asian cases (59). Although Pendred syndrome occurs more frequently in the general population (estimated incidence 7.5-10 per 100,000), CH is a rare association such that Pendrin mutations account for less than 5% of CH (2, 61, 62).

Clinical phenotypes in dyshormonogenesis


Biochemical and radiological hallmarks of specific, genetically ascertained
dyshormonogeneses are summarized in Table 2 however, evidence increasingly
supports a broader phenotypic range in most dyshormonogenesis subtypes than
initially appreciated. This is particularly well-documented for CH associated with
mutations in DUOX2, which were first reported only in 2002. DUOX2 is a thyroidal
NADPH oxidase which generates the thyroidal H$_2$O$_2$ required by TPO as the final
electron acceptor during both iodination of thyroglobulin and coupling of mono and
diiodotyrosine. It is contiguous with DUOX1, (an additional NADPH-oxidase) on the
long arm of chromosome 15, with the DUOXA maturation factor genes occupying the
DUOX intergenic region. DUOX2 is thought to be the dominant isoenzyme in thyroid
hormonogenesis, being expressed at higher levels in thyroid than DUOX1 and
consistent with this, DUOX2 but not DUOX1 mutations are a recognized monogenic
cause of CH. Additionally, DUOX1 null mice do not exhibit hypothyroidism (63).

To date, approximately 100 different DUOX2 mutations have been reported, including
missense, stop-codon, splice-site and in-frame deletion mutations. However, only
around 50% missense mutations have been functionally characterized, leading to
some ambiguity about the number of these which are truly pathogenic. Indeed,
functional evaluation has confirmed that some likely benign polymorphisms have
been misclassified as disease-causing (60). Functional characterization of DUOX2
mutations was initially hampered by failure to achieve plasma membrane expression
of DUOX2 in heterologous cell systems, however, identification and coexpression of
the DUOXA2 maturation factor for DUOX2 enables correct translocation of DUOX2
from the ER and has permitted such evaluation (64). The mechanisms and degree to
which pathogenic mutations exhibit impaired H$_2$O$_2$ generation are variable, including
both complete and partial deficits in H$_2$O$_2$ generation. Truncating mutations are usually predicted to result in impaired enzyme activity due to disruption of the C-terminal NADPH oxidase domain. Cell membrane expression was evaluated for six missense mutations which significantly impair DUOX2 function. All exhibited complete or partial trafficking defects from the endoplasmic reticulum to the cell surface, and decreased plasma membrane expression (65, 66), with preserved or absent intrinsic H$_2$O$_2$ generating activity (66, 67).

Both monoallelic and biallelic DUOX2 mutations have been described, and heterozygous mutations are thought to confer a phenotype due to haploinsufficiency since evidence for dominant negative activity is currently lacking (66). Initially, biallelic DUOX2 mutations were thought to result in permanent CH, and monoallelic mutations to cause transient CH (68). However, subsequent studies have shown almost 40% discordancy with this observation; additionally, penetrance is highly variable and biallelic truncating mutations may be associated with both mild transient and severe permanent CH (69). Although sometimes associated with goitrous dyshormonogenesis, DUOX2 mutations may also cause a resistance to thyrotropin phenotype (70). Additionally, next generation sequencing recently identified frequent DUOX2 NH$_2$-terminal mutations in cases with thyroid ectopy, raising the possibility of a role for DUOX2 in thyroid development, for which the mechanism and putative H$_2$O$_2$-dependency remain unclear (71).

It has been suggested that variants in other H$_2$O$_2$-synthesizing enzymes capable of compensating for DUOX2 deficiency, e.g. DUOX1, may modulate CH severity (2) and the first cases with likely complete DUOX isoenzyme deficiency were recently
reported; although detailed genotype-phenotype segregation studies were not performed, these cases exhibited unusually severe CH consistent with a compensatory role for DUOX1 (72). In contrast, mice homozygous for a DUOX2 point mutation alone, exhibit severe hypothyroidism, suggesting that DUOX1 expression is unable to compensate for the DUOX2 defect in the murine model (73). Evaluation of Italian cases harbouring DUOX2 mutations demonstrated characteristic associated biochemistry including significantly elevated confirmatory TSH and subnormal venous FT4 measurements despite borderline neonatal screening TSH, raising the possibility that cases with DUOX2 mutations could be missed on neonatal screening (60). Clinical and functional data for cases with mutations in DUOX2, the accessory protein for DUOX2, is sparse, but most cases appear to have mild or transient CH and loss-of-function may be associated with either normal protein expression or decreased expression levels of unstable DUOX2 (74, 75).

Thyroid dysfunction associated with NIS or TG mutations can range from severe CH to euthyroid goitre, especially if dietary iodine content is high. TPO mutations usually cause severe CH although milder cases with PIOD have been described, sometimes due to monoallelic defects (55) and both TG and TPO mutations are rarely associated with fetal goitre. Homozygous IYD mutation carriers generally exhibit goitrous congenital or childhood-onset hypothyroidism, and one heterozygote has also been described with hypothyroidism and goitre (55, 76). IYD, NIS and DUOX2 mutations may all present late, following normal neonatal screening TSH results, resulting in neurodevelopmental delay if diagnosis of infantile hypothyroidism is delayed (2, 60, 76, 77).
Evidence for an undiagnosed genetic component in TD

Causative mutations are identified in less than 5% of TD cases, leading to the assumption that TD is a sporadic disease. This notion is supported by a higher than 90% discordance between monozygotic twins with CH (78) and a strong female preponderance of TD, especially thyroid ectopy (79). Since these features are incompatible with simple Mendelian inheritance, it has been hypothesized that somatic mutations restricted to the thyroid or epigenetic events may be implicated.

However, other lines of investigation support a more significant aetiological role for germline mutations in TD than currently diagnosed. Two percent of TD cases in a French National Survey of CH have an affected relative, which is 15-fold greater than predicted by chance alone. Moreover, thyroid developmental abnormalities occur more commonly in euthyroid first degree relatives of CH cases than in controls (80, 81) and the incidence of extrathyroidal developmental malformations is also increased in patients with CH. Additionally, CH occurs more frequently in consanguineous or less genetically diverse populations (82, 83).

Alternative genetic aetiologies in TD and Dyshormonogenetic CH

Recent studies have sought alternative genetic aetiologies for CH, some of which have attempted to reconcile the apparent sporadic occurrence of TD with the data supporting an aetiological role for genetic factors. Potential mechanisms consistent with these observations include two hits, where a germline predisposing mutation occurs in association with an additional genetic or epigenetic alteration within the
thyroid tissue or surrounding structures (84). However, in the only study to investigate this, the gene expression pattern was different in ectopic thyroid, but this was not attributable to significant somatic methylation gene expression profile differences (85). Additionally, frequent somatic mutations were not identified in lymphocyte DNA from monozygotic twins discordant for TD (86) although somatic mosaicism for a PAX8 mutation has been reported (87). Autosomal monoallelic expression has been reported for some genes in both ectopic and eutopic thyroid, (88); however, monoallelic expression of a mutant allele has only been reported for TPO in association with dyshormonogeneic CH (89). Recurrent copy number variants have also not been identified in CH (90).

Next generation sequencing (NGS) technologies have enabled interrogation of the role of oligogenicity in CH, which may also contribute to its apparently sporadic occurrence. An aetiological role for oligogenicity was initially supported by observations that mice with heterozygous TTF1 or PAX8 mutations are euthyroid, but strain-specific TD occurs in mice with combined partial deficiencies of TTF1 and PAX8 (91). Human studies have now confirmed a role for oligogenic inheritance in both CH with eutopic gland-in-situ and TD. In a particularly comprehensive study, 11 CH-associated genes were screened in more than 150 Italian patients with different CH subtypes, with subsequent analysis demonstrating that 23% harboured a likely pathogenic variant in more than one gene (52, 92).

NGS approaches have also enabled the screening of genes classically associated with TD or dyshormonogenesis in mixed CH populations, demonstrating overlap of genetic aetiologies in the two morphological subgroups. Mutations in genes
characteristically associated with TD, (e.g. biallelic FOXE1 mutations), have been reported in association with isolated CH and a normal thyroid gland (52). Conversely, Pendrin mutations have been reported in TD, where secondary atrophy of the thyroid was postulated to occur due to increased oxidative stress (93).

Novel candidate genes for CH have also been identified by exploring mouse and zebrafish models for thyroid development (94). Recently, in murine and human pluripotent stem cell-derived endodermal precursors, expression of NKK2-1 and PAX8 alone, in response to exogenous FGF2 and BMP4 in vitro was found to be sufficient for differentiation into thyroid follicular structures capable of producing thyroid hormones when exposed to thyrotropin (95, 96). This stem cell technology presents an exciting system in which to validate the roles of novel candidate genes for TD in the future as well as raising the future possibility of regenerative therapy for CH.

Isolated Central Congenital Hypothyroidism (CCH)

The Hypothalamic-Pituitary-Thyroid Axis: Positive Regulation of Thyroid Hormone Synthesis

Thyroid hormone biosynthesis is positively regulated by the actions of hypothalamic thyrotropin-releasing hormone (TRH) which stimulates TSH production from the anterior pituitary. TRH is synthesized in the paraventricular nucleus (PVN) of the hypothalamus and following maturation, reaching the thyrotrophs of the anterior pituitary gland via the hypothalamic portal vein. It then binds the TRH receptor (TRHR), a G-protein coupled receptor, which activates a Gq/11 dependent pathway subsequently mobilizing intracellular calcium and activating protein kinase C. TRH
upregulates transcription of the TSH alpha (αGSU) and beta subunit genes (CGA and
TSHB) but also exerts important post-translational effects, facilitating conjugation of
TSH alpha and beta subunits and promoting both secretion of heterodimeric TSH and
its post-translational glycosylation which is required to confer normal bioactivity
(Reviewed in 5, Figure 3).

Genetic ascertainment in CCH due to isolated TSH deficiency has advanced over the
last three decades, and a total of four genes, all with a role in TSH biosynthesis, are
now implicated in its pathogenesis; thyrotropin-releasing hormone receptor (TRHR),
thyroid stimulating hormone beta subunit (TSHB), immunoglobulin superfamily
member 1 gene (IGSF1) and the Transducin Beta Like 1X-Linked gene, TBL1X. (5, 97).

Genetic causes of Isolated Central Congenital Hypothyroidism

TSHB mutations

Biallelic loss-of-function, TSHB mutations result in severe CCH. Therefore if
diagnosis is delayed until children present clinically, the severity of hypothyroidism
frequently results in neurodevelopmental impairment, the extent of which correlates
with the degree of treatment delay. In contrast, developmental outcome is often
improved in cases who are ascertained, diagnosed and treated from birth following
genetic diagnosis in a sibling (98).

Mature TSH comprises a heterodimer of the alpha subunit (αGSU) common to other
glycoprotein hormone (LH, FSH, CG) family members and a beta-subunit which is
TSH specific (TSHB). Key structural features are required to maintain the integrity of
the heterodimer including a ‘seat belt’ formed from the TSH beta subunit which
wraps around the long loop of the alpha-subunit and forms an intra-molecular
disulfide ‘buckle’ to stabilize the heterodimer. Additional alpha-beta subunit
interactions occur around a conserved CAGYC sequence motif.

All reported missense or indel mutations either disrupt key disulphide bridges
required for heterodimeric integrity, truncate the protein or disrupt the CAGYC region
(Figure 4). The most common mutation is a single nucleotide deletion (c373delT)
leading to a cysteine 125 to valine change (p.C125V) and subsequent frameshift and
premature stop codon at position 134 (p.C125Vfs*10) (99) which exhibits a founder
effect in some communities (98). More recently, two TSHB splice-site mutations
(c162G>A, c.162+5 G>A) (100, 101) and two TSHB deletions have been reported (98,
102).

CCH due to TSHB mutations is characterized by profound CH with elevated pituitary
glycoprotein alpha subunit, and severely impaired TSH response to TRH
administration, despite a preserved serum Prolactin rise (103, Figure 5A, B). Serum
TSH levels may be undetectable with mutations (p.G49R, p.Q32*), which disrupt
heterodimer formation between TSHalpha and beta polypeptides, whereas in cases
with mutations resulting in synthesis of non-bioactive heterodimeric TSH (eg p.Q69*,
c.373delT), immunoreactive TSH will be detected in an immunoassay-dependent
manner if epitopes recognized by the anti-TSH monoclonal antibody are preserved
(103, 104).
**TRHR Mutations**

Cases harbouring biallelic loss-of-function mutations in *TRHR* mutations have only been reported in four kindreds. In the first two kindreds, truncating mutations completely abolished TRHR activity (compound heterozygosity for TRHR p.R17*, and an in-frame deletion of three amino acids (S115, I116, and T117) with one substitution (p.A118T), or homozygosis for p.R17*). More recently, an equally deleterious biallelic p.P81R missense variant was reported (105, 106, 107). Homozygous individuals in these families exhibited T4 concentrations 40 to 88% of the lower limit of the normal range and heterozygous carriers were euthyroid (Figure 5A, 6). Where present, the main clinical manifestations comprised growth retardation and delayed bone age. Some affected patients were first diagnosed with CCH in late childhood or adulthood, but did not exhibit significant neurological deficits suggesting sufficient thyroid hormone production in infancy to prevent overt mental retardation. However, even asymptomatic cases exhibited improved quality of life with levothyroxine therapy (105).

Last year, the first kindred, harbouring a TRHR mutation (p.I131T) which resulted in impaired rather than absent signal transduction was reported. Two homozygotes exhibited either moderate CCH on the basis of FT4 levels or isolated hyperthyrotropinaemia and heterozygotes exhibited isolated hyperthyrotropinaemia, for which the mechanism is unclear (108) (Figure 5A, 6). However, since TRH plays a key role post-translational glycosylation of TSH which is required to confer normal bioactivity, it is possible that the high TSH in the homozygotes represents a
compensatory enhanced production of non-bioactive TSH in response to decreased negative feedback by thyroid hormones. This has previously been noted in central hypothyroidism due to TRH deficiency, where increased amounts of bioinactive TSH may be secreted with immature carbohydrate chains and decreased half-life (109).

TRHR is expressed in both thyrotrophs and lactotrophs, therefore intravenous TRH usually stimulates both TSH and prolactin peaks. Both these responses were absent in patients with absent TRHR function but preserved with the milder p.I131T mutation (Figure 5B). One female with the homozygous, p.R17* mutation achieved two normal pregnancies with subsequent lactation prior to her diagnosis, suggesting that TRH action is not obligatory for pregnancy and lactation in humans (105-108).

**IGSF1 mutations**

Loss-of-function mutations in the X-chromosomal immunoglobulin superfamily member 1 (IGSF1) gene are now thought to be the most common genetic abnormality underlying CCH. (110, 111). Since the initial description of IGSF1 deficiency in eleven European kindreds, more than 30 pathogenic IGSF1 mutations have been described (Figure 7). IGSF1 encodes a transmembrane immunoglobulin superfamily glycoprotein and following co-translational proteolysis, its seven carboxyterminal immunoglobulin loops are expressed extracellularly at the plasma membrane. Disease-associated IGSF1 mutations usually impair trafficking and membrane localization of this carboxyterminal domain (110, 111, 112).

Hemizygous males harbouring IGSF1 mutations exhibit a more severe phenotype than
heterozygous females, who may have no overt endocrinopathy. Affected males invariably exhibit CCH, which is usually mild to moderate and associated with a blunted neonatal TSH response to TRH but a low normal response from childhood onwards (Figure 5) (112). Additional endocrine abnormalities include disharmonious pubertal development in the majority of cases with delayed pubertal growth spurt and testosterone rise, but normal onset of testicular growth and subsequent macroorchidism in adulthood. Basal prolactin levels are subnormal in more than 60% males and infrequently, individuals may exhibit transient growth hormone (GH) deficiency in childhood, necessitating GH replacement. In adulthood, IGF-1 levels are paradoxically in the upper half of the reference range or mildly elevated, and acromegaloid features may develop. Heterozygous females harbouring IGSF1 mutations generally exhibit thyroid hormone levels in the lower tertile of the normal range with approximately 20% fulfilling the criteria for central hypothyroidism. Up to 20% demonstrate hypoprolactinaemia and four females reported have required surgery for benign ovarian cysts (110, 111, 112). A typical feature of kindreds harbouring IGSF1 mutations is the new diagnosis of central hypothyroidism in individuals across three generations, following identification of a young affected proband. It is clear that some children and adults diagnosed during family screening exhibit clinical features of untreated endocrinopathy, and benefit from hormone replacement. However, some individuals are apparently healthy, with normal growth and development despite CCH, and in this context, the benefits of levothyroxine treatment remain to be determined (110, 111, 112).

The precise molecular role of IGSF1 and mechanisms underlying the manifestations of the IGSF1 deficiency syndrome remain unclear. IGSF1 mRNA is expressed in
Rathke’s pouch and in adult pituitary gland (111), however a paucity of reliable antibodies has hampered protein expression studies in humans. In rodents, studies using two different antibodies have yielded divergent results, localizing IGSF1 protein either to all cells of the Pou1f1 (Pit1) lineage in murine and rat pituitary (110, 111), or (using a different, commercially available anti-IGSF1 antibody) to thyrotropes and gonadotropes in rats, but not somatotropes or lactotropes (113). Murine studies in two different IGSF1 deficient mouse lines have demonstrated impaired TRH signalling associated with IGSF1 deficiency and decreased pituitary expression of Trhr1 mRNA despite normal TRH synthesis (110, 111). This suggests that impaired TRH signalling may underlie the central hypothyroidism seen in IGSF1 deficiency, which would be consistent with the mild-moderate CCH observed in most humans with hemizygous IGSF1 mutations and blunted neonatal TRH test response. However, the basis for the hypoprolactinaemia and macroorchidism remain unresolved.

**TBL1X (transducin β-like protein 1)**

Loss-of-function missense mutations in *TBL1X* are the most recently reported genetic cause of isolated CCH. Like *IGSF1*, *TBL1X* is also located on the X chromosome and eight males harbouring hemizygous mutations, in addition to eleven females harbouring heterozygous mutations were identified in six unrelated kindreds (97). Penetrance was more variable than in other genetically-mediated forms of CCH, with only six males and three females exhibiting FT4 concentrations below the lower limit of the reference range although FT4 levels in affected adults were significantly lower than in controls. Where present, CCH was isolated, and mild-moderate, with normal
TRH test responses (Figure 5). Mild sensorineural hearing loss also occurred frequently in affected cases (97). Since TBL1X is an essential component of the main nuclear receptor corepressor complex (NCoR/SMRT) involved in T3–regulated gene expression, it is hypothesized that, for negatively regulated genes such as TRH and TSHB, TBL1X may play a role in basal activation, as well as ligand-induced transcriptional repression. Therefore, TBL1X loss-of-function mutations may result in impaired basal activation of these genes.

Screening for primary and central CH

Screening programmes were first introduced with the aim of eliminating the neurodevelopmental sequelae of late treated CH and in this respect have been a huge public health success with the majority of primary CH cases now diagnosed following neonatal screening. Most countries worldwide operate a TSH-based screening strategy where TSH is measured first, usually on a filter paper blood-spot sample, and subsequent FT4 or T4 measurement is only performed in infants with a raised TSH. A minority of countries (some state programmes in the US and Italy, Japan, the Netherlands and Israel) employ a method in which total or FT4 and TSH are either measured simultaneously or in a stepwise manner with T4 measured first. Additional differences in TSH-based screening programme methodology include the timing of the samples (from cord blood to heel prick samples in the second postnatal week), the biochemical assays used and the determined cut points, all of which may confound direct comparison of screening programmes (114, 115).

Each CH screening programme has its own advantages and disadvantages. TSH-based
programmes are the most sensitive for primary CH, and, by definition, detect both subclinical and overt primary CH. However, CCH will only be detected by programmes measuring T4 or FT4 initially, or simultaneously with TSH. A disadvantage of this approach is the high false positive rate, which data from the Netherlands attributes primarily to subnormal total T4 in cases with thyroxine-binding globulin (TBG) deficiency (36% of false positive cases over a one year period) (8). Accordingly, the Netherlands screening strategy includes measurement of TBG as well as TSH, which enables diagnosis of TBG deficiency. However, TBG is only measured in cases with T4 -1.6SD or less due to financial and workload constraints (7, 8).

Premature birth poses problems for both types of screening programme, especially in infants with very low birth weight. Thyroid dysfunction in preterm infants includes transient hypothyroxinemia of prematurity (low T4 with normal TSH), which may result in many false positive diagnoses on T4-based programmes (accounting for 8% false positives in the Dutch screening programme) (8). In order to overcome this problem, the Japanese simultaneous TSH/FT4 system defines positive results from newborns with birth weight <2kg as preliminary, and repeats the measurements at age one month before attributing a definitive diagnosis (116). In the Netherlands, TSH levels are used to recall premature infants, rather than T4 measurements (8). Delayed maturation of the hypothalamic-pituitary-thyroid axis in premature infants may also result in a delayed TSH rise, in which the TSH is initially normal despite primary hypothyroidism but later becomes elevated (117). Since primary CH with delayed TSH rise may therefore evade detection on TSH-based newborn screening programmes, a strategy of second screening is recommended, when the infant is
around one month old, in order to diagnose CH in cases with false negative screening results at birth. Accordingly, in the UK, infants born at less than 32 weeks undergo a repeat TSH screen aged 4 weeks if the first TSH screen is negative.

In TSH-based screening systems, newborn blood spot TSH screening cutpoints remain a continued subject of discussion, exhibiting widespread disparity even within the UK where they remain region-specific (118). The importance of thyroxine in the myelination of the infant brain is undisputed and more recent functional MRI studies have provided further insight into this (119). However, although the rationale for detecting severe CH is unequivocal, the benefit to neurodevelopmental outcomes when treating infants with mild to moderately raised TSH and borderline free T4 (FT4) concentrations is more ambiguous. A recent Belgian study found no relationship between cognitive and psychomotor outcomes of preschool children and screening TSH concentrations (120). However, conversely, a large epidemiological study in Australia did suggest a relationship between educational attainment and neonatal TSH. There was a decrease in attainment as the TSH increased from the 75\textsuperscript{th} centile of screening TSH (121). Although absolute TSH concentrations were not given, the distribution of TSH concentrations in screened newborn infants would suggest that the 75\textsuperscript{th} centile is likely to be below the screening cutpoints of most, if not all, screening laboratories. Both studies have limitations in terms of the chosen developmental assessment tool, the age of child assessment and study design, but the need for future, long term neurodevelopmental studies is clear.

Primary TSH-based screening strategies do not detect CCH at birth. Therefore, although moderate to severe CCH may be diagnosed clinically, due to signs
suggesting hypothyroidism, or due to manifestations of additional pituitary hormone
deficits, e.g. micropenis (hypogonadotrophic hypogonadism), hypoglycaemia or
prolonged neonatal jaundice (central adrenal insufficiency), or postnatal growth
failure (growth hormone deficiency), diagnosis and treatment are often significantly
delayed (122, 9).

There are strong arguments for including T4 measurement in the CH screening
strategy and the most frequently cited counterarguments (the relative rarity of CCH
and the perception that it is usually mild) are becoming questionable. Combined
T4/TSH/TBG evaluation has yielded an incidence of permanent CCH of 1:16,400 to
1:21,000 in Dutch neonates (8, 9) whereas Japanese studies have reported a lower
incidence (1:31,000 to 1:160,000), likely reflecting a less sensitive screening
approach although differences in ethnicity may also be implicated (116, 123).
Therefore, although less common than primary CH, the incidence of CCH in some
regions is comparable to that of other conditions included in newborn screening
programmes, e.g. phenylketonuria. Moreover, previous perceptions that CCH is
usually mild have been refuted by data from the Netherlands demonstrating that more
than 50% of children with CCH exhibit moderate or severe hypothyroidism (124).
Since it is unequivocal that missed diagnosis of severe CCH, such as in the context of
TSHB mutations, or CCH with additional pituitary hormone deficiencies results in
adverse neurodevelopmental outcomes, the potential for delayed diagnosis of
moderate-severe CCH is concerning (124, 98). However, in some contexts (e.g.
IGSF1 deficiency), newly-diagnosed moderate CCH in elderly IGSF1 mutation
carriers, does not seem to have been associated with obvious developmental sequelae
(111). A further benefit of screening for CCH is its potential to trigger early
investigation and detection of concomitant pituitary-adrenal and growth hormone deficiencies, for which treatment may prevent life-threatening consequences. Since seventy-five percent of CCH cases exhibit combined pituitary hormone deficiencies, the significance of this should not be underestimated (5). However, changing from a primary TSH-based screening strategy, to one which identifies CCH whilst maintaining a high sensitivity for detecting primary CH, is not an insignificant undertaking, especially given the need for early detection of false positive results. Increased complexity of the clinical workload and biochemical methodology as well as cost, are all potential barriers to implementation.

Conclusions

Over the last 40 years, neonatal screening for CH represents a major public health success, achieving near elimination of associated severe neurodevelopmental delay. In the future, further studies are needed to address the benefits of early detection and treatment of mild CH, and to provide an evidence base for determining optimal TSH screening cutpoints. Additionally, countries operating primary TSH-based screening systems should be encouraged to revisit the cost-benefits of an additional screening step for central CH in light of current evidence. Although our understanding of the genetic basis for primary and central CH has improved, the basis for TD in particular remains largely uncharacterized and in genetically-ascertained CH, phenotypic variability is poorly accounted for. Further investigation of the contributions of genetic and epigenetic variation, in addition to environmental modifiers, may help elucidate the mechanisms underlying CH as well as providing novel insights into thyroid development.
Declaration of Interest

The authors have nothing to declare

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**Figure Legends**

1. A. Reported thyroid morphologies in the 84 individuals with missense or small indel mutations in \( PAX8 \) categorized as ‘disease-causing’ by HGMD. Numbers refer to the percentage of cases in each morphological category B. Venn diagram summarizing neurological, pulmonary and thyroid phenotypes in 180 individuals harbouring missense or small indel mutations in \( NKX2-1 \) categorized as ‘disease-causing’ by HGMD. Individuals were only included if information was available regarding all three phenotypes (including the absence of phenotype). Numbers refer to
percentages of the cohort.

2. Schematic illustrating thyroid hormone biosynthesis in the thyroid follicular cell, highlighting the position and function of the molecules involved. Mutations in any of these proteins may result in dyshormonogenesis.

3. Diagrammatic representation of the hypothalamic-pituitary-thyroid axis with positive regulation (black) predominantly mediated by thyrotropin-releasing hormone (TRH) and negative (grey) feedback influences, predominantly mediated by thyroid hormone receptor (TR) isoforms $\beta_2$ and $\beta_1$. The inset represents a pituitary thyrotroph, in which the putative sites of action of genes implicated in isolated TSH deficiency are shown. Consequences of mutations in these genes are depicted in the same colour text as the mutant protein.

4 A. Model for heterodimeric thyroid stimulating hormone (TSH) bound to the TSH receptor (TSHR) illustrating the position of reported $TSHB$ mutations associated with CCH. The model was generated using PHYRE for predicting TSHbeta subunit (TSH$\beta$) structure and was modelled onto FSH-FSHR (1xwd) and the TSHR-K1-70FAB (2xwt) structure using PYMOL. Green: TSHR, Red: TSH alpha subunit (TSH$\alpha$, $\alpha$GSU), Blue: TSH$\beta$. Cyan ‘seatbelt’ region, Yellow: conserved cysteines involved in disulphide bridge formation. Spheres: reported TSHB mutations: C105R; C108Y; C125Vfs*10 (yellow) disrupt disulphide bridges, G49R (purple) is located in the conserved CAGYC region; Q69*; F77Sfs*6 (orange) truncate the protein prematurely and E32* and E32K (light blue) are truncating and missense mutations at the same position. The nomenclature of these mutations follows the most recent
HGNC guidelines to include the 20 amino acid signal peptide of TSHB, thus may differ from that cited in the original articles. Nomenclature can be converted to that previously published for missense mutations by subtracting 20 e.g. Q69* new nomenclature = Q49* old nomenclature. B. TRHR structural model generated by homology modeling using the PHYRE server and Pymol showing the positions of the four previously described mutations associated with central hypothyroidism. The truncating mutation (p.R17* truncating the protein in the extracellular domain) and the in-frame deletion of 3 amino acids (Ser115-Thr117) are shown in red; missense changes (p.A118T, p.P81R, p.I131T) are shown in blue.

5. Comparison of endocrinology in individuals with biallelic TSHB or TRHR mutations (TSHB, TRHR), and hetero- or hemizygous IGSF1 or TBL1X mutations (IGSF1, TBL1X), including all reported mutation carriers for whom numerical biochemical data was available. A. Total or FT4 measurements expressed as percentage lower limit of the normal range. B. Peak TSH response to a standard TRH test excluding 1 IGSF1 deficient case for whom results were discrepant at two different ages (125). IGSF1 data excludes neonates <1 month old. Reference ranges are demarcated according to (87). TRHR mutations abolish (black) or partially impair (red) TRHR function. C. Schematic illustrating the protein domain structure of IGSF1 with the internal signal peptide directing cleavage of the carboxy-terminal domain denoted by a blue line. Positions of naturally-occurring mutations associated with congenital central hypothyroidism are denoted; all are located within the carboxyterminal domain. Mutations in black (missense) and red (truncating mutations) are known or likely to affect membrane trafficking or expression, whereas mutations in grey are associated with a characteristic phenotype but do not affect
IGSF1 trafficking in vitro. Four whole gene deletions have also been reported.
Table 1
A summary of the genetic defects implicated in congenital hypothyroidism with thyroid dysgenesis and typical clinical features; M: monoallelic, B: Bilallelic. GIS: thyroid gland in situ, BHC: benign hereditary chorea, IRDS: infant respiratory distress syndrome. * n=1 case ** n=1 case, additional variants: 47, XYY karyotype; and atypical 22q11 deletion. Italics denote less common features.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Family</th>
<th>M/B</th>
<th>Additional clinical features</th>
<th>CH</th>
<th>Radiological features</th>
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<tbody>
<tr>
<td>NKK2-I</td>
<td>Homeodomain-containing transcription factor</td>
<td>M, often de novo</td>
<td>Neurological (BHC, developmental delay, hypotonia), Respiratory (IRDS, recurrent infections)</td>
<td>Subclinical-overt, may be euthyroid GIS, athyreosis and ectopy also reported</td>
<td></td>
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<tr>
<td>PAX8</td>
<td>Paired homeodomain transcription factor</td>
<td>M</td>
<td>Urogenital tract malformations (rare)</td>
<td>Moderate-severe, subclinical euthyroidism or later athyreosis and ectopy onset hypothyroidism also reported</td>
<td></td>
</tr>
<tr>
<td>FOXE1</td>
<td>Forkhead/winged-helix transcription factor</td>
<td>B</td>
<td>Cleft palate, spiky hair (universal), choanal atresia Severe</td>
<td>Athyreosis</td>
<td></td>
</tr>
<tr>
<td>GLIS3</td>
<td>GLI-similar zinc finger protein</td>
<td>B</td>
<td>Neonatal diabetes, dysmorphic facies, renal cystic dysplasia, hepatic fibrosis, congenital glaucoma, learning difficulties and skeletal abnormalities</td>
<td>Severe, TSH resistance GIS, athyreosis</td>
<td></td>
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</tbody>
</table>

** Gene:**
- **NKX2-I:** Homeodomain-containing transcription factor
- **PAX8:** Paired homeodomain transcription factor
- **FOXE1:** Forkhead/winged-helix transcription factor
- **GLIS3:** GLI-similar zinc finger protein

** Family:**
- **NKX2-I**
- **PAX8**
- **FOXE1**
- **GLIS3**

** M/B:**
- **M:** monoallelic
- **B:** bilallelic

** Additional clinical features:**
- Neurological (BHC, developmental delay, hypotonia)
- Respiratory (IRDS, recurrent infections)
- Urogenital tract malformations (rare)
- Cleft palate, spiky hair (universal), choanal atresia

** CH:**
- Subclinical-overt, may be euthyroid
- Moderate-severe
- Severe

** Radiological features:**
- GIS, athyreosis and ectopy also reported
- Hypoplasia/GIS, euthyroidism or later athyreosis and ectopy onset hypothyroidism also reported
- Athyreosis
<table>
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<th>Disease</th>
<th>Spectrum</th>
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<tr>
<td>NKX2</td>
<td>Homeodomain-containing transcription factor</td>
<td>Congenital heart disease</td>
<td>Euthyroid to severe CH, GIS, ectopy, athyreosis</td>
</tr>
<tr>
<td>NKX2-5</td>
<td>Major component of the Chromosomal Passenger Complex</td>
<td>Congenital heart disease*</td>
<td>Euthyroid to severe CH, Athyreosis, ectopy, hemiagenesis, nodules</td>
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<tr>
<td>CDCA8</td>
<td>Chromosomal Passenger Complex</td>
<td>Alagille syndrome/congenital heart disease/isolated CH</td>
<td>Spectrum from subclinical hypothyroidism to GIS/hypoplasia/ectopy</td>
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<tr>
<td>JAG1</td>
<td>Notch ligand</td>
<td>Spectrum from compensated hyperthyrotropinaemia to severe CH, TSH Resistance</td>
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<tr>
<td>NTN1**</td>
<td>Laminin superfamily member</td>
<td>Congenital heart disease</td>
<td>Severe CH, Ectopy</td>
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<td>TSHR</td>
<td>G-protein coupled receptor</td>
<td>Nil</td>
<td>Spectrum from GIS to severe hypoplasia</td>
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Table 2 A summary of the genetic defects implicated in CH with dyshormonogenesis and typical clinical features; M: monoallelic, B: Bilallelic. GIS: thyroid gland in situ, EVA – enlarged vestibular aqueduct. PIOD: partial iodide organification defect, TIOD: total iodide organification defect, * occasional monoallelic cases reported.

<table>
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<th>Function</th>
<th>M/B</th>
<th>Additional clinical features</th>
<th>Radiological features</th>
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<tr>
<td>TG</td>
<td>Glycoprotein upon which thyroid hormones are synthesized and stored</td>
<td>B</td>
<td>Fetal goiter (rare)</td>
<td>GIS/Goitre</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Inappropriately low TG when TSH is elevated.</td>
<td>Spectrum from euthyroidism to severe CH</td>
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<tr>
<td>TPO</td>
<td>Organification of iodide, catalysis of coupling reactions (final step in TH synthesis)</td>
<td>B*</td>
<td>Fetal goitre (rare)</td>
<td>Severe CH</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Sometimes mild CH with monoallelic variants</td>
</tr>
<tr>
<td>DUOX2</td>
<td>H$_2$O$_2$ production, required for iodide organization</td>
<td>M/B</td>
<td>Borderline blood spot</td>
<td>TSH but subnormal venous T4 and significantly elevated TSH at confirmatory testing</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Usually mild-moderate or transient CH</td>
<td>GIS/Goitre</td>
</tr>
<tr>
<td>DUOXA2</td>
<td>Membrane expression and function of DUOX2</td>
<td>M/B</td>
<td>Transient or permanent</td>
<td>Mild/transient CH</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>GIS/Goitre</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>PIOD</td>
</tr>
<tr>
<td>Pendrin</td>
<td>Apical iodide efflux</td>
<td>M</td>
<td>Sensorineural hearing loss with EVA</td>
<td>Euthyroid/mild hypothyroidism</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>GSI/Goitre</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>PIOD</td>
</tr>
<tr>
<td>NIS</td>
<td>Basolateral iodide uptake</td>
<td>M</td>
<td>May present later in childhood resulting in neurodevelopmental delay</td>
<td>Spectrum from euthyroidism to severe CH</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>GSI/Goitre</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Severely impaired thyroid 123I/Tc uptake</td>
</tr>
<tr>
<td>Recycling of unused iodide moieties (MIT and DIT)</td>
<td>Raised urinary MIT and DIT</td>
<td>Spectrum from euthyroidism to Goitre</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-------------------------------------------------</td>
<td>-----------------------------</td>
<td>------------------------------------</td>
<td></td>
<td></td>
</tr>
<tr>
<td>M/B</td>
<td>May present later in severe CH; later Normal onset</td>
<td>Organification of hypothyroidism iodide reported</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Childhood resulting in neurodevelopmental delay</td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>
Table 3
Endocrine, Neuroradiological and extrapituitary manifestations of mutations in genes implicated in CCH in humans. E, Enlarged; N, Normal; TSH thyroid-stimulating hormone, PRL; Prolactin, GH; Growth hormone. AR, Autosomal recessive; XL, X-linked, *Females may also be affected.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Inheritance</th>
<th>Hormone Deficits</th>
<th>TRH Test Responses</th>
<th>MRI</th>
<th>Additional Features</th>
</tr>
</thead>
<tbody>
<tr>
<td>TSHB</td>
<td>AR</td>
<td>TSH</td>
<td>Absent TSH response, preserved PRL response</td>
<td>E, N</td>
<td>-</td>
</tr>
<tr>
<td>TRHR</td>
<td>AR</td>
<td>TSH</td>
<td>TSH &amp; PRL peak absent/preserved</td>
<td>N</td>
<td>-</td>
</tr>
<tr>
<td>TBL1X</td>
<td>XL*</td>
<td>TSH</td>
<td>TSH response normal</td>
<td>N</td>
<td>Sensorineural hearing loss</td>
</tr>
</tbody>
</table>

Isolated TSH Deficiency or combined pituitary hormone deficiency

<table>
<thead>
<tr>
<th>Gene</th>
<th>Inheritance</th>
<th>TRH Test Responses</th>
<th>MRI</th>
<th>Additional Features</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGSF1</td>
<td>XL*</td>
<td>TSH + PRL, GH (transient), Low normal/Normal TSH, Delayed pubertal response</td>
<td>N</td>
<td>Macroorchidism (males), Ovarian cysts (females)</td>
</tr>
</tbody>
</table>
Figure 1

A  Thyroid Morphology associated with PAX8 Mutations

Hypoplasia 58%
GIS 32%
Agenesis 6%
Ectopy 3%
Hemiagenesis 1%

B  Organ Involvement in patients with NKX2-1 Mutations

Brain 90%
Lung 58%

Thyroid 70%

17 24 43
1 6 2
7
Figure 2

Basolateral $I^-$ Uptake

Thyroid Follicular Cell

Circulation

lysosome

Catalyzes organification of iodide & coupling of MIT & DIT to form T3/ T4

$T3 \rightarrow T4$

I$^-$ Recycling

$MIT, DIT \rightarrow Tyrosine$

$I^-$ Organification

$T3 \rightarrow T4$

Recycles unused iodide

Glycoprotein upon which TH synthesis & storage occurs
Figure 3

HYPOTHALAMUS

Paraventricular Nucleus

ANTERIOR PITUITARY

Thyrotroph

THYROID

TSHR

T4 T3

TSH

Central CH

TSHB

TRHR

IGSF1

TBL1X

TRH

TRH expression

basal transcription

TSHB, CGA

TSHα

TSHβ

Conjugation Glycosylation Secretion

TRE

TBL1X Corepressor

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Figure 4

A

- E32K
- E32*
- C125VfsX10
- F77Sfs*6
- G49R

B

- R17
- P81R
- S115, I116, T117
- A118T
- I131T
Figure 5

A. Baseline T4 Levels

B. TRH Tests

C. Diagram showing mutations in the protein sequence.