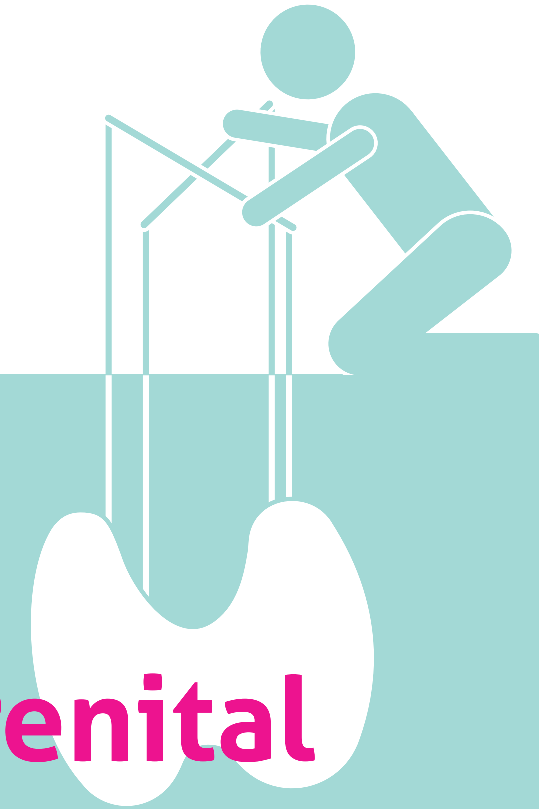
Congenital central hypothyroidism

Zwaveling-Soonawala, N.

CONGENITAL CENTRAL HYPOTHYROIDISM

NITASH ZWAVELING-SOONAWALA



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Congenital Central Hypothyroidism

diagnostics and pathogenesis

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1

General Introduction

GENERAL INTRODUCTION

The hypothalamus-pituitary-thyroid (HPT) axis is responsible for maintaining optimal circulating levels of thyroid hormone. The central regulators of the HPT axis are hypophysiotropic neurons in the hypothalamic paraventricular nucleus (1). These neurons produce the neuropeptide thyrotropin-releasing-hormone (TRH). TRH is secreted into the portal venous system vessels and carried through the pituitary stalk to the anterior pituitary lobe where it stimulates the thyrotrophic cells to produce and secrete thyrotropin (TSH). In turn, TSH stimulates the thyroid gland to produce the thyroid hormones, thyroxine (T4) and triiodothyronine (T3) (1,2).

The HPT axis is a classic example of an endocrine feedback loop (2). Hypothalamic TRH and pituitary TSH secretion are inhibited by T4 and T3. In this way, the HPT axis controls circulating thyroid hormone levels within a narrow range.

In clinical practice the blood TSH concentration is often used as initial measurement to diagnose or rule out thyroid disease. In case of hypothyroidism of thyroidal origin (primary hypothyroidism), decreased T4 and T3 levels lead to increased hypothalamic TRH and pituitary TSH secretion. The increased TSH levels are used to diagnose primary hypothyroidism. Alternatively, in case of hyperthyroidism increased T4 and T3 levels negatively regulate TRH and TSH secretion and the decreased TSH levels are used to diagnose hyperthyroidism.

The measurement of TSH is very useful in identifying hypothyroidism and hyperthyroidism of thyroidal origin but does not aid in diagnosing hypothyroidism of central origin (3). In central hypothyroidism, the problem lies at the level of the hypothalamus and/or pituitary. T4 and T3 levels are decreased while TSH is inappropriately low or normal. Therefore, TSH concentrations have little value in the diagnosis and the key to diagnosing central hypothyroidism is recognizing when thyroid hormone (T4, T3) levels are too low (3,4). In acquired forms of central hypothyroidism the medical history may be helpful (e.g. pituitary surgery, brain irradiation or trauma). The absence of such a medical history makes diagnosing congenital central hypothyroidism especially challenging.

Importance of diagnosing congenital central hypothyroidism

Thyroid hormone is essential for normal brain development starting in the early embryonic period and continuing throughout the first years of life (5-8). While in adult onset hypothyroidism clinical manifestations are usually reversed with appropriate treatment, untreated congenital hypothyroidism (CH) has devastating neurological consequences leading to permanent intellectual and motor disabilities. Children with untreated severe congenital hypothyroidism suffer from mental retardation, short stature, lethargy and obesity. In 1897 Sir William Osler provided the following dramatic description of patients with untreated congenital hypothyroidism (9,10).

"No type of human transformation is more distressing to look at than an aggravated case of cretinism. The stunted stature, the semi-bestial aspect, the blubber lips, retroussé nose sunken at the root, the wide-open mouth, the lolling tongue, the small eyes half-closed with swollen lids, the stolid, expressionless face, the squat figure, the muddy dry skin, combine to make the picture of what has been termed the "pariah of nature". Not the magic wand of Prospero or the brave kiss of the daughter of Hippocrates ever affected such a change as that which we are now enabled to make in these unfortunate victims, doomed heretofore to live in hopeless imbecility, an unspeakable affliction to their parents and their relatives."
[Sir William Osler, 1897]

In the 1890's the first treatment trials in children with CH, using thyroid extract from animals, led to improvement of growth and many other features of untreated CH. These initial treatment protocols, however, were not successful in preventing mental retardation. In 1972 Klein and co-workers demonstrated that the key to preventing brain damage is starting treatment within the first three months of life (11). Since only one third of patients were clinically recognized within the first three months of life, newborn screening programs for congenital hypothyroidism were introduced in the late 1970's. These newborn screening programs have proven to be very effective in preventing brain damage by early detection and treatment. In countries using neonatal screening programs children with CH have near normal development (12,13).

Congenital hypothyroidism may be of thyroidal origin or of central (hypothalamic-pituitary) origin. Congenital thyroidal hypothyroidism (CH-T) is most common with an estimated prevalence of one in 1,500 – 3,000 newborns and accounts for more than 90% of all CH cases. Most of these children (85%) have an abnormal thyroid gland development (thyroid dysgenesis) and a minority (15%) has a defective thyroid hormone synthesis by a structurally normal gland (thyroid dyshormonogenesis) (14).

Congenital central congenital hypothyroidism (CH-C) is much less common. In CH-C the deficient thyroid hormone production is due to insufficient TSH stimulation of an otherwise normal thyroid gland (15). If the defect is limited to pituitary thyrotrophic cells, CH-C may be isolated (isolated CH-C). In most cases CH-C is associated with other pituitary hormone deficiencies. In multiple pituitary hormone deficiency (MPHD), growth hormone deficiency and adrenocorticotrophic hormone (ACTH) deficiency are potentially brain damaging and life threatening, due to hypoglycemia and circulatory insufficiency.

Since the introduction of neonatal screening programs for CH the optimal laboratory strategy has been subject of debate. Worldwide, most national neonatal screening programs for congenital hypothyroidism are TSH-based and effectively detect neonates with CH-T (16). However, neonates with CH-C are missed using this strategy. The Dutch neonatal CH screening program consists of a three-step approach with a primary T4 measurement followed by additional TSH measurement in the lowest 20% T4 concentrations, and an additional measurement of T4 binding globulin (TBG) in the lowest 5%. This screening strategy has proven

to be effective in detecting both CH-T and CH-C (17,18). The incidence of permanent CH-C in the Netherlands is one of the highest in the world, around 1 in 16,000 neonates (19). The majority of these children have MPHD.

A frequently used argument against screening for CH-C is its presumed mild severity that is thought not likely to be associated with brain damage (20-22). Severity of CH is classified as follows, based on initial free T4 (FT4) values; severe CH: FT4 < 5 pmol/l, moderate CH: FT4 5 - <10 pmol/l, mild CH: FT4 ≥ 10 pmol/l (23). To investigate the severity of the hypothyroidism in CH-C we analyzed FT4 concentrations in a large cohort of children with CH-C detected in the Dutch neonatal screening. The results of this study are presented in **Chapter 2** (24).

The severity of CH-C not only lies in the degree of hypothyroidism but also in the additional pituitary hormone deficiencies that may be present. Most patients (around 70%) have multiple pituitary hormone deficiencies including growth hormone deficiency and ACTH deficiency which may be life threatening (18). Data on mortality in CH-C patients, however, is lacking. We investigated the mortality rate and causes of death in our cohort of Dutch patients with early detected CH-C by neonatal screening. The results are presented in **Chapter 3**.

Difficulties in diagnosing congenital central hypothyroidism

TSH levels in CH-C

The defective TSH secretion in central hypothyroidism may be due to *quantitative* TSH deficiency (reduced pituitary TSH secretion), *qualitative* TSH deficiency (reduced TSH bioactivity), or a combination of both.

TSH is a dimeric glycoprotein hormone composed of two subunits, a hormone specific β-subunit and a α-subunit. The α-subunit is shared with follicle stimulating hormone, luteinizing hormone and chorionic gonadotropin. TSH production is regulated by hypothalamic thyrotropin releasing hormone (TRH). In addition to stimulating transcription of the α- and β-subunit genes, TRH mediates glycosylation and conjugation of TSH α and -β subunits, and TSH secretion. Normal TRH stimulation is necessary for normal quantity and quality of TSH. The absence of normal TRH stimulation not only leads to impaired secretion but also to impaired pituitary TSH glycosylation resulting in decreased bioactivity of circulating TSH. As TSH concentrations are measured by immunoassays, which do not take into account bioactivity, TSH levels in central hypothyroidism may be low, normal or even slightly elevated due to the presence of TSH with reduced bioactivity (15,25,26). The combination of low FT4 levels and slightly elevated TSH makes distinguishing central hypothyroidism from mild primary hypothyroidism difficult.

Since there are no reports on the range of TSH levels in patients with CH-C we analyzed TSH levels in a large group of children with CH-C. In addition, we studied whether FT4 levels are helpful in distinguishing patients with CH-C from patients with mild primary CH with similar TSH levels. The results of this study are presented in **Chapter 4**.

When are FT4 levels too low?

Diagnosing central hypothyroidism relies on determining whether FT4 levels are too low. In general, FT4 levels below age specific reference intervals are considered too low, indicating hypothyroidism. However, this does not take into account the individual's T4 set point. Thyroid function parameters such as the TSH, FT4 and FT3 show large inter-individual differences leading to wide laboratory reference intervals while the intra-individual variability is much smaller, suggesting that each individual has his or her own specific HPT axis set point.

Anderson and co-workers found that individual reference ranges for serum T3 and T4 are about half the width of population-based reference ranges (27). Benhadi and co-workers found similar results with a mean intra-individual variation coefficient of 9.9% compared to 16.5% inter-individual variation (28). This means that a test result within the reference interval is not necessarily normal for the individual. Ideally, when diagnosing central hypothyroidism, the individual set point needs to be taken into account. Both genetic and environmental factors seem to contribute to this individual set point. In a large U.K. study in 2,124 female adult twins, environmental factors determined 61% of variation in FT4 and 77% in FT3 (29). This suggests that FT4 and FT3 concentrations are mainly influenced by environmental rather than genetic factors.

The term *set point* describes that an individual trait remains stable over time, which seems to apply to the TSH, FT4 and FT3 concentrations. If indeed these set points are mainly determined by environmental factors, the question arises which environmental factors would have such a major influence that they may be held responsible for determining an individual's set point. We hypothesized that the answer may lie in the fetal environment, which has a major effect on postnatal health (30,31).

To test the hypothesis that the fetal environment to a large extent determines the postnatal thyroid hormone concentration and the T4 set point, we conducted a classical twin-study, comparing the resemblance of neonatal screening blood T4 concentrations in a large sample of mono- and dizygotic twin pairs. The results of this study are presented in **Chapter 5** (32).

In Down syndrome, a high prevalence of mild plasma TSH elevation has been well described, also referred to as subclinical hypothyroidism (33-35). This may suggest an altered HPT axis set point in Down syndrome but may also be indicative of a mild form of Down syndrome-specific congenital hypothyroidism of thyroidal origin. Not only are TSH values slightly elevated, but neonatal T4 concentrations are also lower in Down syndrome compared with non-Down syndrome neonates (35).

Based on the assumption that young children with Down syndrome as a group have a mild form of congenital thyroidal hypothyroidism, it was hypothesized that thyroxine treatment during the first two years of life could improve psychomotor development in children with Down syndrome. Between 1999 and 2003, a randomized clinical trial (RCT) was conducted to study the effects of thyroxine treatment versus placebo on psychomotor development in a cohort of young children with Down syndrome (36). In this RCT thyroxine treatment resulted in somewhat better

motor development and growth compared with placebo treatment at the age of two years. In a follow-up study of this trial we had the opportunity to study the effect of thyroxine vs. placebo treatment during the first two years of life on HPT axis function parameters later in life (37).

Given that both genetic and environmental factors are important in HPT axis set point determination and given that, after the fetal period, the HPT axis continues to mature postnatally, we hypothesized that thyroxine treatment initiated in the neonatal period, resulting in a clearly higher plasma FT4 concentration, may cause an HPT axis set point change that persists later on in life. The results of this study are presented in **Chapter 6** (38).

Genotyping pituitary stalk interruption syndrome

New generation genetic sequencing techniques provide the opportunity to unravel the genetic background of congenital diseases. Given the difficulties in diagnosing central hypothyroidism based on thyroid hormone parameters, genetic confirmation would be of great value. Unfortunately, genetic causes are only found in a minority of CH-C cases (4,39).

Isolated congenital central hypothyroidism may be caused by mutations in the thyrotropin-releasing hormone receptor gene (*TRHR*), thyroid stimulating hormone β-subunit gene (*TSHB*) and the more recently described genes *IGSF1* and *TBL1X* (40-45). *TRHR* and *TSHB* gene mutations are found only rarely. Since the first cases of *IGSF1* gene mutations were reported in 2012, many new cases have been described making *IGSF1* mutations a frequent cause of isolated CH-C (46). *TBL1X* has been described only very recently and its prevalence still needs to be investigated in large cohorts (45).

The majority of CH-C cases is not isolated but occurs within the framework of MPHD. Most patients with MPHD exhibit a pituitary malformation characterized by an absent or thin pituitary stalk, a hypoplastic anterior pituitary lobe and an ectopic posterior pituitary lobe. This malformation is known as pituitary stalk interruption syndrome (PSIS) (47). PSIS often occurs in isolation but may be accompanied by additional midline brain abnormalities (syndromic PSIS).

Various transcription factors are involved in the initial formation of the pituitary gland and the following cellular differentiation. However, mutations in genes encoding these transcription factors are found in less than 5% of PSIS cases, and mostly in syndromic forms (48).

As Mendelian forms of isolated PSIS are detected only rarely, a polygenic and multifactorial etiology should be considered. To provide further evidence for a non-Mendelian, polygenic etiology of isolated PSIS, we performed “whole” exome sequencing (WES) in 20 patients with isolated PSIS and their unaffected parents. In addition to searching for (potentially) pathogenic de novo and biallelic variants, we performed a targeted search in a large panel of genes associated with midline brain development. We included genes with a known association with pituitary formation and also genes associated with other midline brain malformations: holoprosencephaly, hypogonadotropic hypogonadism and absent corpus callosum. The results of this study are presented in **Chapter 7** (49).

KAT6A neurodevelopmental disorder

In the abovementioned exome sequencing study, we identified a child with a pathogenic *KAT6A* gene mutation. This patient had developmental delay and severely delayed speech which had been attributed to hypoglycemic brain damage due to untreated ACTH deficiency and growth hormone deficiency in the first two years of life. In retrospect, the patient fulfilled all the criteria for KAT6A neurodevelopmental disorder and should not have been included in the exome sequencing study on isolated PSIS (50-52). *KAT6A* mutations may, however, be considered a new cause of syndromic PSIS. The case is reported and discussed in **Chapter 8** (53).

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The importance of diagnosing congenital central hypothyroidism

The Severity of Congenital Hypothyroidism of Central Origin Should Not Be Underestimated

2

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ABSTRACT

Context

Congenital hypothyroidism (CH) may be of thyroidal (CH-T) or central origin (CH-C). Worldwide, most neonatal screening programs are TSH-based and effectively detect CH-T. Only a few screening programs measure total or free T4 and TSH simultaneously or stepwise, enabling detection of CH-T as well as CH-C. A frequently used argument against screening for CH-C is its presumed mild hypothyroid character. In the recently published European Society for Pediatric Endocrinology (ESPE) CH consensus guidelines on screening, diagnosis, and management, severity of CH is classified based on initial free T4 (FT4) concentrations.

Objective

Our objective was to assess disease severity of CH-C compared with CH-T in a Dutch cohort of CH patients.

Methods

Pretreatment FT4 concentrations were analyzed in all children with CH detected by the Dutch neonatal T4+TSH+T4-binding-globulin (TBG) screening between 1995 and 2011. Disease severity was classified using the FT4-based ESPE classification.

Results

Between 1995 and 2011, 1288 children were diagnosed with CH. Data of 1200 (143 CH-C and 1057 CH-T) were available for analysis. Based on FT4 concentrations, 4 children with CH-C (2.8%) had severe, 75 (52.4%) moderate, and 64 (44.8%) mild CH. In the CH-T group, 280 children (26.5%) had severe, 341 (32.3%) moderate, and 436 (41.2%) mild CH.

Conclusion

Our results indicate that, based on initial FT4 values, severe CH was much more prevalent in CH-T compared with CH-C. However, CH-C itself should not be considered as only mild because more than half of CH-C patients have moderate CH with initial FT4 below 10 pmol/L (0.78 ng/dl).

INTRODUCTION

Congenital hypothyroidism (CH) is among the most common preventable causes of mental retardation with a reported incidence of 1 in 2500 to 3500 live births (1, 2). Neonatal screening programs allow for early detection and treatment of CH and have proven to be successful in preventing brain damage (1, 2).

In the January 2014 issue of this journal, the newest European Society for Pediatric Endocrinology (ESPE) consensus guidelines on screening, diagnosis and management of CH were published (3). Although CH may be of thyroidal (CH-T) or central origin (CH-C, also known as hypothalamic-pituitary CH), these ESPE guidelines focus on management of CH-T. This is not surprising because CH-T is much more common than CH-C (reported prevalence of CH-C in The Netherlands is 1 in 16 000) (4, 5).

Worldwide, most neonatal screening programs are TSH-based and effectively detect only CH-T. Only a few screening programs in some countries employ a method in which total T4 or free T4 (FT4) and TSH are measured simultaneously or stepwise (T4+TSH method) enabling detection of CH-T as well as CH-C (2, 4–8).

Whether neonatal screening programs should also focus on detecting CH-C besides CH-T remains a subject of debate (6). Regarding this issue, the recent ESPE consensus guidelines state that there is some published evidence to suggest neonatal screening for CH-C may fulfil criteria for disease screening, namely: 1) CH-C is a relatively frequent disease with an incidence similar to that of phenylketonuria in some populations, 2) screening tests are available and inexpensive, 3) treatment is available and effective, and 4) the risks of an unfavorable outcome in cases of delayed diagnosis are well known. However, it is also acknowledged that outcome studies showing that “screening is superior to detection through clinical presentation” are lacking (3).

A frequently used argument against screening for CH-C is its presumed only mild hypothyroid character that is thought not likely to be associated with an increased risk of hypothyroidism-induced brain damage (1).

In the ESPE consensus guidelines, the following classification of severity of CH is proposed based on initial FT4 values: severe CH, FT4 <5 pmol/L (<0.39 ng/dl); moderate CH, FT4 5 to <10 pmol/L (0.39–<0.78 ng/dl); and mild CH, FT4 ≥10 (≥0.78 ng/dL) (3). Using this classification, we assessed disease severity of CH-C compared with CH-T in a cohort of Dutch CH patients detected by neonatal screening.

MATERIALS AND METHODS

In 1995, the existing Dutch T4+TSH method (primary total T4 determination with TSH determination in the lowest 20% of T4 concentrations) was extended with measurement of T4-binding globulin (TBG) in the lowest 5% of T4 concentrations (4). This enabled calculation of the T4 to

TBG ratio and, thus, estimation of FT4 concentration. This 3-step screening approach proved to be effective in detecting CH-C (4, 5). Children with an abnormal screening result are referred to a pediatric endocrinologist or pediatrician for further evaluation, including FT4 measurement in a venous blood sample. Because many prematurely born children (gestational age ≤36 weeks in combination with a birth weight ≤2500 g) suffer from hypothyroxinemia of prematurity, T4 measurement in this group of children is always combined with TSH measurement, and referral is based solely on the TSH concentration. In this way, the number of false-positive results and the false classification of normal premature infants as CH-C is prevented.

Since the start of the Dutch neonatal screening program in 1981, the Netherlands Organization for Applied Scientific Research (TNO) Leiden registers all children with abnormal CH screening results and sends out questionnaires to pediatricians to collect data on laboratory results and diagnosis. A first questionnaire is sent out in the first months after a child is referred and includes questions on whether the diagnosis is CH-T or CH-C and whether the diagnosis is thought to be transient or permanent. A second questionnaire is sent out at the age of 4 years to reassess the diagnosis. Using the TNO database, we analyzed initial FT4 values of all children diagnosed with permanent CH between 1995 and 2011. Diagnosis was based on questionnaire 2 when available; otherwise, the diagnosis was based on questionnaire 1. Patients registered as transient CH were excluded from the analysis. The use of the TNO database was in accordance with the Privacy Regulations of the Privacy Committee of the Dutch CH Screening Board. Because children are referred to various hospitals throughout The Netherlands, FT4 levels are measured in various laboratories. Between 1995 and 2011, FT4 was measured by standard immunoassay in all cases. Although supplied by different manufacturers, in the vast majority of these assays, the adult reference range was 10 to 22 pmol/L (0.78–1.71 ng/dL). Based on this reference range and in line with the ESPE guidelines, we classified CH as severe (FT4 <5 pmol/L [<0.39 ng/dL]), moderate (FT4 5–<10 pmol/L [0.39–<0.78 ng/dL]), or mild (FT4 ≥10 [≥0.78 ng/dL]) (3).

RESULTS

Between 1995 and 2011, 1288 children were diagnosed with CH. Eighty-eight children were excluded because of unknown CH etiology (n = 19) or missing FT4 concentrations (n = 69). In these cases, TNO failed to receive these data from the treating pediatricians. Of the remaining 1200 children, 143 had CH-C and 1057 had CH-T.

The CH-C group included 12 children (8.8%) born prematurely (<37 weeks, but >2500 g). The CH-T group included 106 (10.4%) prematurely born children. TSH concentrations at neonatal screening were <18 mIU/L (serum) in the CH-C group, except for 1 patient with a value of 25 mIU/L. In the CH-T group screening TSH was <18 mIU/L in 21% and ≥18 mIU/ in 79% of cases.

FT4 was measured at an average age of 14 days (interquartile range, 8 days) for CH-C and 9 days (interquartile range, 5 days) for CH-T. Based on FT4 concentrations, 4 children (2.8%) in the CH-C group were classified as severe CH, 75 (52.4%) as moderate CH, and 64 (44.8%) as mild CH. In the CH-T group, 280 children (26.5%) classified as severe CH, 341 (32.3%) as moderate CH, and 436 (41.2%) as mild CH (Table).

For 692 children (89 CH-C and 603 CH-T), results from questionnaire number 2 were available. Analysis of the FT4 concentrations in this subgroup of children with confirmed permanent CH at age 4 years was similar to the analysis in the total group (CH-C: severe, 3.4%; moderate, 56.2%; mild, 40.4%; CH-T: severe, 30.5%; moderate, 36.8%; mild, 32.7%).

Table 1. First pre-treatment plasma free T4 (FT4) concentrations at diagnostic work up of children with congenital hypothyroidism of central origin (CHC) and thyroidal origin (CHT) detected by the Dutch neonatal screening program between 1995 and 2011.

Initial FT4, pmol/l[a]	CHC (143 patients)	CHT (1057 patients)
< 5	4 (2.8%)	280 (26.5%)
5 - < 10	75 (52.4%)	341 (32.3%)
≥ 10	64 (44.8%)	436 (41.2%)

[a]To convert pmol/l to ng/dL divide by 12.87

DISCUSSION

Our main finding is that, based on initial FT4 values, most patients with CH-C (55.2%) and CH-T (58.8%) were classified as severe or moderate CH. The prevalence of mild CH was similar for both groups (44.8% for CH-C, 41.2% for CH-T).

CH-C is generally considered to be milder than CH-T. To our knowledge, this is the first report on initial FT4 values in a large cohort of CH-C patients. Indeed, severe CH was much more prevalent in CH-T (26.5%) compared with CH-C (2.8%), supporting the presumption that CH-C is milder than CH-T. However, CH-C itself should not be considered as only mild because more than half of CH-C patients have moderate CH with initial FT4 values below 10 pmol/L.

The clinical relevance of moderate CH is not questioned, and the ESPE consensus guidelines for CH advise that treatment with T4 should be started immediately when FT4 values are below norms for age to prevent brain damage due to thyroid hormone deficiency (3). Unfortunately, studies of neurocognitive development in patients with early treated CH-C are lacking.

van Tijn et al (5) reported on a 2-year cohort of children with CH-C detected by the Dutch neonatal screening program. Of 144 children with CH diagnosed by the neonatal screening program, 13.3% had CH-C. This is similar to the 12% CH-C in this 12-year cohort. van Tijn et al (5) reported that 78% of CH-C patients had additional pituitary hormone deficiencies. In a recent report on 34 children with late diagnosed CH-C, developmental delay was seen in 19 (56%) of these 34 children (9). Almost all children (98%) had multiple pituitary hormone deficiencies, such as ACTH and GH deficiency. Given the fact that ACTH and GH deficiency can cause hypoglycemic brain damage, it is difficult to delineate the exact cause and the precise role of thyroid hormone deficiency in this developmental delay. However, given our finding that most neonates with CH-C present with moderate hypothyroidism (FT4 <10 pmol/L) it is not unlikely that part of the delay might have been prevented by early detection and treatment with T4. Early detection of CH-C also allows for earlier diagnosis and treatment of ACTH and GH deficiency and, with that, reduction of the number of hypoglycemic episodes. This might further improve neurocognitive outcome and even reduce mortality.

A limitation of our study is that our results are based on FT4 values measured in many different laboratories and collected by surveys sent out to pediatricians only. The fact that FT4 was not always measured by the same immunoassay at exactly the same age may make our results a little less precise. Second, it should be taken into account that FT4 values are highest in the first 1 to 2 weeks after birth and decrease thereafter (10). Because FT4 was measured at a somewhat earlier age in CH-T cases (9 days of age) as compared with CH-C cases (14 days of age), this may have affected the classification of severity resulting in a possible underestimation of the severity of CH-C compared with CH-T. A third limitation is that further data on the exact cause of CH-C and on the presence or absence of additional pituitary hormone deficiencies are missing. The same applies to data on the presence or absence of clinical signs or symptoms of hypopituitarism, making it impossible to estimate whether patients would have been diagnosed because of such features.

The Dutch T4+TSH+TBG method enables calculation of the T4 to TBG ratio and reduces the number of false-positives due to harmless TBG deficiency. This approach has proven to be very effective in detecting CH-C with a rise in the detection rate of permanent CH-C in The Netherlands from 1 in 25000 to 1 in 16000 (4, 5).

Internationally, it is recognized that the stepwise Dutch T4+TSH+TBG method enables early detection and treatment and reduces neonatal mortality and morbidity associated with pituitary hormone deficiencies and central nervous system developmental damage associated with untreated CH (11). In the most recent literature update by the U.S. Preventive Services Task Force on screening for CH it was stated that “the U.S. may want to consider expanding screening strategies that incorporate central CH screening,” referring to our Dutch screening program (12).

In the past 2 decades, lowering of TSH cut-off levels in TSH-based screening programs has led to the detection of very mild cases of CH-T with normal FT4 levels and only mild TSH

elevation (so-called hyperthyrotropinemia), leading to questions about the significance of these findings and the unnecessary burden it puts on parents who are faced with such a finding in their child (3, 13). Another advantage of the Dutch stepwise approach in which an initial T4 measurement is followed by a TSH measurement only in the lowest 20% of T4 values is that it will reduce the number of such very mild cases.

We do acknowledge that, depending on local logistic circumstances, adapting an existing screening program will have financial consequences. The introduction of the T4 to TBG ratio into a program using a primary T4 with supplemental TSH approach generated an extra cost of $11 206 per additional case detected and the costs of the T4+TSH+TBG method were considered to be acceptable (4).

We conclude that these results indicate that, although severe CH is more prevalent in CH-T compared with CH-C, CH-C itself should not be considered as only mild because more than half of CH-C patients have moderate CH with initial FT4 below 10 pmol/L. This is a strong argument for adapting existing neonatal CH screening programs in a way that detection of both CH-T and CH-C is possible.

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Mortality in Children with Early Detected Congenital Central Hypothyroidism

3

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ABSTRACT

Context

Approximately 60-80% of patients with congenital central hypothyroidism (CH-C) have multiple pituitary hormone deficiencies (MPHD), making CH-C a potentially life-threatening disease. Data on mortality in CH-C patients, however, are lacking.

Objective

To study the mortality rate in early detected and treated pediatric CH-C patients in the Netherlands and to investigate whether causes of death were related to pituitary hormone deficiencies.

Methods

Overall mortality rate, infant mortality rate and under-5 mortality rate were calculated in all children with CH-C detected by neonatal screening between 1-1-1995 and 1-1-2013. Medical charts were reviewed to establish causes of death.

Results

139 children with CH-C were identified, of which 138 could be traced (82 MPHD/56 isolated CHC). Total observation time was 1414 years with a median follow up duration of 10.2 years. The overall mortality rate was 10.9% (15/138). Infant mortality rate (IMR) and under-5 mortality rate were 65.2/1000 (9/138) and 101.4/1000 (14/138), respectively, compared to an IMR of 4.7/1000 and under-5 mortality of 5.4/1000 live born children in the Netherlands during the same time period ($p<0.0001$). Main causes of death were severe congenital malformations in six patients, asphyxia in two patients, and congenital or early neonatal infection in two patients. Pituitary hormone deficiency was noted as cause of death in only one infant.

Conclusion

We report an increased mortality rate in early detected CH-C patients which does not seem to be related to endocrine disease. This suggests that mortality due to pituitary insufficiency is low in an early detected and treated CH-C population.

INTRODUCTION

Congenital hypothyroidism (CH) may be of thyroidal origin (CH-T) or central, i.e. hypothalamic-pituitary, origin (CH-C). CH-C is less common than CH-T, with an estimated prevalence of 1 in 16000 versus 1 in 3000 live births (1). From a public health perspective, however, CH-C is an important disease because of associated potentially life-threatening multiple pituitary hormone deficiencies (MPHD). Especially undiagnosed central adrenal insufficiency and growth hormone deficiency may cause neonatal hypoglycemia and/or circulatory insufficiency (2). Approximately 60-80% of CH-C patients have MPHD (2).

Worldwide, most neonatal CH screening programs are thyrotropin (TSH)-based and effectively detect CH-T, but not CH-C. Only a few countries use screening methods in which besides TSH also total or free thyroxine (T4) is measured, allowing CH-C detection. Since 1995 the Dutch neonatal CH screening program consists of a three-step approach: measuring T4 in all newborns with TSH determination in the lowest 20% of T4 concentrations and thyroxine-binding globulin (TBG) measurement in the lowest 5% of T4 concentrations. This screening approach proves to be very effective in detecting CH-C. As a result, the reported prevalence of CH-C in the Netherlands is one of the world's highest (2). Early detection and treatment of CH-C is assumed to reduce morbidity and mortality, especially in children with MPHD (2,3). However, actual data on morbidity and mortality in early and late diagnosed CH-C, with or without MPHD, are lacking.

Currently, we are conducting a nationwide follow-up study on morbidity and developmental outcome in children with CH-C, detected by neonatal screening since 1995. During the initial part of the study, i.e. tracing all Dutch CH-C patients, we observed a high pediatric mortality rate. This was in contrast with the assumption that early detection and treatment reduces mortality. To gain more insight into the cause(s) of death among the deceased children, we reviewed their medical charts.

METHODS

Since the start of the Dutch neonatal screening program in 1981, the Netherlands Organization for Scientific Research (TNO Leiden) registers all abnormal neonatal screening results for CH plus final diagnoses by sending out questionnaires to treating pediatricians at several time points. The first questionnaire is sent out in the first month after a child is referred, the second questionnaire at the age of four years. To verify the diagnosis of CH-C and to trace all patients with CH-C detected by screening since 1995 for a long-term follow-up study, we sent out a third questionnaire between 2013 and 2015. Subsequently, we received several notifications of deaths of CH-C patients, prompting us to develop the current study to collect data on mortality. If a patient had died we asked to be sent all medical details to be able to assess the cause of death. Medical charts of deceased patients were reviewed by two clinicians (NZS and JCN). The

year 1995 was chosen as the start of the study period, because then measurement of TBG in the lowest 5% T4 was added to the T4-reflex TSH strategy in the Netherlands, and because of anticipated difficulties in tracing data from patients born between 1981 and 1995. The use of the TNO database was in accordance with the Privacy Regulations of the Privacy Committee of the Dutch CH Screening Board. The study protocol was approved by the local medical ethics committee.

Statistical analysis

Our primary outcome was mortality rate, specified into infant mortality rate (IMR, mortality under 1 year of age) and under-5 mortality rate (probability of dying between birth and exactly age 5, expressed per 1000 live births). Under-5 mortality was chosen since all children were followed until at least the age of 5, while follow up until the age of 18 is not available for the entire cohort. Infant and under-5 mortality rates among children with CH-C ± MPHD were compared to mortality rates in the Netherlands in the same time period, i.e. between 1-1-1995 and 1-1-2013, as registered by Statistics Netherlands (CBS). Children with CH-C were excluded from Dutch birth and mortality rates. Length of follow-up was defined as time period between the birth date and the date of sending out the third questionnaire or the date of the patient's hospital visit in context of our current follow-up study.

RESULTS

The TNO database contained 141 patients classified as CH-C and detected by neonatal screening between January 1, 1995 and January 1, 2013. Total observation time was 1414 years, with a median follow up duration of 10.2 years.

Two patients did not have CH-C and one MPHD patient could not be traced. Of the remaining 138 patients, 82 patients had MPHD (69.5% male) and 56 patients had isolated CH-C (82.4% male). Three patients had isolated CH-C combined with a congenital disorder of glycosylation syndrome (CDG).

In the studied cohort fifteen patients (10 MPHD and 5 isolated CH-C) had died (mortality rate 10.9%; 108.6 per 1000).

Nine patients died within the first year of life, resulting in an IMR of 65.2 per 1000. The Dutch IMR in the same period was 4.7 per 1000 live born children (Odds ratio 14.5, 95% confidence interval 7.6–29.3, $p<0.0001$). Fourteen patients died before the age of 5, corresponding with an under-5 mortality of 101.4 per 1000 live born children, compared to 5.4 per 1000 live born children in the Netherlands during the same time period (Odds ratio 21.1, 95% confidence interval 12.1-36.6, $p<0.0001$).

Patient characteristics and causes of death are summarized in Table 1, which shows several congenital malformations, birth asphyxia and infections as leading causes of death. In only one patient cause of death was attributed to pituitary hormone deficiency (case 9).

Additional diagnostic information was available for two patients with isolated CH-C. In case number 7 CH-C was most likely caused by severe encephalopathy, following birth asphyxia. In case number 8 brain imaging (MRI) was normal, but a thyrotropin releasing hormone (TRH) test confirmed the diagnosis of isolated CH-C.

DISCUSSION

To our knowledge this is the first report on mortality in pediatric CH-C patients detected by neonatal screening. The overall mortality rate was 10.9% (15/138), while infant mortality rate and under-5 mortality were 6.5% (9/138) and 10.1% (14/138), respectively. Severe congenital cerebral malformations, congenital heart defects, birth asphyxia and infections were important causes of death, while hypopituitarism was not. Overall, CH-C was considered a comorbidity rather than the primary diagnosis in this group of deceased patients.

It is important to realize that this is a report on mortality in an early detected CH-C cohort originating from a screened population. Our results indicate that mortality due to pituitary deficiencies is low in early treated patients. Despite early detection and treatment one patient had died due to acute adrenal insufficiency during an infection, emphasizing the severity of MPHD. Unfortunately, mortality rates in unscreened populations are lacking. Early death in children with unrecognized CH-C might partly explain the lower observed CH-C prevalence in these countries (7).

An estimation of how many deaths or hypoglycemic events were actually prevented by early detection would be very valuable. Unfortunately, these data are not available. We are currently performing a long term follow up study of all children with CH-C detected by neonatal screening from 1995 onwards. In this study data on perinatal history, including occurrence of hypoglycemia, are being collected.

While the Dutch neonatal screening effectively detects children with CH-C, missed cases might occur. Missed cases are not systematically registered, although pediatricians are encouraged to report these patients to TNO. In a recent study among Dutch children with non-acquired, presumed isolated growth hormone deficiency 29 cases of probable CH-C, missed by neonatal screening, were detected over a time period of ten years (8). This suggests that the Dutch neonatal screening misses at least 2.9 children with congenital central hypothyroidism per year. Extrapolating these data to our cohort indicates that approximately 52 children were missed by neonatal screening in this 18 year period, thus missing 27% (52/191) of all CH-C cases. From countries using different T4-based screening programs, higher percentages of missed cases have been reported. For example, in a cohort of 42 pediatric CH-C patients, 81% had been missed

Table 1. Cause of death and diagnosis in 15 children with CH-C detected by neonatal screening

Case	Sex	GA (wk)	BW (kg)	Age at death (y)	Cause of death	Diagnoses	Treatment		
							L-T4	HCT	GH
1	M	38 2/7	4.3	0.1	Renal and respiratory insufficiency	Joubert syndrome, intestinal malrotation, severe cerebral malformation, MPHD	Yes	Yes	No
2	M	36 6/7	2.3	0.1	Birth asphyxia, intracerebral bleeding	6p deletion syndrome, aortic coarctation, MPHD	Yes	No	No
3	M	38 1/7	3.0	0.1	Vena cava inferior thrombosis	Probable CDG syndrome, MPHD	Yes	Yes	No
4	M	37 1/7	2.8	0.1	Respiratory insufficiency	Congenital toxoplasmosis, MPHD	Yes	Yes	No
5	F	42 1/7	3.8	0.3	Cerebral malformation and insufficiency	Holoprosencephaly with hydrocephalus, MPHD	No (parents declined endocrine treatment)		
6	F	40 1/7	4.0	0.4	Circulatory insufficiency	Congenital heart disease, NEC, isolated CH-C	Yes	No	No
7	F	37 1/7	2.1	0.4	Respiratory insufficiency during aspiration pneumonia	Birth asphyxia after placental abruption, severe encephalopathy, isolated CH-C	Yes	No	No
8	M	34 4/7	2.7	0.6	Respiratory insufficiency	Severe lung hypoplasia, renal dysplasia, isolated CH-C	Yes	No	No
9	M	34 5/7	2.8	0.9	Cardiorespiratory arrest during adrenal crisis	Congenital pituitary malformation, MPHD	Yes	Yes	Not yet
10	M	37 5/7	2.9	1.0	Status epilepticus during meningitis	Epilepsy, MPHD	Yes	Yes	Not yet
11	F	39	1.5	1.6	Respiratory insufficiency	Partial trisomy 19p and deletion Xp22, truncus arteriosus, holoprosencephaly, MPHD (central hypothyroidism and -diabetes insipidus)	Yes	No	No

Table 1. Continued

Case	Sex	GA (wk)	BW (kg)	Age at death (y)	Cause of death	Diagnoses	Treatment		
							L-T4	HCT	GH
12	M	42	3.9	2.1	Nephrotic syndrome and infection	Congenital heart disease, cystic kidneys, epilepsy, MPHD	Yes	Yes	No
13	F	37	3.2	6.7	Respiratory insufficiency during pneumonia with sepsis	Bacterial meningitis with hypothalamic infarcts, West syndrome, MPHD	Yes	Yes	No
14	M	40 1/7	2.9	1.1	Cardiorespiratory arrest during sepsis	CDG syndrome, isolated CH-C	Yes	No	No
15	F	37	2.1	1.7	Sepsis with multi organ failure	CDG syndrome, isolated CH-C	Yes	No	No

Abbreviations: BW, birth weight; CDG, congenital disorder of glycosylation; F, female; GA, gestational age; GH, growth hormone; HCT, hydrocortisone; L-T4, levothyroxine; M, male; NEC, necrotic enterocolitis.

with a neonatal screening strategy that used a "fixed" T4 concentration cut off of 5 mcg/dL, which is approximately 65 nmol/L and corresponds to approximately -2 SD (9). When imputing the estimated number of missed Dutch children in our calculations, the mortality rate declines (15/191; 7.9%) but is still considerably higher than in the general Dutch pediatric population.

This cohort study provides new demographic data on children with CH-C. A strong male predominance was seen for both isolated CH-C and MPHD, as has previously been reported in smaller studies (3,4). Recently, several X-linked genetic causes for isolated CH-C have been identified, e.g. mutations in the *IGSF1* and *TBL1X* genes, which may explain the male predominance (10,11). In almost 60% of our cases CH-C occurred within the frame-work of MPHD. In previous reports the proportion of children with MPHD ranges from 58-78% (3–5).

Our cohort included three patients with CDG. CDG form a group of inborn errors in protein and lipid glycosylation. As glycosylation is crucial in myriad processes, symptoms are highly variable and diagnosis is often delayed (12). Abnormal results for neonatal CH screening, however, can be seen in children with CDG and might allow early diagnosis especially when combined with other CDG symptoms or dysmorphic features (12,13). Abnormal thyroid function tests in CDG patients are caused by altered glycosylation of TSH and TBG. Abnormalities most often consist of low free T4 with elevated TSH, or low total T4 caused by partial TBG deficiency (14). When measuring free T4 and TSH concentrations using equilibrium dialysis, values in the normal range have been reported (15). Although it has been concluded previously that most CDG patients can be considered chemically euthyroid (14), patients with clinical symptoms of hypothyroidism requiring thyroxine supplementation have been reported as well (16). Whether these patients should be classified as having isolated CH-C is debatable.

This study has its limitations. CH-C is not an easy diagnosis, because it heavily relies on the serum or plasma free T4 concentration (given the hypothalamic-pituitary origin, measurement of TSH does not contribute to the diagnosis). Since patients were diagnosed by various pediatricians, misdiagnosis cannot be ruled out. In addition, in the deceased patients with presumed isolated CH-C and other "severe illnesses" the abnormal thyroid function tests might be explained by non-thyroidal illness (17). Because of these patients' early death, the permanence of isolated CH-C could not be verified after the first two to three years of life. We did, however, review medical charts of all deceased patients including their neonatal screening results, laboratory values and medication history.

Given the association between hypopituitarism and cerebral anomalies an increased mortality in this group of patients was to be expected and therefore the results of this study are not surprising. However, at the same time, by tracing all patients with CH-C ± MPHD detected by neonatal screening since 1995, we were able to show that the mortality in hypopituitarism without concurrent diagnoses is low: in our cohort only one patient died.

In conclusion, we report an increased mortality in early detected CH-C patients which does not seem to be related to endocrine disease. This suggests that mortality due to pituitary deficiencies is low in an early detected and treated CH-C population while mortality rate for CH-C patients in unscreened populations remains unknown.

N.Z.-S. and J.C.N. contributed equally to the study

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Difficulties in diagnosing congenital central hypothyroidism

TSH and FT4 concentrations in Congenital Central Hypothyroidism and Mild Congenital Thyroidal Hypothyroidism

4

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ABSTRACT

Context

In central hypothyroidism (CeH), free thyroxine (FT4) concentrations are low, whereas thyrotropin (TSH) concentrations may be low, normal, or even slightly elevated due to reduced bioactivity. Congenital CeH (CCeH) may be isolated or part of multiple pituitary hormone deficiencies (MPHD).

Objective

We tested our hypotheses that (1) TSH concentrations have a more U-shaped distribution in children with CCeH compared with children with a normally functioning hypothalamic-pituitary-thyroid axis and (2) TSH concentrations in children with CCeH with MPHD are higher compared with children with isolated CCeH. We also studied whether FT4 levels are helpful in distinguishing CCeH from mild congenital hypothyroidism of thyroidal origin (CH-T).

Methods

Dutch neonatal screening TSH and first diagnostic TSH and FT4 were analyzed in all children diagnosed with permanent CCeH between 1995 and 2012. Controls were children with T4-binding globulin deficiency. FT4 concentrations in CCeH were compared with those in CH-T with TSH values in the same range as those of CCeH.

Results

We studied 120 children with CCeH (isolated CCeH, n = 50; MPHD, n = 70) and 350 control subjects. Screening TSH concentrations were not significantly different (P = 0.055), but diagnostic TSH values were significantly different between the CCeH group and the control group (P = 0.037). TSH was significantly higher in MPHD compared with isolated CCeH (P = 0.004). FT4 concentrations were significantly lower in CCeH compared with mild CH-T (P < 0.0005).

Conclusion

TSH values in CCeH have a more U-shaped distribution compared with controls with the highest TSH concentrations in MPHD. FT4 levels were significantly lower in CCeH compared with CH-T.

INTRODUCTION

Central hypothyroidism (CeH) is characterized by suboptimal thyroid hormone production due to insufficient stimulation by thyrotropin (TSH) of an otherwise normal thyroid gland (1). CeH may be caused by congenital or acquired disorders of the pituitary gland or hypothalamus, resulting in altered TSH production (1).

TSH is a dimeric glycoprotein hormone composed of two subunits: a hormone-specific β-subunit and an α-subunit that is shared with follicle-stimulating hormone, luteinizing hormone, and chorionic gonadotropin. TSH production is regulated by hypothalamic TSH-releasing hormone (TRH). In addition to stimulating transcription of the α- and β-subunit genes, TRH mediates glycosylation and conjugation of TSH-α and -β subunits and TSH secretion. The absence of normal TRH stimulation leads to impaired pituitary TSH glycosylation, resulting in decreased bioactivity of circulating TSH (1–3).

The biochemical diagnosis CeH is usually made when a low serum free thyroxine (FT4) concentration is accompanied by a "normal" or low TSH concentration. However, due to the presence of TSH with reduced bioactivity, TSH concentrations as measured by immunoassays are not always normal or low but may also be slightly elevated. This sometimes makes distinguishing CeH from mild primary hypothyroidism a challenge.

In previous studies, TSH concentrations in CeH have been reported to range from well below the reference range up to ~11 mIU/L (upper limit of reference range, 4.0) (4–6). Most of the patients in these reports had acquired/adult-onset CeH (*e.g.*, due to pituitary tumors). There are no reports on TSH concentrations in large groups of patients with congenital CeH (CCeH).

Worldwide, most neonatal screening programs for congenital hypothyroidism are TSH based and detect congenital hypothyroidism of thyroidal origin (CH-T) (7). Only a few countries use a screening method in which, in addition to TSH, total T4 or FT4 are measured (T4+TSH method), enabling detection of both CH-T and CCeH. In the Netherlands, a three-step approach is used in which the initial T4 measurement is followed by measurement of TSH and T4-binding globulin (TBG). This approach has proven to be effective in detecting CCeH and CH-T (8, 9). In the last decade, lowering of the TSH cutoff in TSH-based neonatal screening programs has led to an increase in the detection of mild CH-T accompanied by slightly elevated TSH concentrations, which may be difficult to distinguish from CCeH.

CCeH may be due to isolated TSH deficiency or due to altered TSH secretion within the framework of multiple pituitary hormone deficiencies (MPHD). In general, TSH values in genetic forms of isolated CCeH have been reported to range from undetectable to normal (10–17). In MPHD, TSH values may be low in pituitary defects but higher in hypothalamic defects. The most common cause of congenital MPHD is a malformation of the pituitary gland, the so-called "pituitary stalk interruption syndrome" (PSIS) (18). In PSIS the pituitary stalk malformation thwarts hypothalamic TRH stimulation of pituitary thyrotrope cells, resulting in impaired TSH

glycosylation and reduced bioactivity. In this condition TSH, as measured by immunoassays, may be elevated (9).

Because TSH levels in CCeH may be low but also may be elevated (especially in MPHD associated with PSIS), we expected the overall range of TSH concentrations in children with CCeH to have a more U-shaped distribution compared with children with a normally functioning hypothalamus-pituitary-thyroid (HPT) axis. Because CCeH within MPHD is most often caused by PSIS and accompanied by TSH with reduced bioactivity, we hypothesized that TSH concentrations in CCeH within MPHD are higher than in isolated CCeH.

The goals of this study were to study whether TSH concentrations in children with CCeH have a more U-shaped distribution compared with the TSH distribution in children with a normally functioning HPT axis and to test the hypothesis that TSH concentrations in children with CCeH within the framework of MPHD are higher compared with children with isolated CCeH. In addition, we studied whether FT4 levels are helpful in distinguishing CCeH from mild CH-T.

MATERIALS AND METHODS

Dutch neonatal screening program

The Dutch neonatal screening program consists of a three-step T4-TSH-TBG approach. The initial measurement is total T4, which is expressed as standard deviation (SD) of the daily mean. In the lowest 20% (T4 ≤−0.8 SD) additional TSH measurements are done, and in the lowest 5% (T4 ≤−1.6 SD) additional TBG measurements are done. This approach enables calculation of the T4/TBG ratio as an estimation of the FT4 concentration. TBG measurement reduces the number of false positives due to harmless TBG deficiency.

Up until 30 June 2012, the T4/TBG ratio in the Dutch screening program was only used for T4 results between −3.0 and −1.6 SD. Children with T4 ≤−3.0 SD were immediately referred because in this sample a large proportion of children have a serious form of CH-T or CCeH, and it was considered that waiting for TSH and TBG results would lead to too much delay in treatment. Because of this approach, a large number of children with TBG deficiency were referred and underwent a diagnostic work-up consisting of TSH and FT4 measurement. For children born at ≤36 weeks of gestation with a birth weight ≤2500 g, referral is based only on the TSH concentration to avoid unnecessary referral (false-positive results) for a low T4 due to transient hypothyroxinemia of prematurity.

Data retrieval

In the Netherlands, all children with an abnormal neonatal screening result are referred to a pediatric endocrinologist or general pediatrician. TNO Leiden registers all children with an abnormal congenital hypothyroidism screening result and sends out questionnaires to pediatricians to collect data on confirmatory laboratory results and diagnosis. The first questionnaire

is sent out shortly after referral; the second questionnaire is sent out when the children are 4 to 5 years of age to reassess the diagnosis. Between 2013 and 2015, a third questionnaire was sent out to pediatricians caring for patients born between 1995 and 2012 with a diagnosis of CCeH based on the two first questionnaires. The third questionnaire requested for further specification of the diagnosis (*e.g.*, whether the child has isolated CCeH or MPHD). MPHD was defined as CeH in combination with at least one more pituitary hormone deficiency.

Using the TNO database, we analyzed screening TSH concentrations and the first diagnostic/pretreatment TSH concentrations of all children diagnosed with permanent CCeH between 1 January 1995 and 13 December 2012. Because children with normal screening results are not referred for diagnostic work-up, data from these children were not available. Results of children with false-positive screening results were available, but this was considered an inappropriate control group because it may contain children with transient forms of congenital hypothyroidism. Instead, as a control group, we used children who were referred for a low screening T4 but who at diagnostic work-up had TBG deficiency instead of congenital hypothyroidism. Within this group, we used a low cutoff for TBG (TBG concentration <7.5 mg/L serum = <140 nmol/L as measured at diagnostic work-up). All children with a birth weight <2500 g were excluded from the study because TSH concentrations may be slightly higher in premature and low-birth-weight infants.

Screening TSH was measured at five national dedicated screening laboratories with an immunoassay supplied by Perkin Elmer (Waltham, Massachusetts) or Brahms (Waltham, Massachusetts). Assays were standardized using external control samples, allowing use of the same cutoff values. Because children are referred to various hospitals in the Netherlands, pretreatment TSH was measured in various laboratories. Although TSH tests are supplied by different manufacturers, all TSH measurements are performed by immunoassays, with an adult reference range between 0.4 and 4.0 mIU/L in the vast majority of tests.

We hypothesized that in CCeH, TSH values are more often somewhat lower as well as more often somewhat higher (U-shaped distribution) compared with control subjects. Therefore, we categorized screening TSH values as well as diagnostic TSH values. The categorized TSH values of children with CCeH and control subjects were compared. Furthermore, we hypothesized that TSH concentrations in children with CCeH within the framework of MPHD would be higher compared with children with isolated CCeH. Therefore, for the comparison between MPHD and isolated CCeH we did not categorize the TSH values. For categorized data, we used Pearson's $\chi 2$ test or, in case of expected counts <5, we used Fisher's exact test. For continuous data, the Mann-Whitney *U* test was used. A *P* value <0.05 (two-sided) was considered statistically significant.

To test whether FT4 levels may help distinguish CCeH from mild CH-T with comparable TSH levels, we compared FT4 levels of the children with CCeH with FT4 levels of children with CH-T who had TSH levels in the same range as children with CCeH.

The use of the TNO database was in accordance with the Privacy Regulations of the Privacy Committee of the Dutch CH Screening Board.

RESULTS

Between 1995 and 2012, 131 children with CCeH were detected by neonatal screening, 383 children were diagnosed with TBG deficiency, and 1022 children were diagnosed with CH-T. After excluding children with a birth weight <2500 g, the groups consisted of 120 children with CCeH (isolated CCeH, n = 50; MPHD, n = 70) and 350 control subjects with TBG deficiency. The highest measured diagnostic TSH in CCeH was 12.9 mIU/L. Selection of patients with CH-T was made on the basis of TSH comparable to that of the CCeH group. This resulted in the inclusion of 20 cases of CH-T with a first diagnostic TSH <13 mIU/L. In the MPHD group, besides CCeH, the following patterns of pituitary deficiencies were reported: 1 × growth hormone (GH) + adrenocorticotropin (ACTH) + gonadotropin + vasopressin; 2 × GH + ACTH + vasopressin; 21 × GH + ACTH + gonadotropin; 31 × GH + ACTH; 1 × GH + gonadotropin; 1 × ACTH + gonadotropin; 5 × GH; 5 × ACTH; 1 × gonadotropin; two missing data. In 39 cases, information on gonadotropin deficiency was missing or reported as not available. Screening TSH was measured at a median age of 5 days (interquartile range, 2 days), and the first diagnostic TSH and FT4 were measured at a median of 9 days (interquartile range, 5 days). Screening TSH was missing in two cases of CCeH, in one case of MPHD, and in eight control subjects. Diagnostic TSH was missing in one case of MPHD. Diagnostic FT4 was missing in one case of CCeH and in one case of MPHD.

There was a higher percentage of lower (<3 mIU/L serum) as well as higher (≥7 mIU/L serum) screening TSH values in neonates with CCeH compared with the control group. However, these screening TSH concentrations were not statistically significantly different in the CCeH group compared with the control group (Fisher exact test; $P = 0.055$) (Table 1). TSH values at screening were not significantly different in the subgroup of isolated CCeH (median, 3 mIU/L serum) compared with the subgroup with MPHD (median, 3.5 mIU/L serum) (Mann-Whitney *U* test; $P = 0.568$). Diagnostic TSH values were statistically significantly different between the total CCeH group and the control group (Fisher exact test; $P = 0.037$), with somewhat higher percentages of low and (rather) high TSH values in the CCeH group (Table 2; Fig. 1). In a subgroup analysis of MPHD vs isolated CCeH, diagnostic TSH values were higher in the subgroup of MPHD (median, 4.4 mIU/L serum) than in the subgroup of isolated CCeH (median, 3.1 mIU/L serum) (Mann-Whitney *U* test; $P = 0.004$). The distribution of TSH values in MPHD compared with isolated CCeH was shifted to the right, with the highest TSH values in the MPHD group (Table 2; Fig. 2).

Magnetic resonance imaging data were not systematically stored in the database but were available in 36 cases (32 patients with pituitary malformations, three patients with a normal

Table 1. Screening TSH concentrations in children with CCeH, including subgroups with isolated CCeH and CCeH within the framework of MPHD, and a control group consisting of children with TBG deficiency.

TSH (mIU/l)	Total CCeH (n=117)	Isolated CCeH (n=49)	MPHD (n=68)	Control Group (n=342)
<3	35 (29.9%)	17 (34.7%)	18 (26.5%)	97 (28.4%)
3-6.9	77 (65.8%)	29 (59.2%)	48 (70.6%)	227 (66.4%)
7-10.9	1 (0.9%)	1 (2.0%)	0	15 (4.4%)
11-14.9	4 (3.4%)	2 (4.1%)	2 (2.9%)	2 (0.6%)
≥15	0	0	0	1 (0.3%)

Table 2. First diagnostic TSH concentrations in children with CCeH, including subgroups with isolated CCeH and CCeH within the framework of MPHD, and a control group consisting of children with TBG deficiency.

TSH (mIU/l)	Total CCeH (n=119)	Isolated CCeH (n=50)	MPHD (n=69)	Control Group (n=350)
<0.5	3 (2.5%)	2 (4%)	1 (1.4%)	2 (0.6%)
0.5-2.5	33 (27.7%)	19 (38%)	14 (20.3%)	69 (19.7%)
2.6-5	49 (41.2%)	18 (36%)	31 (44.9%)	198 (56.6%)
5.1-7.5	28 (23.5%)	11 (22%)	17 (24.6%)	64 (18.3%)
7.6-10	4 (3.4%)	0	4 (5.8%)	11 (3.1%)
>10	2 (1.7%)	0	2 (2.9%)	6 (1.7%)

pituitary anatomy, and one patient with uncertain pituitary anatomy). We did have information on the diagnosis of the six children with the highest TSH concentrations (>7.5 mIU/L) at first diagnostic work-up (Table 3). Five of these children had a pituitary malformation, and one had CHARGE syndrome without further data on pituitary anatomy.

In Fig. 3, TSH and FT4 values of the 20 patients with mild CH-T (red) are depicted together with values in isolated CCeH (green) and MPHD (blue). Because distinguishing mild CH-T and CCeH may be difficult and misclassification is possible, we used the available data in the TNO database to review the CH classification. Of the 20 patients with mild CH-T (red circles), the diagnosis CH-T seemed valid in 14 cases based on either additional diagnostic blood tests, imaging (thyroid ultrasound and/or scintigraphy), or available follow-up data at 5 years of age. However, in five cases (solid red dots), we were not sure about the diagnosis. At initial diagnosis and at follow-up

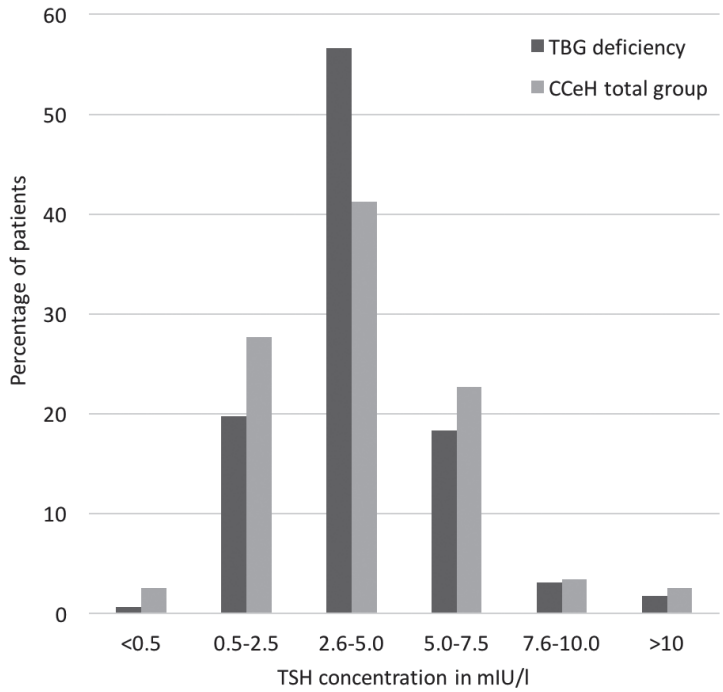

Figure 1. Distribution of the first diagnostic TSH concentrations in the total CCeH group (isolated + MPHD) and TBG deficiency as control group.

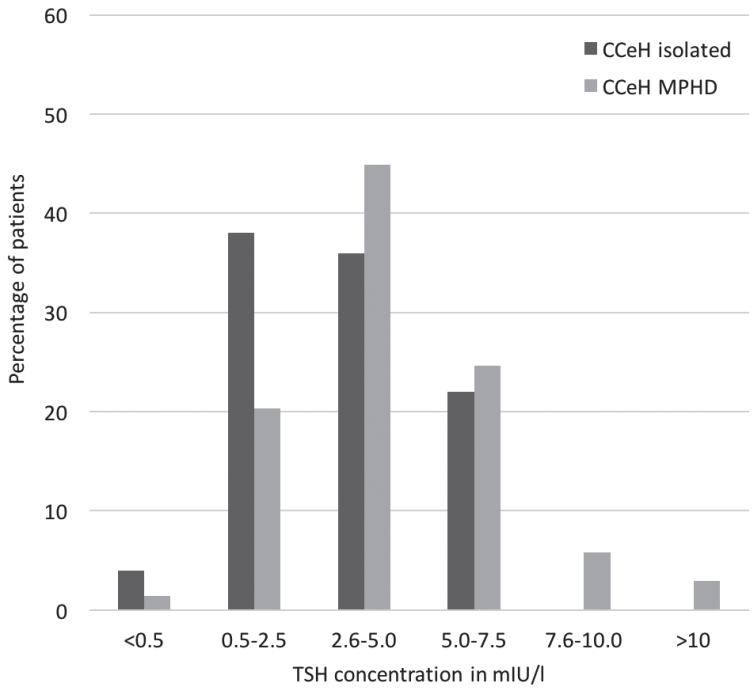

Figure 2. Distribution of the first diagnostic TSH concentrations in the isolated CCeH group, and in the CCeH within the framework of MPHD group.

Table 3. TSH and FT4 concentrations at first diagnostic work-up in six individuals with the highest TSH concentrations, together with their diagnosis.

	TSH (mIU/l)	FT4 (pmol/l)[a]	Diagnosis
1	7.8	10.1	CHARGE syndrome
2	8.3	5.7	pituitary stalk interruption syndrome
3	8.8	8.1	pituitary stalk interruption syndrome
4	9.0	5.9	Septo-optic-dysplasia
5	10.2	7.8	pituitary stalk interruption syndrome
6	12.9	10.1	pituitary stalk interruption syndrome

[a] divide by 12.87 to convert pmol/l to ng/dl

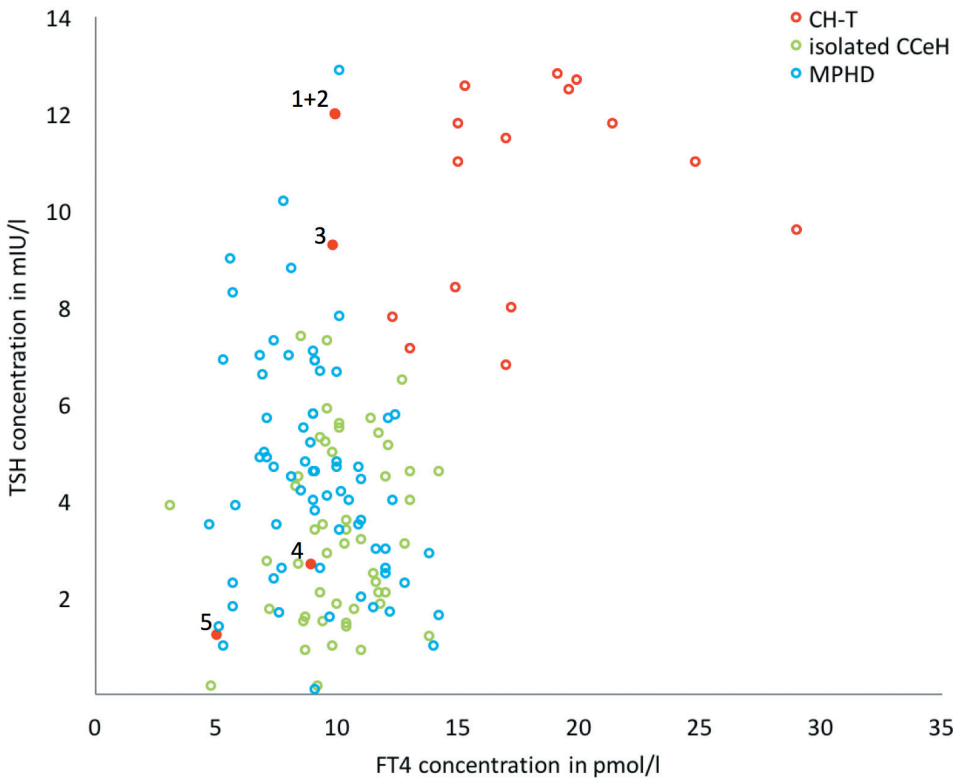

Figure 3. First diagnostic TSH and FT4 concentrations in isolated CCeH (n=49; green circles), CCeH within the framework of MPHD (n=69; blue circles) and in mild CH-T (TSH < 13 mIU/l) (n=20; red circles and red solid dots). Numbered red solid dots (1-5) are cases possibly misclassified as CH-T.

around 5 years, these cases were reported to be CH-T by their treating pediatricians. However, in cases 1, 2, and 3 (solid red dots 1, 2, and 3), the available data did not allow for distinction between CCeH and CH-T, and in cases 4 and 5 (solid red dots 5 and 6), CCeH seemed to be more likely. Therefore, among cases 1 through 5, some may be misclassified as CH-T.

FT4 levels were significantly lower in CCeH compared with mild CH-T (Mann-Whitney *U* test; $P < 0.0005$). Within CCeH, FT4 levels were significantly higher in isolated CCeH compared with MPHD (Mann-Whitney *U* test; $P = 0.008$).

DISCUSSION

In this study, we found serum TSH concentrations in neonates with CCeH to be significantly different from TSH concentrations of neonates with TBG deficiency at the time of the first diagnostic laboratory testing after referral for an abnormal neonatal screening result. Compared with neonates with a normally functioning HPT axis, neonates with CCeH had higher percentages of low and high TSH values, whereas the percentage of "normal" values was lower. The same pattern between the groups was found for TSH values based on screening. However, this difference was not statistically significant. Further analyses showed that the higher percentage of low TSH serum values could be mainly attributed to neonates with isolated CCeH, whereas the higher percentage of high values could be mostly attributed to neonates with CCeH within the framework of MPHD. Both findings supported our hypotheses. The highest measured diagnostic TSH in CCeH was 12.9 mIU/L. FT4 levels were significantly lower in CCeH compared with CH-T, with comparable TSH values.

CCeH is characterized by suboptimal thyroid hormone production due to insufficient stimulation by TSH of an otherwise normal thyroid gland (1). The TSH insufficiency may be due to hypothalamic or pituitary pathology leading to a qualitative (*i.e.*, decreased bioactivity) or quantitative defect in TSH production. TSH deficiency can be isolated if the defect is limited to thyrotroph function (isolated CCeH) or may be associated with MPHD (19, 20). Isolated CCeH is less common and may be caused by mutations in the TRH receptor gene (*TRHR*), the thyroid-stimulating hormone β-subunit gene (*TSHB*), and the recently described *IGSF1* and *TBL1X* genes (10–13, 15, 16). In the majority of cases, CCeH occurs with other pituitary hormone deficiencies (9, 19, 20). Various genetic defects in transcription factors involved in pituitary development and differentiation have been described (*POU1F1*, *PROP1*, *HESX1*, *LHX3*, *LHX4*, *OTX2*, *SOX3*, *GLI2*), but in most cases of MPHD the cause is unknown (21, 22). In the majority of these cases, a pituitary malformation is seen on magnetic resonance imaging consisting of an absent or thin pituitary stalk and a hypoplastic anterior and ectopic posterior pituitary lobe, also known as PSIS (9, 18). In these cases, the pituitary stalk malformation results in a lack of hypothalamic TRH stimulation, resulting in impaired TSH glycosylation and secretion. In our study, TSH concentrations were higher in patients with MPHD and CCeH compared with patients

with isolated CCeH, suggesting that in the MPHD group the TSH deficiency was more often of hypothalamic origin than in the isolated CCeH group. We did not have information on possible genetic etiologies of the isolated CCeH cases. In general, TSH values in genetic forms of isolated CCeH have been reported to be undetectable to normal. Isolated CCeH due to *TRHR* gene defects have only been described in three cases, and in these cases TSH was low/normal (10, 11). In *TSHB* gene defects, TSH concentrations are usually undetectable but may be low/normal due to bioinactive TSH (12–14). In the newly described genetic forms of X-linked CeH, *IGSF1* and *TBL1X* gene defects, TSH concentrations were reported to be within normal reference ranges (15–17).

Although isolated CCeH is reported to be less frequent than CCeH within MPHD, in our study population the percentage of patients with isolated CCeH was relatively high (50/119 = 42%). Because diagnosing isolated CCeH relies solely on finding too low FT4 concentrations and because proper neonatal reference values for FT4 are missing, we cannot rule out misinterpretation and overrepresentation of these patients. However, it is likely that the high prevalence of isolated CCeH is the result of the unique Dutch neonatal screening program.

The difference in TSH concentrations between MPHD with CCeH and isolated CCeH patients was only clearly detected in the first diagnostic TSH measurements and not in the neonatal screening measurements. A probable explanation for this is that screening TSH is performed on blood samples collected on filter paper from a heel puncture with a larger measurement variation than TSH measurements performed in a venous blood sample. The larger measurement error leads to a lower statistical power to detect differences in screening TSH levels compared with venous TSH levels between the subgroups.

Two children with CCeH within the framework of MPHD had TSH concentrations >10 mIU/L, with the highest measured screening TSH of 14 mIU/L and a pretreatment value of 12.9 mIU/L. In these cases, TSH could have been (or may also be) interpreted as suggestive for mild CH-T. This emphasizes the importance of careful interpretation of slightly elevated TSH levels. Because proper reference values for FT4 in the neonatal period are not available, diagnosing CCeH remains a challenge, especially in cases of isolated TSH deficiency. FT4 levels in CCeH were on average lower compared with FT4 in CH-T with TSH values in the same range of those with CCeH. If FT4 levels are below the reference range, CCeH must be considered. Additional analysis (*e.g.*, thyroid imaging, pituitary function testing) and careful monitoring of thyroid function tests in the first months of life may provide evidence for a more certain diagnosis.

Our finding of TSH values well above the reference range is in line with reports of TSH values reported in cases of acquired CeH (4–6). In a report on a 2-year Dutch cohort of CCeH (April 1994 to April 1996) in 19 Dutch children with CCeH, first diagnostic TSH concentrations ranged from <0.05 to 10 mIU/L, similar to our results (9). We did not find any reports specifying TSH values in isolated CCeH vs MPHD.

Strengths of our study are that data were extracted from a unique, large national cohort of children with CCeH detected by neonatal screening and that, by sending out a third questionnaire, we verified the diagnosis of permanent CeH at a later age, with the oldest

patients being 20 years of age. Furthermore, we were able to include a control group with normal HPT axis because in TBG deficiency only total T4 levels are low and the HPT axis is normal, with normal serum FT4 and TSH concentrations. A limitation of our study was that the CCeH and CH-T diagnoses were made by a rather large group of pediatricians. Although it seems reasonable to assume that for MPHD diagnoses are reliable, this is less certain for isolated CCeH and mild CH-T, so misinterpretation and misclassification cannot be ruled out. Nonthyroidal illness (accompanied by low TSH and FT4) may also be a differential diagnosis in these cases.

In summary, TSH values in CCeH are more often lower or higher compared with control subjects. TSH concentrations were higher in CCeH within the framework of MPHD compared with isolated CCeH, probably indicating the hypothalamic origin of the hypothyroidism in most patients with MPHD. Patients with CCeH may have TSH concentrations >10 mIU/L. FT4 levels were lower in CCeH compared with CH-T and may be helpful in distinguishing CCeH from mild CH-T.

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Genetics versus environment in T4-setpoint determination

Fetal Environment Is a Major Determinant of the Neonatal Blood Thyroxine Level: Results of a Large Dutch Twin Study

5

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ABSTRACT

Context

The interindividual variability in thyroid hormone function parameters is much larger than the intraindividual variability, suggesting an individual set point for these parameters. There is evidence to suggest that environmental factors are more important than genetic factors in the determination of this individual set point.

Objective

This study aimed to quantify the effect of genetic factors and (fetal) environment on the early postnatal blood T_4 concentration.

Methods

This was a classical twin study comparing the resemblance of neonatal screening blood T_4 concentrations in 1264 mono- and 2566 dizygotic twin pairs retrieved from the population-based Netherlands Twin Register. Maximum-likelihood estimates of variance explained by genetic and environmental influences were obtained by structural equation modeling in data from full-term and preterm twin pairs.

Results

In full-term infants, genetic factors explained 40%/31% of the variance in standardized T_4 scores in boys/girls, and shared environment, 27%/22%. The remaining variance of 33%/47% was due to environmental factors not shared by twins. For preterm infants, genetic factors explained 34%/0% of the variance in boys/girls, shared environment 31%/57%, and unique environment 35%/43%. In very preterm twins, no significant contribution of genetic factors was observed.

Conclusion

Environment explains a large proportion of the resemblance of the postnatal blood T_4 concentration in twin pairs. Because we analyzed neonatal screening results, the fetal environment is the most likely candidate for these environmental influences. Genetic influences on the T_4 set point diminished with declining gestational age, especially in girls. This may be due to major environmental influences such as immaturity and nonthyroidal illness in very preterm infants.

INTRODUCTION

In healthy individuals, thyroid function parameters such as the plasma TSH, free T_4 (FT_4) and free T_3 (FT_3) concentrations show large interindividual differences leading to wide laboratory reference intervals. Their intraindividual variability, however, is much smaller, suggesting that each individual has his or her own specific hypothalamus-pituitary-thyroid axis set point (1, 2).

Previous twin studies have shown that both genetic and environmental factors seem to contribute to this individual set point (3–8). In a large UK study of 2124 female adult twins, environmental factors determined 61% of variation in FT_4 and 77% in FT_3 (3). This suggests that FT_4 and FT_3 concentrations are mainly influenced by environmental rather than genetic factors.

The term set point describes that an individual trait remains stable over time, which seems to apply to the TSH, FT_4, and FT_3 concentrations (2). If indeed these set points are mainly determined by environmental factors, the question arises which environmental factors would have such a major influence that they may be held responsible for determining an individual's set point. We hypothesized that the answer may lie in the fetal environment, which has a major effect on postnatal health (9, 10).

The fetal and maternal thyroid hormone state has been shown to influence postnatal thyroid hormone levels. One example is infants born to inadequately treated mothers with Graves' disease. Kempers et al (11) described congenital central hypothyroidism (low plasma FT_4 concentrations in the presence of normal TSH concentrations in the neonatal period), and ultimately loss of thyroid function and even morphology in these infants. Another example is severe primary congenital hypothyroidism (CH), eg, thyroid agenesis. When treating patients with this condition with levothyroxine, it is often necessary to aim for and keep plasma FT_4 concentrations above the age-specific reference range interval to achieve a normal TSH concentration (12–14). This suggests that the pituitary-thyroid axis set point is altered, possibly caused by a hypothyroid intrauterine environment. Recently, it was shown that in normal pregnancies higher maternal T_4 concentrations (within the normal range) are associated with lower birth weight as well as with higher T_4 cord blood concentrations (15, 16).

To test the hypothesis that the fetal environment to a large extent determines the postnatal thyroid hormone concentration and the T_4 set point, we conducted a classical twin study, comparing the resemblance of neonatal screening blood T_4 concentrations in a large sample of mono- (MZ) and dizygotic (DZ) twin pairs.

SUBJECTS AND METHODS

This study was based on record linkage of two databases: the Dutch neonatal CH screening results, stored at the National Institute for Public Health and Environment (Rijksinstituut voor Volksgezondheid en Milieuhygiene, RIVM) and The Netherlands Twin Register (NTR).

In the Dutch neonatal CH screening program blood is drawn on average on the fifth day after birth. The program consists of a three-step-approach with primary T_4 measurement in filter paper blood spots followed by additional TSH measurement in the lowest 20% T_4 concentrations, and additional measurement of T_4 binding globulin in the lowest 5%. T_4 concentrations are expressed as SD score to the daily mean of the values for the neonatal screening series of that day, which we will refer to as "standardized T_4 scores." For children born at least 36 weeks of gestation in combination with a birth weight less than or equal to 2500 g, T_4 measurement is always combined with TSH measurement, and referral is based only on the TSH concentration to avoid unnecessary referral due to transient hypothyroxinemia of prematurity (THOP). Permission for retrieval of screening results for this specific study was granted by the national neonatal screening program committee, which is linked to the RIVM.

The NTR has been recruiting newborn twins in The Netherlands since 1989. Depending on birth cohort, between 25 and 40% of all multiple births in The Netherlands are registered by the NTR. The general aim of the NTR is to study behavioral and emotional development from birth onward, and parents and teachers are asked to provide information via surveys, started shortly after birth (17, 18).

Given that screening results were stored in a single national RIVM database only from 2006 onward, we selected twin pairs from the NTR database born between 2006 and 2011. In this 2006–2011 cohort, 4663 mothers had returned the first survey, which is collected after registration of the twins and includes information on maternal age at birth, gestational age, sex, birth weight, birth order, and zygosity. In this first survey mothers are asked for permission to link data to external databases in The Netherlands, and 4146 mothers (88.9%) gave this permission. If data were missing for gestational age (31 pairs), birth weight (93 pairs), or zygosity (147 pairs), or if infants were born abroad (22 pairs), pairs were not selected for linkage to screening results. The final NTR selection included 3853 twin pairs (7706 twins).

For 20% of the same-sex twins, classification of zygosity was based on items about physical similarity and frequency of confusion of the twins by parents and strangers, which are collected in a survey at the age of 3 years. A comparison with zygosity based on genomic polymorphisms revealed that this procedure correctly classifies zygosity in 93% of the cases (19). When this information was not available, zygosity was determined by a single item in a survey at the age of 2 years, indicating how much the children look alike (41%). This question correctly classifies zygosity in 92% of the cases (20). For the remaining part of the sample (39%), the zygosity was based on the maternal answer on the question whether the zygosity of the twin pair was MZ, DZ or unknown.

Statistical analyses

Means were estimated by SPSS version 21.0 (Statistical Packages for Social Sciences; SPSS Inc., Chicago, IL). To test effects of maternal age, gestational age, birth weight, and sex on standardized T_4 scores, we applied a linear mixed-effects model. The model included these factors plus time (day) of screening and birth order as predictors for the standardized T_4 scores. To correct for the dependence of the data from twin pairs, family was included as a random factor.

Genetic analysis

The relative contribution of genetic and environmental factors to the variance of the standardized T_4 scores can be inferred using data from genetically related subjects such as MZ and DZ twins. The logic of the twin method is to compare the resemblance for a certain trait in MZ twin pairs, who are genetically almost 100% identical, with resemblance in DZ pairs, who share on average 50% of their segregating genes. If the MZ resemblance, often expressed in correlations, is twice as large as the DZ resemblance, the trait is influenced by genetic factors, because the only difference between the two zygosity groups is in genetic relatedness. Shared environmental experiences, such as the intrauterine environment, will affect both MZ and DZ twins in the same way, and is a source for resemblance, which does not depend on zygosity. If a trait is influenced by shared environment, it is expected that the DZ correlation is the same or larger than half the MZ correlation. Experiences not shared by twin pairs, called unique environment, will cause twins within MZ and DZ pairs to differ from each other and will make them more dissimilar. Examples of circumstances referred to as unique environment are differential placental implantation site, differences in transplacental transmission of nutrients and other agents, and differences in presentation during birth. Measurement error is also a source of unique environment (21).

With structural equation modeling, maximum likelihood estimates of the influence of additive genetic factors (A), common environment (C) shared by twins, and nonshared environment (E) were obtained for 3 groups: full-term born twins (≥37 wk), preterm-born twins (≥32 to <37 wk), and very preterm-born twins (<32 wk). To test whether the genetic and environmental factors differ among the three groups, the absolute genetic and environmental influences were constrained to be equal across the full-term, preterm, and very preterm groups. A significant decrease in goodness of fit implies that there are significant differences in genetic and/or environmental influences across the full-term and (very) preterm-born twins and the constraint is not allowed. Models were compared by likelihood-ratio tests. The difference in likelihood follows a χ^2 distribution with −2 degrees of freedom (df) equal to the difference between the df for the full and the reduced model (22). The estimation of the twin correlations and genetic analyses were performed using Mx Software (Richmond, VA) (23). All analyses included sex and gestational age as a fixed covariate.

RESULTS

Linkage results

The record linkage was successful in 7665 of the 7706 children. Of this group, 28 children were excluded because of lacking or uncertain CH screening results, three pairs (n = 6) who were original triplets, and there were four families with two pairs of twins, for which we included only one pair (n = 8). This left 7623 children (98.9% of the original selection of 7706 children of the NTR cohort), consisting of 3793 complete pairs and 37 incomplete pairs. There were 653 monozygotic male pairs (MZM), 630 dizygotic male pairs (DZM), 611 monozygotic female pairs (MZF), 656 dizygotic female pairs (DZF), and 1280 dizygotic opposite sex (male–female) pairs. Data on thyroid status of the mother or on other health problems of mother and fetus were not available.

Descriptive statistics

The standardized T_4 scores of first- and second-born twins showed normal distributions with a mean standardized T_4 score lower than the population reference mean: –0.57 (SD = 1.10) (Figure 1). Table 1 shows standardized T_4 scores according to maternal age, gestational age, birth weight, and sex. The results of the linear mixed-effects model revealed significant effects of gestational age (F = 174.066; df = 2; *P* < .0001), birth weight (F = 70.423; df = 5; *P* < .0001), sex (F = 46.808; df = 1; *P* < .0001) and birth order (F = 27.291; df = 1; *P* < .0001). There was no effect of maternal age at birth (F < 1; df = 3; *P* = .544). Post hoc tests revealed that the standardized T4 scores were significantly lower in children with a gestational age below 37 weeks compared with children with a gestational age of 37 weeks or more. Children with a birth weight below 2500 g had lower standardized T_4 scores compared with children with birth weight of 2500 g or higher. In addition, boys had lower standardized T4 scores than girls, standardized T_4 scores of

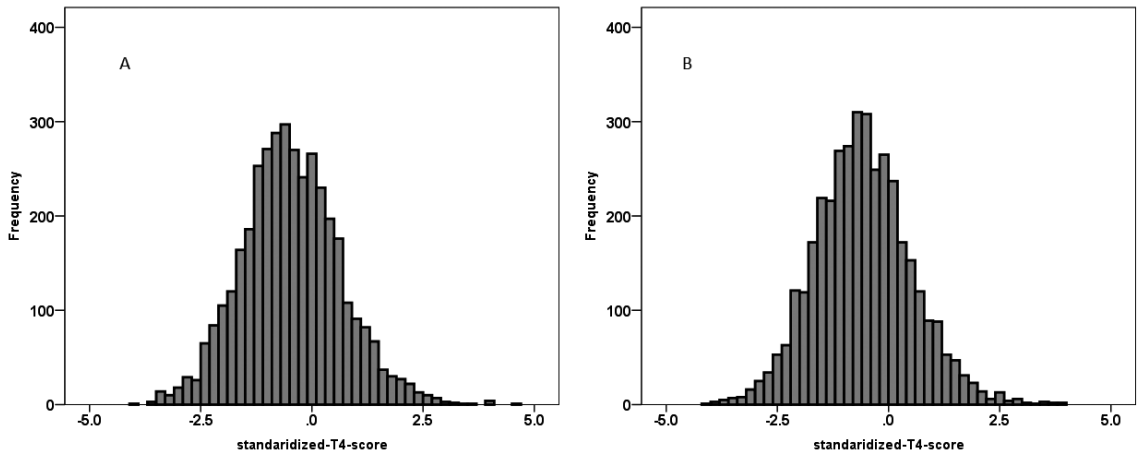

Figure 1. Distribution of standardized T_4 SD scores in (A) the firstborn twin (n = 3820; mean, -0.52; SD = 1.104) and (B) second-born twin (n = 3803; mean, -0.62; SD, 1.086).

firstborn twins were slightly higher than that of second-born twins. Information on screening date was available for 85% of the children. In this group 72% of the children were screened at the fourth day, 90.6% before the sixth day of life and only 1% after 1 week. A later screening day was associated with a lower standardized T_4 score ($F = 7.173$; $P < .007$). When including screening day in the model, the effects of other covariates remained the same.

Table 1. Means and standard deviations (SD) of standardized-T4-SD-scores across groups for maternal age, gestational age, birth weight, and sex for first and second born twins.

	First born			Second born		
	N	Mean	SD	N	Mean	SD
Maternal age, y						
<29.24	938	-0.59	1.08	938	-0.69	1.14
>=29.24 and age <32.04	924	-0.54	1.10	916	-0.68	1.07
>=32.04 and age <35.13	978	-0.51	1.14	972	-0.62	1.06
>=35.13	960	-0.44	1.10	957	-0.52	1.06
Gestational age, wk						
Full-term (≥ 37)	2111	-0.14	0.96	2106	-0.23	0.93
Preterm (>32 and <37)	1402	-0.76	1.01	1391	-0.90	1.00
Very preterm (≤ 32)	307	-2.04	0.83	306	-2.08	0.78
Birth weight, g						
<1500	190	-2.24	0.83	224	-2.15	0.85
1500-1999	458	-1.40	0.83	524	-1.41	0.84
2000-2499	1095	-0.55	1.00	1175	-0.63	0.98
2500-2999	1383	-0.20	0.95	1310	-0.28	0.94
3000-3499	618	-0.07	0.98	507	-0.08	0.93
>=3500	76	0.06	0.99	63	-0.01	0.91
Sex						
Boys	1939	-0.56	1.11	1888	-0.68	1.07
Girls	1881	-0.48	1.10	1915	-0.57	1.10

Twin correlations

The results were taken forward in the genetic analyses (ie, different mean scores for first and second-born twins, a fixed effect of sex and gestational age). Twin correlations were obtained separately among the three groups: full-term twins, preterm twins, and very preterm twins. The twin correlations were corrected for gestational age and are given in Table 2. The full-term group showed stronger correlation in MZ than in DZ twins. However, the DZ correlations are more than half the MZ correlations, indicating an additional influence of shared environmental factors. For the preterm and the very preterm twin pairs, the difference between MZ and DZ twin

Table 2. Gestational age-corrected twin correlations (r) and variance estimates for standardized-T4-scores in full-term, preterm and very preterm infants.

	N	r	T1 var	T2 var
Full-term (≥ 37 wk)				
MZM	308	0.689	0.9335	0.9334
DZM	378	0.479	0.9413	0.9108
MZF	278	0.532	0.8876	0.8771
DZF	395	0.371	0.8424	0.9420
DOS	757	0.409	0.8374	0.8040
Preterm (>32 to <37 wk)				
MZM	281	0.6605	0.8720	0.8269
DZM	207	0.424	0.7626	0.7838
MZF	265	0.5598	0.9381	0.9103
DZF	217	0.601	0.7270	0.7080
DOS	435	0.449	0.8520	0.7929
Very preterm (≤32 wk)				
MZM	64	0.548	0.4250	0.4812
DZM	45	0.405	0.3155	0.4618
MZF	68	0.545	0.5014	0.4021
DZF	44	0.506	0.4680	0.5212
DOS	88	0.431	0.4410	0.6227

Abbreviations: MZM, monozygotic males; DZM, dizygotic males; MZF, monozygotic females; DZF, dizygotic females; DOS, dizygotic opposite sex; T1var, gestational age-corrected variation for twin 1; T2var, gestational age-corrected variation for twin 2.

correlations is less substantial than that in the full-term group, indicating that the resemblance in twins could be attributed more to shared environmental factors and less to genetic factors.

With structural equation models, we estimated the effects of genetic and environmental influences for full-term and preterm groups separately. Constraining the influence of genetic and environmental factors to be equal across the three groups revealed significantly deteriorated fits compared with the full model (Table 3), indicating that the influences of genetic and environmental factors were different across these three groups. In a next series of analyses, the constraints of equal genetic and environmental variances across the three groups were performed separately for boys and girls (Table 3). These results showed that the group differences in genetic and environmental influences on standardized T_4 scores were significant for girls but not for boys.

Table 3. Results of model fitting for standardized-T4-scores in a multigroup analysis: ACE estimates were allowed to be different in the 3 groups of full-term, preterm and very preterm infants.

	Goodness of fit test		Model fit comparison			
Model	χ^2	**df**	**vs model**	$\Delta\chi^2$	**Δdf**	***p***
Full model ACE estimates were different in the 3 groups of term, preterm, and very preterm and were different in boys and girls	18962.907	7593				
Constraining A, C, or E to be equal in the 3 groups both for boys and girls						
As FM but A equal across groups	18977.181	7597	1	14.274	4	0.006
As FM but C equal across groups	18976.014	7597	1	13.108	4	0.011
As FM but E equal across groups	18980.002	7597	1	17.096	4	0.002
Constraining A, C, or E to be equal in the groups only in boys						
As FM but A equal across groups	18967.675	7595	1	4.769	2	0.092
As FM but C equal across groups	18963.823	7595	1	0.916	2	0.633
As FM but E equal across groups	18967.202	7595	1	4.295	2	0.117
Constraining A, C, or E to be equal in the 3 groups only in girls						
As FM but A equal across groups	18970.795	7595	1	7.889	2	0.019
As FM but C equal across groups	18974.697	7595	1	11.791	2	0.003
As FM but E equal across groups	18975.499	7595	1	12.593	2	0.002

Abbreviations: A, additive genetic factors; C, shared environmental factors; E, unique environmental factors.

In Table 4 heritability estimates (and their 95% confidence interval [CI]) for standardized T_4 scores are shown. In full-term (≥37 wk) boy twins, additive genetic factors explained 40% of the variance in standardized T_4 scores, whereas shared environmental factors explained 27% and unique environmental effects explained 33%. In full-term girl twins additive genetic factors explained 31% of the variance in standardized T_4 scores, shared environmental factors explained 22% and unique environmental effects explained 47%. In preterm (>32 to <37 wk) boy twins, additive genetic factors explained 34% of the variance, shared environmental factors 31%, and unique environmental factors 35%; whereas in preterm girl twins no effect of additive genetic factors was seen and only shared (57%) and unique environmental factors (43%) were involved. In the very preterm (≤32 wk) twins for both boys and girls, the contribution of genetic factors was not significant. Shared (23% and 49% for boys and girls, respectively) and unique environmental factors (45% and 39% for boys and girls, respectively) contributed to the variance of standardized T_4 scores.

Table 4. Estimates (and 95% CI) of genetic and environmental influences for standardized-T4-scores corrected for gestational age in the full-term, preterm, and very preterm infants.

Gestational Age		**a^2**	**c^2**	**e^2**
Full-term	boys	0.4015 (0.24-0.55)	0.2739 (0.15-0.41)	0.3246 (0.28-0.38)
	girls	0.3104 (0.11-0.47)	0.2149 (0.10-0.37)	0.4747 (0.41-0.56)
Preterm	boys	0.3403 (0.19-0.50)	0.3092 (0.19-0.44)	0.3504 (0.30-0.42)
	girls	0.0040 (0-0.10)	0.5719 (0.47-0.63)	0.4241 (0.37-0.48)
Very preterm	boys	0.3240 (0-0.60)	0.2295 (0.02-0.61)	0.4466 (0.32-0.64)
	girls	0.1219 (0-0.61)	0.4870 (0.05-0.69)	0.3911 (0.27-0.55)

Abbreviations: a^2, relative contribution of additive genetic factors; c^2, relative contribution of shared environmental factors; e^2, relative contribution of unique environment.

DISCUSSION

In this study neonatal screening blood T_4 concentrations of a large cohort of MZ and DZ twin pairs were compared and heritability was calculated. In full-term infants genetic factors were found to explain 31–40% of the variation in postnatal T_4 concentrations, and environmental factors (unique and shared) were responsible for 60–69% of the variation. So although genetics do influence postnatal T_4 concentrations, the environment seems to play an important role. Because blood T_4 concentrations were measured on average on the fifth day of life, the fetal

environment is the most likely candidate for the shared environmental influences on postnatal T_4 concentrations.

Several studies have attempted to estimate the contribution of genetic vs environmental influences on the individual T_4 set point, using either twin- or family-based study designs (3–7). The heritability estimates from these studies ranged from 32–65%, and our findings of 31–40% heritability fall within this range. The range of heritability's is rather large and may be explained by differences in study designs, sample sizes, sex, ages, and ethnicity (8). Although all previous studies were performed in adult samples, our heritability's in the subgroup of full-term infants were very comparable. For example, Panicker et al (3) reported a heritability of 39% for FT_4 in female adult twin pairs. This suggests that the contribution of genetic vs environmental influences in full-term infants remains stable throughout life. Genetic studies have revealed several candidate genes that may be involved in T_4 set point determination. However, the contribution of each of these genes to the variability in thyroid hormone concentrations was small (8, 24–26). This may mean that other genes play a role, but it is also possible that nongenomic mechanisms play an important role in T_4 set point determination. During embryonic development, driven by environmental forces, the epigenome undergoes modification, including DNA methylation and histone acetylation, leading to altered gene expression. This has been demonstrated for maternal diet and has been proposed to underlie the fetal origins of adult disease hypothesis (27–30). We hypothesize that the mechanism underlying T_4 set point determination also lies in epigenetic modifications taking place during the fetal period.

The large variation in gestational ages enabled us to study the effect of prematurity. In preterm infants, at least in girls, the estimated heritability was lower than in full-term infants. In fact, the heritability seemed to diminish with declining gestational age. In preterm infants born between 32 and 37 weeks' gestation, heritability decreased to 34% in boys and was not significant in girls. For very-preterm infants (≤32 wk gestation) variation in standardized T_4 scores was completely determined by environmental factors.

Preterm infants have lower umbilical cord T_4 concentrations than full-term infants, that correlate with gestational age and birth weight (31). Our results are in line with this, with the lowest standardized T_4 scores in the most preterm and lowest birth weight infants. Within the first 72 hours of life, full-term infants display a TSH surge that is accompanied by increases in plasma T_4 and T_3 concentrations. In preterm infants the postnatal TSH surge is less pronounced, and in very preterm infants it is often absent. In the latter group of infants the T_4 concentration may even decrease, which is referred to as THOP. Multiple factors are involved in THOP such as immaturity of the hypothalamic-pituitary-thyroid-axis, limited thyroid gland reserve, early loss of transplacental T_4 supply, and acute illness (31–33). Any illness on itself has a major influence on thyroid function, a phenomenon known as nonthyroidal illness syndrome (NTI, also known as euthyroid sick syndrome). NTI leads to decreased T_4 and T_3 concentrations and in premature infants NTI-like changes contribute to THOP (34). In this study for the preterm

infants, and especially for the very-preterm infants, immaturity and illness seem to have such a major influence on the thyroid hormone parameters that the genetic influence on the postnatal blood T_4 concentration seems to be "overruled." This may explain the apparent "decrease" of heritability with declining gestation age found in our study. The hypothalamic-pituitary-thyroid negative feedback axis matures with time. In full-term infants the axis is mature at birth but in very premature infants the maturation continues during the first 6 weeks of postnatal life (35). Given that we analyzed T_4 values measured within the first week of life, these values do not depict the actual T_4 set point, especially in the very preterm infants.

Other intriguing findings in this study were significantly lower standardized T_4 scores in boys compared with girls, and significantly lower standardized T_4 scores in second-born compared with first-born infants. The explanation for the sex difference may lie in the so-called male disadvantage hypothesis that was introduced in 1971 by Naeye et al (36) as an explanation for the increased perinatal morbidity that is observed in boys compared with girls. This poorer neonatal outcome in boys has been confirmed in various other studies (37–40). Perhaps the lower standardized T_4 scores found in boys suggest a more pronounced THOP due to more neonatal morbidity.

The lower standardized T_4 scores in second-born twins may be explained by the disadvantage brought to the second-born twin due to a prolonged twin-to-twin delivery time index. This is an independent risk factor for an adverse neonatal outcome of the second-born twin and is associated with lower umbilical arterial cord pH (41–43). The lower standardized T_4 scores in second twins may be the result of increased morbidity compared with firstborn twins.

Because we analyzed neonatal screening standardized T_4 scores (taken on average on the fifth day after birth), a limitation of our study is that results may be influenced by environmental factors occurring during the first days of life. Ideally, we should have analyzed plasma T_4 concentrations taken directly postpartum by umbilical cord sampling. Although the TSH surge observed in full-term infants during the first 72 hours after birth is usually on the decline at the time of sampling for the neonatal screening, the surge itself is driven by a major environmental factor (exposure to the cold extra uterine environment leading to stimulation of peripheral and central hypothalamic cold receptors). This could have led to an overestimation of the environmental influence for the full-term infants. Another limitation is that we used neonatal screening results, and hence could only analyze blood total T_4 concentrations and not FT_4 concentrations. Total T_4 concentrations are influenced by T_4 binding globulin concentrations and we were unable to correct for this.

In conclusion, in this study of neonatal T_4 screening results in a large Dutch twin cohort we found that environmental factors were the major factor influencing variability in postnatal blood T_4 concentrations. Given that we analyzed neonatal screening results, this emphasizes the importance of the fetal environment for the postnatal T_4 concentration. This suggests that the mechanism underlying T_4 set point determination may lie in epigenetic modifications taking

place in the fetal period. Heritability for T_4 set point diminished, at least for girls, with declining gestational age, most likely due to the major environmental influences of immaturity and illness in very preterm infants.

N.Z.-S. and C.E.M.v.B. contributed equally to the study.
D.I.B. and A.S.P.v.T. jointly directed this study.

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Early Thyroxine Treatment in Down syndrome and Thyroid Function Later in Life

6

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ABSTRACT

Objective

The hypothalamus–pituitary–thyroid (HPT) axis set point develops during the fetal period and first two years of life. We hypothesized that thyroxine treatment during these first two years, in the context of a randomized controlled trial (RCT) in children with Down syndrome, may have influenced the HPT axis set point and may also have influenced the development of Down syndrome-associated autoimmune thyroiditis.

Methods

We included 123 children with Down syndrome 8.7 years after the end of an RCT comparing thyroxine treatment vs placebo and performed thyroid function tests and thyroid ultrasound. We analyzed TSH and FT4 concentrations in the subgroup of 71 children who were currently not on thyroid medication and had no evidence of autoimmune thyroiditis.

Results

TSH concentrations did not differ, but FT4 was significantly higher in the thyroxine-treated group than that in the placebo group (14.1 vs 13.0 pmol/L; $P = 0.02$). There was an increase in anti-TPO positivity, from 1% at age 12 months to 6% at age 24 months and 25% at age 10.7 years with a greater percentage of children with anti-TPO positivity in the placebo group (32%) compared with the thyroxine-treated group (18.5%) ($P = 0.12$). Thyroid volume at age 10.7 years (mean: 3.4 mL; range: 0.5–7.5 mL) was significantly lower ($P < 0.01$) compared with reference values (5.5 mL; range: 3–9 mL) and was similar in the thyroxine and placebo group.

Conclusion

Thyroxine treatment during the first two years of life led to a mild increase in FT4 almost 9 years later on and may point to an interesting new mechanism influencing the maturing HPT axis set point. Furthermore, there was a trend toward less development of thyroid autoimmunity in the thyroxine treatment group, suggesting a protective effect of the early thyroxine treatment. Lastly, thyroid volume was low possibly reflecting Down-specific thyroid hypoplasia.

INTRODUCTION

Down syndrome is characterized by an extra copy of chromosome 21 and is associated with various congenital malformations such as heart defects and digestive system malformations. Thyroid dysfunction is also frequent in Down syndrome, including congenital primary (or thyroidal) hypothyroidism and acquired autoimmune thyroid dysfunction (1). Besides clinically evident forms of hypothyroidism, subclinical hypothyroidism with only very mild thyrotropin (TSH) elevation is also frequently encountered in Down syndrome (1, 2, 3). As Down syndrome neonates not only have mildly elevated TSH levels but also lower T4 concentrations compared with non-Down syndrome neonates, it has been suggested that there may be an altered hypothalamic–pituitary–thyroid (HPT) axis set point in Down syndrome or it may indicate a mild form of Down syndrome-specific congenital hypothyroidism of thyroidal origin (1, 2, 3).

Based on the assumption that young children with Down syndrome as a group have a mild form of congenital thyroidal hypothyroidism, it was hypothesized that thyroxine treatment during the first two years of life could improve the psychomotor development in children with Down syndrome. Between 1999 and 2003, a randomized clinical trial (RCT) was conducted to study the effects of thyroxine treatment vs placebo during the first two years of life on psychomotor development in a cohort of children with Down syndrome (4). In this RCT, thyroxine treatment resulted in somewhat better motor development and growth compared with placebo treatment at the age of 2 years (4). As the primary outcome of this study was early mental and motor development, treatment was discontinued at the age of two years. In the Netherlands, publication of the trial results in 2005 led to a debate on whether thyroxine treatment should be started in all neonates with Down syndrome. With only modest improvements in motor development (0.7 months reduction in delay) and growth (0.9 cm height gain), but no clear positive effect on mental development, the benefits of early started treatment were considered not to outweigh the burden of daily treatment (4).

In a follow-up study performed at the age of 10.7 years, no developmental improvement was found. In that same follow-up study, we had the opportunity to study the effect of thyroxine vs. placebo treatment during the first two years of life on the HPT axis and the development of thyroid autoimmunity later on in life (5).

The HPT axis set point develops during the fetal period followed by slowly decreasing TSH and minimal change in FT4 concentrations in the first two years of life. After that period, the FT4 set point remains stable into adulthood (6, 7, 8). As there is evidence suggesting that environmental factors may be even more important than genetic factors in determining HPT axis set point (9, 10), we hypothesized that thyroxine treatment in the first two years of life, resulting in a clearly higher plasma FT4 concentration in the period that the HPT axis is not completely mature, may have caused an HPT axis set point change that persists later on in life.

Autoimmune thyroid disease is reported to be common in Down syndrome after the age of eight years, with a gradually increasing concentration of thyroid autoantibodies (11, 12). As

early thyroxine treatment in autoimmune thyroiditis has been reported to delay the disease progression, we studied the development of thyroid autoimmunity in this cohort of very early treated children with Down syndrome (13, 14).

Finally, the follow-up study enabled us to measure thyroid gland volumes in a substantial number of children with DS within a narrow age range.

SUBJECT AND METHODS

In this follow-up study in children with Down syndrome, thyroid hormone parameters including anti-TPO antibodies were analyzed at a mean age of 10.7 years, 8.7 years after the end of a single-center RCT in which the effects of thyroxine vs placebo administration between the neonatal period and the age of two years were compared (4, 5). The initial study was conducted between June 1999 and October 2003. Down syndrome neonates were randomized to receive either thyroxine or placebo, and treatment was continued until the age of 24 months. Thyroxine doses were adjusted to reach and maintain normal plasma TSH (0.4–4.0 IU/L) and high-normal plasma-free T4 (FT4) concentrations (18–24 pmol/L). The daily thyroxine dose decreased from 8 µg/kg at randomization to a little more than 4 µg/kg at age 9 months.

The institutional Medical Ethics Committee approved both the initial and follow-up study. All children who completed the original trial were eligible for inclusion in the follow-up study (Fig. 1). Results on psychomotor development and growth were published previously (5). The purpose of this part of the study was to analyze the effect of thyroxine vs placebo on the thyroid hormone parameters 8.7 years after the end of the trial. Plasma TSH and free thyroxine (FT4) concentration were measured in venous blood two months and 8.7 years after cessation of the trial medication. Anti-TPO antibodies were measured at ages 12 months, 24 months and 10.7 years. Ultrasound of the thyroid gland, to examine the presence of signs of thyroiditis, was performed at the age of 10.7 years.

Measurements

Thyroid function parameters

Plasma TSH was measured with an electrochemiluminescence immunoassay (E170, Roche Diagnostics). The detection limit was 0.01 U/L, and the total assay variation was less than 5%. The TSH reference interval was 0.5–5.0 U/L. FT4 was measured by time-resolved fluoroimmuno-assay (PerkinElmer). The detection limit was 2.0 pmol/L, and the total assay variation was 5–8%. The FT4 reference interval was 10.0–23.0 pmol/L. Anti-TPO was measured by chemiluminescence immunoassay (LUMI-test anti-TPO, Thermo Fisher Scientific). The detection limit was 30 kU/L and the total assay variation was 8–12%. An anti-TPO concentration of ≥60 kU/L was rated as a positive test result.

Ultrasonography

Two experienced pediatric radiologists who were blind to the treatment mode during the trial, performed the thyroid ultrasonography using a Philips iU22 in combination with an L17-5 broadband linear array transducer (Philips Medical Systems). Besides thyroid volume (length × width × depth × 0.52 for both thyroid lobes, where the volume of the isthmus was not taken into account), echogenicity, ultrasonographic texture (homogeneous and heterogeneous), irregularities (nodules and cysts) and vascularization were assessed. Autoimmune thyroiditis was defined as an anti-TPO concentration ≥60 kU/L or ultrasonographic signs of thyroiditis. Thyroid volume was compared to published reference values for Dutch children of the same age (15).

Statistical analysis

Results were compared using chi-square test, Fisher's exact test, the independent samples t-test + one-sample t-test (group statistics, thyroid hormone concentrations and thyroid volume) and binomial test (thyroid volume data compared with Dutch reference data). Statistical analyses were carried out with IBM SPSS Statistics 23.0.0.0. P values <0.05 were considered statistically significant.

RESULTS

Participants

One hundred and eighty-one children completed the original RCT of whom 123 participated in the follow-up study (Fig. 1). Analyses to trace possible selection bias revealed that in the thyroxine group, the children participating in the follow-up study showed a smaller delay in mental development at 24 months than the total thyroxine group that completed the original RCT (difference: 0.6 months, 95% confidence interval (CI): −1.2 to 0.0, P = 0.04). The baseline characteristics of the 123 children in the follow-up study are given in Table 1 and were similar in the thyroxine and placebo groups. There were no significant differences in comorbidity, including cardiac, pulmonary or neurological disease (5). During the RCT, daily thyroxine doses were adjusted at regular intervals to maintain plasma TSH in the reference interval and FT4 concentrations in the higher end of the reference interval. In the follow-up study, measurements were not available in all patients, mainly due to lack to cooperate with blood withdrawal. TSH and FT4 concentrations were available in 113 children (91%; 59 in the thyroxine group (29 males/30 females) and 54 children in the placebo group (28 males/26 females)).

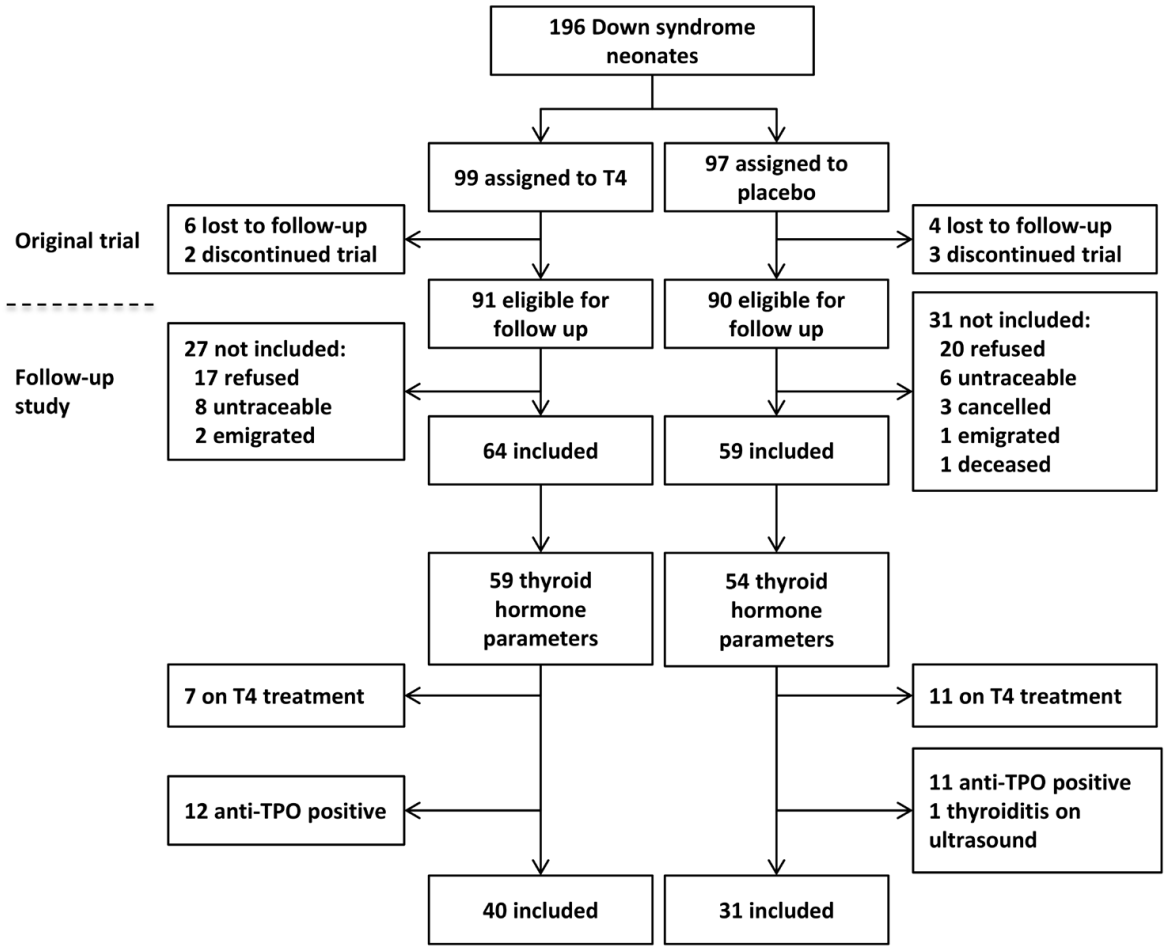

Figure 1. Flow diagram representing the original trial and the follow-up study at the age of 10.7 years

Thyroid hormone parameters

TSH and FT4 concentrations were analyzed in the subgroup of children currently not on thyroid medication and without evidence of autoimmune thyroiditis. This group consisted of 71 children; 40 in the thyroxine group (17 males/23 females) and 31 in the placebo group (14 males/17 females) (Fig. 1).

At original trial entry, no significant differences were seen in either TSH or FT4. As expected, during the trial, at ages 6, 12 and 24 months, FT4 were significantly higher and TSH concentrations significantly lower in the thyroxine-treated group compared with that in the placebo group (Table 2). Two months after cessation of trial medication, at age 26 months, TSH was significantly higher in the thyroxine-treated group (6.9 U/L) compared with that in the placebo group (5.0 U/L) ($P = 0.002$), whereas FT4 concentrations did not differ ($P = 0.40$). At the follow-up age of 10.7 years, TSH concentrations did not differ ($P = 0.83$), but FT4 was

Table 1. Baseline characteristics of the thyroxine and placebo groups in the follow-up study, given for the total follow-up group (n=123) and the subgroup without evidence of auto-immune thyroiditis, currently not treated with thyroxine (n=71).

	Total follow-up group		Subgroup without treatment/autoimmunity	
Characteristics	**Thyroxine group** N=64	**Placebo group** N=59	**Thyroxine group** N=40	**Placebo group** N=31
Mean age at follow-up, years (SD)	10.7 (0.04)	10.7 (0.08)	10.7 (0.04)	10.7 (0.07)
Male/female, N	33/31	31/28	17/23	14/17
Gestational age at birth, weeks (SD)	38.3 (1.4)	38.6 (1.3)	38.3 (1.4)	38.5 (1.3)
Mean birth weight, grams (SD)	3058 (535)	3073 (531)	3127 (559)	3085 (490)
Age at trial entry, days (SD)	24.3 (3.3)	24.2 (3.3)	24.1 (2.7)	23.5 (3.4)
TSH at trial entry, mU/L (SD)	6.8 (3.3)	6.0 (2.7)	7.1 (3.7)	6.2 (2.6)
FT_4 at trial entry, pmol/L (SD)	18.6 (3.4)	18.5 (2.8)	18.5 (2.7)	18.7 (2.4)
Hypothyroidism at follow-up, N (%)	7 (11.1)	10 (17.2)	-	-
Hyperthyroidism at follow-up, N (%)	0 (0.0)	1 (1.7)	-	-
Celiac disease	3	2	0	1
Signs of puberty, male/female	15/9	15/8	9/7	5/5
Mental Age Equivalent, mean (SD)	51.6 (10.7)	50.9 (13.6)	53.6 (9.7)	52.3 (11.9)

significantly higher in the thyroxine-treated group compared with that in the placebo group (14.1 and 13.0 pmol/L respectively, P = 0.02) (Table 2, Fig. 2). Log-linear regression analysis of TSH and FT4 concentrations was not possible due to the small sample size.

TSH and FT4 concentrations in the subgroups of thyroxine- and placebo-treated children (N = 40 and 31 respectively) were also compared with TSH and FT4 in the total groups of thyroxine- and placebo-treated children during the trial (N = 99 and 97 respectively; ages 6, 12 and 24 months), and no statistically significant differences were found (differences in FT4 between subgroup of 40 thyroxine-treated patients compared with total group of 99 thyroxine-treated patients in original trial: trial age 6 months: mean difference: −0.12 pmol/L (95% CI: −1.71 to 1.47), P = 0.88; trial age 12 months: mean difference: 0.043 pmol/L (95% CI: –1.47 to 1.55), P = 0.96; trial age 24 months: mean difference: –0.25 pmol/L (95% CI: –1.48 to 0.98), P = 0.68; differences in FT4 between subgroup of 31 placebo treated patients compared with the total

Table 2. Plasma TSH and FT4 concentrations in the subgroup of patients without evidence of auto-immune thyroiditis, currently not treated with thyroxine. Data are given in mean (SD), TSH in mU/L, FT4 in pmol/L (to convert FT4 to ng/dl divide by 12.87).

		Thyroxine group N=40	**Placebogroup** N=31	**Mean difference**	**P-value**
Trial entry age 0.8 mo	TSH	7.1 (3.7)	6.2 (2.6)	0.9	0.28
	FT4	18.5 (2.7)	18.7 (2.4)	-0.2	0.78
During trial age 6 mo	TSH	1.3 (1.5)	5.2 (2.7)	-3.9	**<0.001**
	FT4	21.9 (5.0)	14.5 (1.9)	7.4	**<0.001**
During trial age 12 mo	TSH	1.4 (1.4)	4.9 (2.5)	-3.5	**<0.001**
	FT4	20.8 (4.7)	14.1 (2.1)	6.7	**<0.001**
During trial age 24 mo	TSH	1.6 (1.8)	5.2 (1.9)	-3.6	**<0.001**
	FT4	20.4 (3.8)	13.9 (1.8)	6.5	**<0.001**
After trial age 26 mo	TSH	6.9 (3.3)	5.0 (1.8)	1.9	**0.002**
	FT4	14.5 (3.0)	14.0 (1.9)	0.5	0.40
After trial age 10.7 yrs	TSH	3.8 (2.5)	3.9 (1.8)	-0.1	0.83
	FT4	14.1 (2.6)	13.0 (1.6)	1.1	**0.02**

group of 97 placebo treated patients in the original trial: trial age 6 months: mean difference: −0.07 pmol/L (95% CI: −0.77 to 0.63), P = 0.84; trial age 12 months: mean difference: −0.25 pmol/L (95% CI: −1.03 to 0.54), P = 0.53; trial age 24 months: mean difference: −0.18 pmol/L (95% CI: −0.86 to 0.49), P = 0.58).

Thyroid autoimmunity

Anti-TPO was measured in 104 children. The proportion of children with positive anti-TPO at the age of 12 months, 24 months and 10.7 years were 1, 6 and 25% respectively. Ultrasound of the thyroid gland was performed in 120 children. Clear signs of thyroiditis were present in seven children (four in the thyroxine group and three in the placebo group). Except for one child, all these children were positive for anti-TPO. Based on these findings, 8.7 years after the end of the RCT, 26 children could be classified as having thyroid autoimmunity. This included ten out of 54 (18.5%) in the thyroxine group and 16 out of 50 (32%) in the placebo group (Fig. 3). Ten of them were on thyroid medication (4 in the thyroxine group, 6 in the placebo group). Treatment had been started by the patients' own pediatrician and indications for treatment were overt hypothyroidism in nine children and hyperthyroidism in one child (in the placebo group; block

and replace treatment with carbimazole and thyroxine). No difference was found in prevalence of anti-TPO between boys and girls.

Thyroid volume

For the total group of 120 children (61 thyroxine group/ 59 placebo group, three missing data) thyroid volume assessed by ultrasonography was 3.56 mL (s.d. 1.48) in the thyroxine group and a similar 3.64 mL (s.d. 1.63) in the placebo group (P = 0.77). Also for the subgroup of children without evidence of autoimmune thyroiditis, thyroid volume was similar; thyroxine group (n = 38) 3.46 mL (s.d.1.14) and placebo group (n = 31) 3.42 mL (s.d. 1.29) (P = 0.88). No difference was found in thyroid volume between boys and girls.

When comparing thyroid volume of the subgroup without evidence of autoimmune thyroiditis (n = 69) to the Dutch reference range at age 11 years (median: 5.5, range: 3–9 mL), the mean volume of 3.4 mL (s.d. 1.2; range: 0.5–7.5 mL) was significantly lower (P < 0.01) with a thyroid volume below 3 mL in 38 per cent of cases and below 5.5 mL in 97 per cent of cases (P < 0.01) (15).

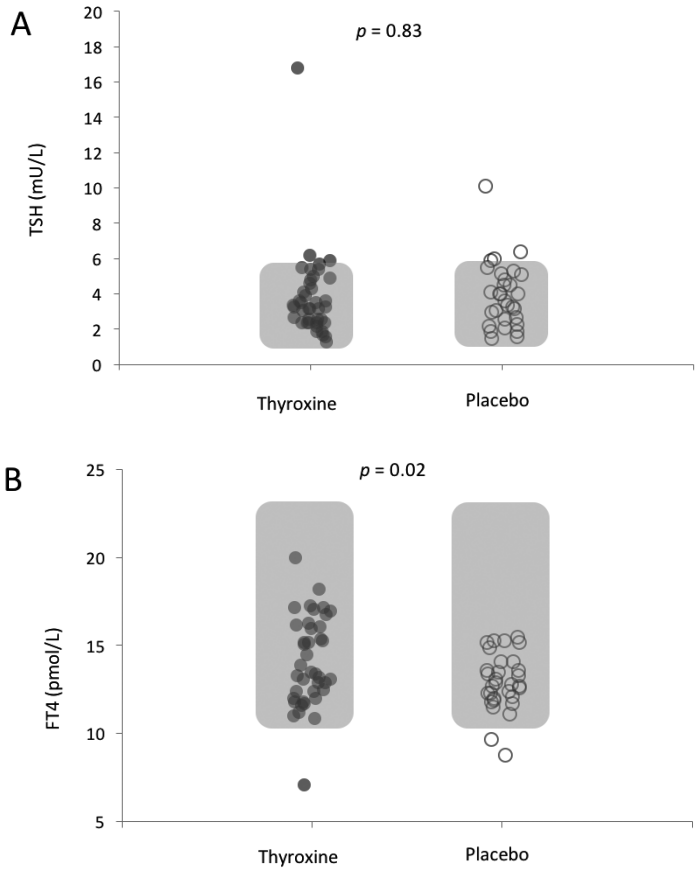

Figure 2. Scatter plot for (A) plasma TSH and (B) FT4 concentrations for the thyroxine (41) and placebo (31) groups within the subgroup of 71 patients without evidence of auto-immune thyroiditis, currently not treated with thyroxine, at age 10.7 years. (reference range in grey: TSH 0.5-5.0 mU/l, FT4 10-23 pmol/l).

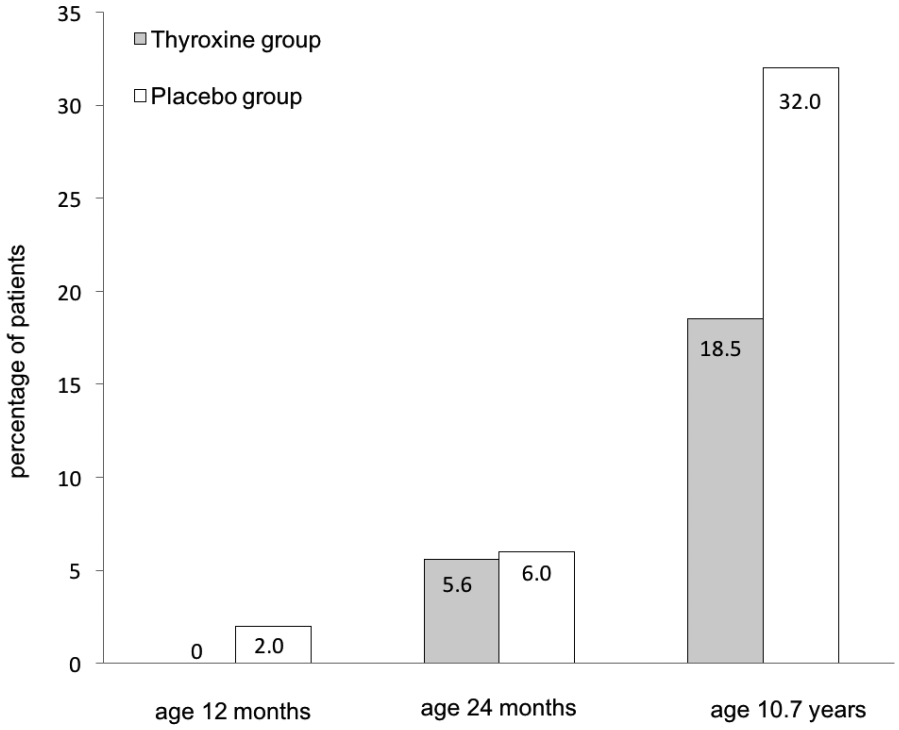

Figure 3. Percentage of patients positive for anti-TPO in the thyroxine (n=54) and placebo groups (n=50). *P*-values: age 12 months *P*=0.48, age 24 months *P*=1.00, age 10.7 years *P*=0.12

DISCUSSION

In this follow-up study of an RCT in which children with Down syndrome were treated with thyroxine or placebo between the neonatal period and the age of two years, we found a slightly, but statistically significant higher FT4 concentration (mean difference 1.14 pmol/L, P = 0.02) at the age of 10.7 years in the thyroxine group. This slightly higher FT4 concentration in combination with a similar TSH concentration suggests a change in the HPT axis set point induced by the thyroxine treatment during the first two years of life. In addition, there was an increasing incidence of thyroid autoimmunity, measured by the presence of anti-TPO, from 1% at age 12 months to 6% at age 24 months and 25% at age 10.7 years, and there was a trend toward a greater percentage of children with anti-TPO positivity in the placebo group (32%) compared with the thyroxine-treated group (18.5%) although not statistically significant (P = 0.12 at age 10.7 years). Finally, thyroid volume at age 10.7 years (mean: 3.4 mL; range: 0.5–7.5 mL) was significantly lower (P < 0.01) compared with reference values (5.5 mL; range: 3–9 mL) and was similar in the thyroxine and placebo group.

In healthy individuals, plasma FT4 and TSH concentrations show inter-individual differences leading to wide reference intervals. The intra-individual variability in plasma FT4 and TSH, however, is much smaller, suggesting that each individual has his or her own specific hypothalamus–pituitary–thyroid (HPT) axis set point that seems to remain stable (16, 17).

The HPT axis set point develops during the fetal period followed by slowly decreasing TSH and minimal change in FT4 concentrations in the first 2 years of life (6, 7, 8). During the fetal period, the HPT axis seems susceptible to permanent changes driven by environmental factors. An example of the fetal environment influencing the postnatal (F)T4 set point is the biochemical phenotype of infants born to inadequately treated mothers with Graves' disease. In these infants, Kempers et al. described normal TSH concentrations accompanied by low FT4 concentrations (18). Another example is severe primary congenital hypothyroidism (CH), e.g. thyroid agenesis. When treating patients with this condition with thyroxine, achieving a normal TSH concentration is accompanied by FT4 concentrations above the age-specific reference range interval, suggesting an altered HPT axis set point possibly caused by a hypothyroid intra-uterine environment (19, 20). In this study, early intervention with thyroxine treatment after the fetal period seems to have led to an altered TSH-FT4 set point. During the trial, TSH concentrations were significantly lower and FT4 significantly higher (~7 pmol/L) in the thyroxine treatment group ($P < 0.001$) (Table 2). Two months after treatment, TSH was significantly higher ($P = 0.002$) in the thyroxine-treated group, probably due to a recovery of the HPT axis after a long period of thyroxine supplementation.

In general, the relationship between TSH and FT4 is presumed to be log-linear (21). Although it has been suggested that the TSH-FT4 relationship may be more complex and nonlinear (22). Due to the small sample size, log-linear regression analysis was not possible in our study. Physiologically, transient set point changes occur during fasting and as a result of diurnal variation, whereas a permanent set point shift takes place with aging characterized by relatively higher TSH concentrations. Certain non-physiological circumstances may also cause short-term alterations in TSH/FT4 set point, including the effects of acute inflammation on the HPT axis leading to decreased FT4 levels accompanied by normal TSH levels (23, 24, 25). These changes, however, are temporary and do not persist when circumstances are reversed. In our study, the set point change is observed many years after the discontinuation of thyroxine treatment. The mechanism responsible for this persistent set point change may lie in epigenetic modifications of genes involved in TSH/FT4 set point development induced by the thyroxine treatment. In a twin study of neonatal screening T4 results, we were able to emphasize the importance of the fetal environment in neonatal T4 set point determination and hypothesized that the mechanism underlying T4 set point determination lies in epigenetic modifications during the fetal period influencing the maturing HPT axis (10). Perhaps thyroxine treatment during the first two years of life, in a period in which the HPT axis is not yet completely mature, may also lead to epigenetic modifications inducing a set point change that persists later on in life.

We do acknowledge that in both groups FT4 concentrations were within the normal range and that the observed difference in FT4 between the thyroxine and placebo treated groups is small, based on a single measurement, and probably, without clinical consequences. However, the FT4 difference did reach statistical significance and may point to an interesting new mechanism influencing the maturing HPT axis set point.

At age 10.7 years, 25% of the children with Down syndrome in this study were anti-TPO positive. This is in line with previous findings in children with Down syndrome and emphasizes the much higher prevalence of thyroid autoimmunity in Down syndrome compared with non-Down syndrome children (11, 12). In comparison, in a study of 440 healthy school children with a similar mean age to our study group, anti-TPO antibodies were found in only 3.2% for boys and 5.8% for girls (26). In our study, anti-TPO positivity increased with age with a prevalence of 1% at age 12 months, 6% at age 24 months and 25% at age 10.7 years. In a ten-year longitudinal study of thyroid function in children with Down's syndrome, a similar increase in anti-thyroglobulin antibodies (3% at age 1 year, 25% at age 10 years) and anti-TPO (5% at age 1 year, 37% at age 10 years) was observed (27). Combined, this suggests an increase in anti-TPO positivity of approximately 2.2–3.6% per year. Although not statistically significant ($P = 0.12$), there was a trend toward a greater percentage of children with thyroid autoimmunity in the placebo group (32%) compared with the thyroxine-treated group (18.5%) in our study and may indicate an effect of thyroxine therapy on the development of autoimmune thyroiditis. A possible protective effect of levothyroxine treatment in anti-TPO positive (non-Down syndrome) patients has been described in previous studies (13, 14). The mechanism might be decreased presentation of thyroidal autoantigens due to decreased TSH levels during levothyroxine treatment.

Thyroid volume was similar in the thyroxine-treated and placebo-treated groups, also for the subgroup of children without evidence of autoimmune thyroiditis. However, the observed thyroid volume of approximately 3.4 mL was significantly lower than the reference value of 5.5 mL (range: 3–9 mL) for Dutch children at age 11 years ($P < 0.01$) (15). We found several reports on thyroid ultrasonography findings in autoimmune thyroiditis in Down syndrome, but hardly any studies on thyroid volume in the absence of autoimmunity. Cebeci et al. reported a high percentage of thyroid hypoplasia in children with Down syndrome, but these children were all on thyroid medication because of congenital or acquired hypothyroidism (28). Lughetti et al. reported on thyroid ultrasonography results at age 10 years in Down syndrome (90% was classified as normal and 10% as hypoplasia) but did not mention the actual measurements (27). Our finding of overall reduced thyroid volume in children with Down syndrome also in the subgroup without thyroid autoimmunity supports the hypothesis that the observed TSH elevation in Down syndrome may be due to a subtle form of congenital hypothyroidism associated with mild thyroid hypoplasia. Very recently thyroid dysgenesis was described in a Down syndrome murine model (transgenic Dyrk1A mice) although thyroid volume in these mice was increased (29).

A limitation of this study is that we measured FT4 and TSH at only one point of time later in life. Another limitation is that results from children with Down syndrome cannot be simply extrapolated to the general population. A third limitation is that there may be a selection bias as the thyroxine treated children participating in the follow-up study had a smaller delay in the

development than the total thyroxine group from the original RCT. Further analysis showed that this could not be explained by difference in FT4 concentrations during the T4 treatment in the trial. Important strengths of this study are that the study groups were initially enrolled in an RCT resulting in groups with similar baseline characteristics and that all children were studied within a very small age range. Other strengths are the longitudinal character and the long period of follow-up.

In conclusion, in this study in children with Down syndrome, thyroxine treatment during the first two years of life seems to have resulted in a small change in the FT4 set point measurable almost 9 years later. We hypothesize that thyroxine treatment, in the period that the HPT is not yet completely mature, may have induced epigenetic modifications leading to an FT4 set point change that persists later on in life. Furthermore, there was a trend toward less development of thyroid autoimmunity in the thyroxine treatment group vs the placebo group, suggesting a protective effect of the early thyroxine treatment. Lastly, thyroid volume was lower compared to reference values suggesting Down-specific thyroid hypoplasia.

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IV

Genotyping pituitary stalk interruption syndrome

Clues for Polygenic Inheritance of Pituitary Stalk Interruption Syndrome from Exome Sequencing in 20 Patients

7

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ABSTRACT

Context

Pituitary stalk interruption syndrome (PSIS) consists of a small/absent anterior pituitary lobe, an interrupted/absent pituitary stalk, and an ectopic posterior pituitary lobe. Mendelian forms of PSIS are detected infrequently (<5%), and a polygenic etiology has been suggested. *GLI2* variants have been reported at a relatively high frequency in PSIS.

Objective

To provide further evidence for a non-Mendelian, polygenic etiology of PSIS.

Methods

Exome sequencing (trio approach) in 20 patients with isolated PSIS. In addition to searching for (potentially) pathogenic de novo and biallelic variants, a targeted search was performed in a panel of genes associated with midline brain development (223 genes). For *GLI2* variants, both (potentially) pathogenic and relatively rare variants (<5% in the general population) were studied. The frequency of *GLI2* variants was compared with that of a reference population.

Results

We found four additional candidate genes for isolated PSIS (*DCHS1*, *ROBO2*, *CCDC88C*, and *KIF14*) and one for syndromic PSIS (*KAT6A*). Eleven *GLI2* variants were present in six patients. A higher frequency of a combination of two *GLI2* variants (M1352V + D1520N) was found in the study group compared with a reference population (10% vs 0.68%). (Potentially) pathogenic variants were identified in genes associated with midline brain anomalies, including holoprosencephaly, hypogonadotropic hypogonadism, and absent corpus callosum and in genes involved in ciliopathies.

Conclusion

Combinations of variants in genes associated with midline brain anomalies are frequently present in PSIS and sustain the hypothesis of a polygenic cause of PSIS.

INTRODUCTION

Pituitary stalk interruption syndrome (PSIS) is a congenital anomaly of the pituitary gland consisting of a classic triad of magnetic resonance imaging (MRI) findings: (1) small or absent anterior pituitary lobe, (2) interrupted or absent pituitary stalk, and (3) ectopic posterior pituitary lobe. PSIS may be isolated or may occur in association with other midline brain malformations (syndromic PSIS) (1, 2).

The clinical consequences of PSIS vary from isolated growth hormone (GH) deficiency to multiple pituitary hormone deficiencies (MPHD) in which two or more anterior pituitary lobe functions [production of GH, thyrotropin, corticotropin, and gonadotropins (luteinizing hormone + follicle-stimulating hormone)] may be insufficient. Prolactin level is usually elevated because of lack of hypothalamic dopaminergic inhibition. In isolated PSIS, typically only anterior pituitary hormone deficiencies occur, whereas in syndromic PSIS, posterior pituitary hormone deficiency (antidiuretic hormone) also may be present, with central diabetes insipidus as a clinical consequence (1, 2).

The pituitary lobes and stalk have different embryological origins. The anterior lobe derives from the oral ectoderm, whereas the posterior lobe and pituitary stalk derive from the neural ectoderm. Various transcription factors are involved in the initial formation of the pituitary gland and the following cellular differentiation (3). Important signaling pathways involved in pituitary development are Wnt, Notch, Sonic Hedgehog (Shh), bone morphogenetic proteins (BMPs), and fibroblast growth factor (FGF). Mutations in genes encoding transcription factors in these pathways are found in <5% of PSIS cases and most often in association with other extrapituitary anomalies (4–8).

In a recent review, Fang et al. (9) reported 30 candidate genes for PSIS and MPHD. Various genetic studies showed an association between PSIS and genes involved in holoprosencephaly, which is characterized by severely disturbed midline brain development, suggesting that PSIS may represent a mild form of this disorder (7). In particular, *GLI2* variants, which are associated with holoprosencephaly, have been reported at a relatively high frequency in isolated PSIS (10–14). Furthermore, genes involved in hypogonadotropic hypogonadism (*PROKR2*, *FGF8*, *FGFR1*) have been associated with MPHD (6, 15–17). However, clinical expression is variable, including unaffected mutation-carrying relatives, suggesting involvement of additional genetic or environmental factors.

Most studies searching for genetic causes of MPHD have been done in heterogeneous patient populations, including patients with other midline brain malformations, and most studies have focused on pathogenic mutations in specific genes, assuming a Mendelian inheritance (5). Because Mendelian forms of PSIS are detected only rarely, a polygenic and multifactorial etiology for PSIS should be considered as well (9). A polygenic inheritance has also been suggested for holoprosencephaly (18). In a very recent study using exome sequencing in 24 Chinese patients with isolated PSIS, heterozygous mutations, mostly in genes associated with Notch,

Shh, and Wnt signaling pathways, were identified in 22 patients. Most patients (20 of 24) had more than one mutation, suggesting polygenic involvement. No mutations were found in genes known to be associated with pituitary malformation. Because parents were not included in this Chinese study, the authors were unable to determine whether mutations were de novo (19).

To provide further evidence for a non-Mendelian, polygenic etiology of PSIS, we performed whole exome sequencing in 20 patients with isolated PSIS and their unaffected parents. In addition to searching for (potentially) pathogenic de novo and biallelic variants, we performed a targeted search in a large panel of genes associated with midline brain development. We included not only genes with a known association with pituitary formation (37 genes) but also genes associated with other midline brain malformations: holoprosencephaly (18 genes), hypogonadotropic hypogonadism (34 genes), and absent corpus callosum (134 genes). For each patient, all pathogenic variants and variants of unknown clinical significance (potentially pathogenic) were listed. Because rare *GLI2* variants have been frequently reported in PSIS, we searched for (potentially) pathogenic *GLI2* and for all relatively rare *GLI2* variants (reported frequency <5% in the general population). Although rare *GLI2* variants have been reported in association with PSIS, the frequency of (combinations of) these rare variants in the general population has not been investigated. To verify whether these rare *GLI2* variants are indeed more common in PSIS, we compared the frequency of these variants in our study group with the frequency in a reference population.

PATIENTS AND METHODS

Patients

A database containing patients with congenital central hypothyroidism, located at the Department of Pediatric Endocrinology of the Academic Medical Center, Amsterdam, was searched for all patients with isolated PSIS. Most patients had been diagnosed after an abnormal result in the Dutch thyroxine-based neonatal screening program for congenital hypothyroidism (20). We selected 20 patients who had two or more pituitary hormone deficiencies and were still being treated at the Academic Medical Center. The diagnosis of PSIS was based on the three classic MRI findings. Patients with other (midline) brain abnormalities or with dysmorphic features or other pathologies suggestive of syndromic PSIS were excluded. The study was approved by the local medical ethics committee (NL47088.018.13). After informed consent was obtained, a venous blood sample was collected from all patients and their unaffected parents.

Molecular studies

Whole exome sequencing and variant calling were performed by the Beijing Genomic Institute. The capture used to enrich for exome sequences was the Agilent SureSelect Human All Exon V5 (50M) kit, and sequencing was done on either an Illumina or Complete Genomics platform.

Variant prioritization

Variant prioritization was done using the Cartagenia Bench Laboratory NGS (Agilent). Variants were considered potentially pathogenic when either resulting in truncation of the protein, predicted to result in a possibly/probably pathogenic variant by Polyphen2, or predicted to affect splicing and present at a frequency of <1% in the general population [based on the dbSNP database (dbSNP build 141 GRCh37.p13), ESP6500 (http://evs.gs.washington.edu/EVS/), 1000 Genomes Project (1000 Genomes phase 3 release, version 5.20130502), GoNL (http://www.nlgenome.nl/), and >900 in-house reference samples]. The validity of variants was judged by visual inspection of read data in Integrative Genomics Viewer or was confirmed by Sanger sequencing. A target gene panel for MPHD, holoprosencephaly, hypogonadotropic hypogonadism, and absent corpus callosum was made on the basis of results of a literature search (Table 1) (3, 8, 9, 18, 21, 22).

The exome data set was analyzed stepwise: First, we searched for potentially pathogenic variants in 37 genes known to be associated with pituitary development or function. Next, we searched for new candidate genes by identifying any potentially pathogenic *de novo*, homo-

Table 1. Gene panels used in targeted search.

Genes evaluated as part of pituitary panel (n=37) *ARNT2, BMP2, BMP4, CDON*, FGF8*, FGF10, FGF18, FGFR1*, GATA2, GLI 1, GLI2*, GLI3, GLI4, GLI5, GLI6, GPR161, HESX1, IGSF1, LHX3, LHX4, NR5A1, OTX2, PAX6, PITX1, PITX2, POU1F1, PROP1, SHH, SIX1, SIX2, SIX3, SIX4, SIX5, SIX6, SOX1, SOX2, SOX3, TBX19, TGIF, WNT5**
Genes evaluated as part of holoprosencephaly panel (n=18) *CDON*, DISP1, DLL1, FGF8*, FGFR1*, FOXH1, GAS1, GLI2*, HHAT, NODAL, PTCH1, SHH, SIX3, STIL, SUFU, TDGF1, TGIF1, ZIC2*
Genes evaluated as part of hypogonadotropic hypogonadism panel (n=34) *AXL, CCDC141, CHD7, DMXL2, FEZF1, FGF17, FGF8*, FGFR1*, GNRH1, GNRHR, HESX1, HS6ST1, KAL1, KISS1, KISS1R, LEP, LEPR, NR0B1, NSMF, OL14RD, OTUD4, PCSK1, PNPLA6, PROK2, PROKR2, RNF216, SEMA3A, SEMA3E, SEMA7A, SOX10, STS, TAC3, TACR3, WDR11*
Genes evaluated as part of corpus callosum agenesis panel (n=134) *AHI1, AKT3, AMPD2, ANOP1, ARID1B, ARL13B, ARX, ASPM, ATR, ATRX, B9D1, B9D2, BCOR, BMP4, C12ORF57, CASK, CC2D2A, CENPJ, CEP152, CEP290, CEP41, CEP63, CREBBP, CTBP1, AS1, DCX, DHCR24, DHCR7, DIS3L2, DISC1, EFNB1, EOMES, EP300, EPG5, FGF8, FGFR1, FGFR2, FH, FKRP, FKTN, FLNA, GLI3, GPSM2, GTDC2, HCCS, HESX1, HS6ST1, HYLS1, IGBP1, IGF1, INPP5E, ISPD, KAT6B, KCC3, KIF7, L1CAM, LARGE, LRP2, MED12, MID1, MKS1, NDE1, NFIX, NIN, NPHP1, NPHP3, NSD1, OFD1, OTX1, PAX6, PDHA1, PDHB, POMGNT1, POMT1, POMT2, PYCR1, RAB18, RAB3GAP1, RAB3GAP2, RBBP8, RBM10, RELN, RNU4ATAC, RPGRIP1L, RPS6KA3, SCKL3, SOX2, SPG11, STRA6, TCF4, TCTN1, TCTN2, TCTN3, TMEM138, TMEM216, TMEM237, TMEM67, TUBA1A, TUBB2B, TUBB3, VAX1, WDR62, ZEB2*

*Genes with asterisk included in more than one list

zygous, or compound heterozygous variants, including X-linked mutations in males. For each gene, a PubMed search was performed to determine function and/or brain expression pattern. Thereafter, we searched for potentially pathogenic variants in the panel of genes involved in midline brain pathologies: holoprosencephaly, hypogonadotropic hypogonadism, and absent corpus callosum. Lastly, we identified all *GLI2* variants with a reported minor allele frequency (MAF) <0.05. The frequency of *GLI2* variants with a MAF <0.05 was compared with the frequency of *GLI2* variants with a MAF <0.05 in the 1000 Genomes database. Specific combinations of *GLI2* variants, detected in the current study, were also searched for in the 1000 Genomes database. Because of the ethnic diversity of our study population, we used the 1000 Genomes database as an international reference population.

Modelling and molecular dynamic study

Three-dimensional models were constructed for the wild-type DCHS1 and DCHS1 I780V, P1519S, L2132P, H2729L mutants and for wild-type roundabout guidance receptor 2 (ROBO2; isoform 2a) and R314Q mutants. Full-length secondary structure predictions and the identification of most suitable templates were performed using the ROSETTA-based Robetta Web server (http://robetta.bakerlab.org). Using the homology modelling program Modeler 9v14 (www.salilab.org/modeller/), four pairs of models of the three extracellular domains (wild-type and mutants; residue range: 680 to 886, 1318 to 1641, 2062 to 2270, and 2536 to 2867) were generated considering previously published structural DCHS1 and ROBO2 data and Robetta outputs (23–26). Modelling was performed with the default parameters using the allHmodel protocol to include hydrogen atoms and the HETATM protocol to include Ca2+ with restricting Ca2+-binding residues side-chain distance and with thorough molecular dynamics optimization and refinement protocol. To study the mutational effects on structure, the molecular dynamics of each model pair was simulated using a NAMD 2.11 (http://www.ks.uiuc.edu/Research/namd/) plug-in in the VMD v1.9.2.27 program (http://www.ks.uiuc.edu/Research/vmd/) for 100 ns. Intermediate and final structures were evaluated in PyMol.

RESULTS

We studied 20 unrelated patients (14 males) with an age range of 3 to 28 years (Table 2). Age at diagnosis ranged from 2 weeks to 5 years. Eleven children had been diagnosed with congenital central hypothyroidism after detection by neonatal screening, seven children after referral for growth retardation, one child for prolonged jaundice, and another child for neonatal hypotonia. Twelve patients had deficiency of four anterior pituitary hormones, seven patients had three hormone deficiencies, and one patient had two hormone deficiencies. All patients had GH deficiency. Central adrenal insufficiency was present in 19 patients, and hypogonadotropic hypogonadism was present in three patients of prepubertal age and in nine patients of

Table 2. Clinical characteristics of studied patients with isolated PSIS.

Case	Sex	Age (yrs)	Age at diagnosis	Presentation	Pituitary deficiencies	MRI findings
1	M	8	2 weeks	Abnormal NS	GH; TSH; ACTH; LH/FSH (tested at 3 months)	EPP, absent stalk, small AP
2	F	25	5 years	Growth retardation	GH; TSH; ACTH; LH/FSH	EPP, absent stalk, absent AP
3	M	28	3 months	Prolonged jaundice	GH; TSH; ACTH; LH/FSH	EPP, absent stalk, small AP
4	M	3	2 weeks	Abnormal NS	GH; TSH; ACTH; LH/FSH (tested at 3 months)	EPP, interrupted stalk, small AP
5	M	26	2 weeks	Hypotonia,	GH; TSH; ACTH	EPP, small stalk, small AP
6	F	10	2 years	Growth retardation	GH; TSH; ACTH; LH/FSH unknown	EPP, absent stalk, small AP
7	F	9	2 weeks	Abnormal NS	GH; TSH; ACTH; LH/FSH unknown	EPP, absent stalk, absent AP
8	F	16	1 month	Abnormal NS	GH; TSH; ACTH LH/FSH	EPP, absent stalk, small AP
9	M	23	6 months	Abnormal NS	GH; TSH; ACTH	EPP, absent stalk, small AP
10	F	9	2.5 years	Growth retardation	GH; TSH; ACTH; LH/FSH unknown	EPP, absent stalk, small AP
11	M	18	2 weeks	Abnormal NS	GH; TSH; ACTH; LH/FSH	EPP, absent stalk, small AP
12	M	13	2 years	Growth retardation	GH; TSH	EPP, absent stalk, normal AP
13	M	23	6 weeks	Abnormal NS	GH; TSH; ACTH; LH/FSH	EPP, absent stalk, normal AP
14	M	18	4 years	Growth retardation	GH; TSH; ACTH; LH/FSH	EPP, absent stalk, absent AP
15	M	19	3 weeks	Abnormal NS	GH; TSH; ACTH; LH/FSH	EPP, absent stalk, small AP
16	M	17	3 weeks	Abnormal NS	GH; TSH; ACTH; LH/FSH	EPP, small stalk, small AP
17	F	5	3 weeks	Abnormal NS	GH; TSH; ACTH; LH/FSH (tested at 3 months)	EPP, absent stalk, small AP

Table 2. Continued

Case	Sex	Age (yrs)	Age at diagnosis	Presentation	Pituitary deficiencies	MRI findings
18	M	5	1.5 years	Growth retardation	GH; TSH; ACTH; LH/FSH unknown	EPP, absent stalk, small AP
19	M	8	2.5 years	Growth retardation	GH; TSH; ACTH; LH/FSH unknown	EPP, small stalk, small AP
20	M	20	2 weeks	Abnormal NS	GH; TSH; ACTH; LH/FSH	EPP, absent stalk, small AP

Abbreviations: ACTH, adrenocorticotropic hormone; AP, anterior pituitary; EPP, ectopic posterior pituitary; FSH, follicle-stimulating hormone; GH, growth hormone; LH, luteinizing hormone; NS, neonatal screening result; TSH, thyrotropin.

postpubertal age. Three children had normal pubertal development, and in five prepubertal children, information on the status of the hypothalamic-pituitary-gonadal axis was not available. No patient had central diabetes insipidus. The medical history of parents was negative for endocrine deficiencies, and all parents had normal development, growth, and fertility.

For each patient, all pathogenic and potentially pathogenic variants and all *GLI2* variants with an MAF <0.05 are shown in Tables 3 and 4.

Variants in pituitary genes

In the panel of 37 genes associated with pituitary development, no pathogenic variants were found. However, several rare, potentially pathogenic variants were present in *GLI2*, *GLI3*, *BMP4*, *IGSF1*, *CHD4*, *ARNT2*, *SIX6* (two patients, same variant), and *FGF8*.

New candidate genes

De novo or heterozygous mutations were found in five genes (in six patients) with a known expression/function in the brain: *ROBO2*, *KAT6A*, *KIF14*, compound heterozygosity for *DCHS1* (two patients), and compound heterozygosity for *CCDC88C*.

Protein modelling for both cases of the compound heterozygous *DCHS1* mutations indicated disrupted interaction with the ligand FAT4, making functional consequences likely (Fig. 1). Protein modelling for the *ROBO2* mutation indicated that the mutation was in the most mobile region of the ROBO2 protein. The mutation causes an extra H-bond, which solidifies the ROBO2 protein, probably leading to less adaptability to environmental influences and in interactions with the ligand SLIT (Fig. 2).

Table 3. Exome sequencing results of studied patients with PSIS including variants, reported allele frequency, classification according to ACMG guidelines, *in silico* prediction (Polyphen 2) and phenotypes known to be caused by variants in each gene.

Case	Gene	Variant	dbSNP	Inheri-tance	Variant clas-sification	MAF (GnomAD)	*In Silico* Prediction	Gene panel	Known phenotype (MIM number)	Information on specific variant
3	*GLI2*	c.4054A>G, p.M1352V (missense)	rs149140724	Mother	Benign	0.0099	Benign	PIT+HPE	Holoprosencephaly 9 (MIM 610829), Culler-Jones syndrome(MIM 615849)	*Same two GLI2 variants in case 14* Combination of these two variants previously described in PSIS patients, including functional study showing reduced transcriptional activity and reduced luciferase activity ((10, 13, 27)
	GLI2	c.4558G>A, p.D1520N (missense)	rs114814747	Mother	VUS	0.0101	Probably damaging			
4	*DHCR7*	c.452G>A, p.W151* (nonsense)	rs11555217	Mother	Pathogenic	0.0007	Damaging	CCA	Smith-Lemli-Opitz syndrome (MIM 270400	*Same mutation in patient 18* Homozygosity described in patients with Smith-Lemli-Opitz syndrome
	RELN	c.9646G>A, p.E3216K (missense)	-	Mother	VUS	N	Benign	CCA	Lissencephaly 2 (MIM 257320)	
	IGSF1	c.498G>C, p.E166D	rs201255931	Mother	VUS	0.0005	Probably damaging	PIT	XL central hypothyroidism (MIM 300888)	
5	*INPP5E*	c.902T>C, p.L301P (missense)	-	Mother	VUS	N	Probably damaging	CCA	Joubert syndrome 1 (MIM 213300)	

Table 3. Continued

Case	Gene	Variant	dbSNP	Inheritance	Variant classification	MAF (GnomAD)	*In Silico* Prediction	Gene panel	Known phenotype (MIM number)	Information on specific variant
6	*KAT6A*	c.235C>T, p.A79* (nonsense)	-	*De novo*	Pathogenic	N	Damaging	Not from panel	KAT6A neurodevelopmental syndrome (MIM 616268)	(28)
	GLI3	c.539G>A, p.R180Q (missense)	rs140772904	Father	VUS	0.00004	Possibly damaging	PIT	Greig cephalopolysyndactyly syndrome (MIM 175700), Pallister-Hall syndrome (MIM 146510), Postaxial polydactyly (MIM 174200), Preaxial polydactyly (MIM 174700)	
	BMP4	c.804_815del-CCGGCCCCTCCT, p.R269_L272del (deletion)	-	Father	VUS	N		PIT	Syndromic microphthalmia type 6 (MIM607932)	
	GLI2	c.1761G>A, p.T587T (synonymous)	rs61732852	Mother	Likely benign	0.0119		PIT+HPE	Holoprosencephaly 9 (MIM 610829), Culler-Jones syndrome (MIM 615849)	
7	*DCHS1*	c.6395T>C p.L2132P (missense)	-	Father	VUS	N	Probably damaging	Not from panel	Van Maldergem syndrome (MIM 601390), AD mitral valve prolapse (MIM 607829)	
	DCHS1	c.2338A>G p.I780V (missense)	rs145735483	Mother	VUS	0.0003	Benign			
	GLI2	c.1294G>A, p.V432M (missense)	rs142296407	Mother	VUS	0.0015	Possibly damaging	PIT+HPE	Holoprosencephaly 9 (MIM 610829), Culler-Jones syndrome (MIM 615849)	Same variant described in patients with MPHD/PSIS (ref 10, 11, 13)

Case	Gene	Variant	dbSNP	Inheritance	Variant classification	MAF (GnomAD)	*In Silico* Prediction	Gene panel	Known phenotype (MIM number)	Information on specific variant
8	*NDE*	c.488T>A, p.L163Q (missense)	-	Father	VUS	N	Probably damaging	CCA	Lissencephaly 4 (MIM 614019)	
	OTUD4	c.823G>T, p.V275L (missense)	-	Mother	VUS	N	Probably damaging	HH	Hypogonadotropism (MIM 611744))	
9	*ROBO2*	c.914G>A, p.R314Q (missense)	-	De novo	VUS	N	Probably damaging	Not from panel	Vesicoureteral reflux 2 (MIM 610878)	
10	*GLI2*	c.963C>G, p.P321P (synonymous)	rs149894186	Mother	Benign	0.0046		PIT+HPE	Holoprosencephaly 9 (MIM 610829), Culler-Jones syndrome (MIM 615849)	
	GLI2	c.2088A>G, p.A696A (synonymous)	rs146059306	Mother	Benign	0.0009				
	GLI2	c.2262G>T, p.R754R (synonymous)	rs142856393	Father	Benign	0.0011				

Table 3. Continued

Case	Gene	Variant	dbSNP	Inheritance	Variant classification	MAF (GnomAD)	*In Silico* Prediction	Gene panel	Known phenotype (MIM number)	Information on specific variant
11	*ASPM*	c.10161+5G>C (insertion)	-	Mother	VUS/Likely pathogenic (altered splicing?)	N		CCA	Primary autosomal recessive microcephaly (MIM 608716)	
	FGF8	c.77C>T, p.P26L (missense)	rs137852660	Mother	VUS	0.0019	Benign	PIT+HH	Hypogonadotropic hypogonadism 6 (MIM 612702)	Heterozygosity described in a patient with HH and partial empty sella (Falardeau *et al.* (29))
	CHD4	c.2374C>T, p.R792W (missense)	-	Father	VUS	N	Probably damaging	PIT	Sifrim-Hitz-Weiss syndrome (MIM 617159)	
12	*CC2D2A*	c.3055C>T, p.R1019* (nonsense)	rs370880399	Mother	Pathogenic	0.0001	Damaging	CCA	Joubert syndrome 9 (MIM 612285), Meckel syndrome 6 (MIM 612284)	Variant reported in compound heterozygosity with another variant in a number of patients with Joubert syndrome
	NR0B1	c.315G>C, p.W105C (missense)	rs132630327	Mother	Pathogenic	0.00001	Damaging	HH	Congenital adrenal hypoplasia (MIM 300200), 46XY reversal 2(MIM 300018)	Same mutation reported in patient with isolated mineralocorticoid deficiency (30)
	ARNT2	c.1707G>T, p.Q569H (missense)	rs145379118	Father	VUS	0.0033	Possibly damaging	PIT	Webb-Dattani syndrome (MIM 615926)	

Case	Gene	Variant	dbSNP	Inheri-tance	Variant clas-sification	MAF (GnomAD)	*In Silico* Prediction	Gene panel	Known phenotype (MIM number)	Information on specific variant
13	*PROK2*	c.163delA p.I55* (nonsense)	rs554675432	Father	Pathogenic	0.0001	Damaging	HH	Hypogonadotropic hypogonadism 4 (MIM 610628)	Homozygosity reported in patients with anosmic hypogonadotropic hypogonadism (31, 32)
	B9D1	c.151T>C, p.S51P (missense)	rs546359789	Father	VUS	0.00006	Probably damaging	CCA	Joubert syndrome 27 (MIM 617120), Meckel syndrome 9 (MIM 614209).	Variant reported in compound heterozygosity with another variant in a patient with Joubert syndrome (Srour *et al.* (33))
14	*GLI2*	c.4054A>G, p.M1352V (missense)	rs149140724	Father	Benign	0.0099	Benign	PIT+HPE	Holoprosencephaly 9 (MIM 610829), Culler-Jones syndrome (MIM 615849)	*See case 3 (same combination of GLI2 variants)*
	GLI2	c.4558G>A, p.D1520N (missense)	rs114814747	Father	VUS	0.0101	Probably damaging			
	GLI2	c.1944C>T, p.T648T (synonymous)	rs13008360	Both parents	Benign	0.0253				
	CHD4	c.5149C>T, p.R1717W (missense)	-	Father	VUS	N	Probably damaging	PIT	Sifrim-Hitz-Weiss syndrome (MIM 617159)	
	SIX6	c.385G>A, p.E129K (missense)	rs146737847	Mother	VUS	0.0040	Probably damaging	PIT	Optic disc anomalies + retina/ macula dystrophy (MIM 212550)	*Same variant in patient 19* Variant previously described with reduced function (Carnes *et al.* (34))
15	*KIF14*	c.3728A>G, p.K1243R	-	De novo	VUS	N	Probably damaging	Not from panel	Meckel syndrome 12 (MIM 616258)	

Table 3. Continued

Case	Gene	Variant	dbSNP	Inheritance	Variant classification	MAF (GnomAD)	*In Silico* Prediction	Gene panel	Known phenotype (MIM number)	Information on specific variant
16	*GLI2*	c.4145G>A, p.R1382H (missense)	rs200080112	Mother	VUS	0.0002	Probably damaging	PIT+HPE	Holoprosencephaly 9 (MIM 610829), Culler-Jones syndrome (MIM 615849)	
17	*CCDC88C*	c.3895C>T, p.R1299C (missense)	rs142539336	Father	VUS	0.0049	Possibly damaging	Not from panel	Non-syndromic hydrocephalus (MIM 616053)	
	CCDC88C	c.1984G>A, p.E662K (missense)	not in dbSNP	Mother	VUS	0.0001	Benign			
	TACR3	c.659T>C, p.L220P (missense)		Mother	VUS	N	Probably damaging	HH	Hypogonadotrophic hypogonadism (MIM 614840)	
18	*DHCR7*	c.452G>A, p.W151* (nonsense)	rs11555217	Mother	Pathogenic	0.0007	Damaging	CCA	Smith-Lemli-Opitz syndrome (MIM 270400)	*Same mutation in patient 4* Homozygosity described in patients with Smith-Lemli-Opitz syndrome
	CHD7	c.1324G>A, p.A442T (missense)	rs368086966	Mother	VUS	0.0002	Benign	HH	CHARGE syndrome (MIM 214800), Hypogonadotropic hypogonadism (MIM 612370)	

Case	Gene	Variant	dbSNP	Inheritance	Variant classification	MAF (GnomAD)	*In Silico* Prediction	Gene panel	Known phenotype (MIM number)	Information on specific variant
19	*DCHS1*	c.8186A>T, p.H2729L (missense)	rs148148252	Father	VUS/likely pathogenic	0.0003	Probably damaging	Not from panel	Van Maldergem syndrome (MIM 601390), AD mitral valve prolapse (MIM 607829)	
	DCHS1	c.4555C>T, p.P1519S (missense)	rs199544459	Mother	VUS	0.0017	Benign			
	SIX6	c.385G>A, p.E129K (missense)	rs146737847	Mother	VUS	0.0040	Probably damaging	PIT	Optic disc anomalies + retina/ macula dystrophy (MIM 212550)	*Same variant in patient 14* Variant previously described with reduced function (Carnes *et al.* (34))
	BMP4	c.1001C>A, p.A334D (missense)	rs550409227	Mother	VUS	0.000008	Probably damaging	PIT	Syndromic microphthalmia type 6 (MIM607932)	
20	*SLC12A6*	c.1787C>T, p.P596L (missense)	-	Mother	VUS/Likely Pathogenic	N	Possibly damaging	CCA	AR corpus callosum agenesis + peripheral neuropathy (MIM218000)	
	CC2D2A	c.3865A>G, p.T1289A (missense)	-	Mother	VUS	N	Possibly damaging	CCA	Joubert syndrome (MIM 612285), Meckel syndrome (MIM 612284)	

Abbreviations: ACMG, The American College of Medical Genetics and Genomics; AD, autosomal dominant; AR, autosomal recessive; CCA, corpus callosum agenesis; dbSNP, Single Nucleotide Polymorphism Database; HH, hypogonadotropic hypogonadism; HPE, holoprosencephaly; MAF, minor allele frequency; MIM, numerical assignment in Mendelian Inheritance in Man catalog; MPHD, multiple pituitary hormone deficiency; N, variant not previously reported; PIT, pituitary; VUS, variant of unknown significance.

Variants in genes associated with midline brain pathologies

In the panel of genes associated with holoprosencephaly, 11 variants in *GLI2* were found but no potential pathogenic variants in any of the other candidate genes. In the panel for hypogonadotropic hypogonadism, pathogenic mutations were found in *NR0B1* and *PROK2*, and potentially pathogenic variants were found in *OTUD4*, *FGF8*, *TACR3*, and *CHD7*. In the panel for absent corpus callosum, pathogenic mutations were found in *DHCR7* (two patients, identical mutation) and *CC2D2A*, and variants of unknown significance, predicted to potentially be damaging, were found in *RELN*, *INPP5E*, *NDE*, *ASPM*, *B9D1*, *CC2D2A* (two patients), and *SLC12A6*. All mutations found in the target panels were inherited from an unaffected parent (Table 3).

We detected 11 *GLI2* variants (six missense, five synonymous) with a MAF <0.05 in six patients; all were inherited from an unaffected parent. The total prevalence of *GLI2* variants with a MAF <0.05 was 55% in our study population (11 variants in 20 individuals), which was comparable to the 58.6% prevalence of *GLI2* variants with a MAF <0.05 in the 1000 Genomes database (1468 variants in 2504 individuals). Two of the 20 study patients had a combination of the missense mutations M1352V + D1520N. In the 1000 Genomes database, this specific combination was found in only 17 of 2504 individuals (0.68%).

DISCUSSION

In this exome sequencing study of 20 unrelated patients with isolated PSIS and their unaffected parents, we identified five additional candidate genes for PSIS: *DCHS1*, *ROBO2*, *CCDC88C*, *KIF14*, and *KAT6A*. In addition, by using a target gene panel, we found potentially pathogenic variants in genes involved in midline brain formation in a panel of genes known to be associated with pituitary formation (in 8 of 37 genes) and in a panel of genes associated with holoprosencephaly, hypogonadotropic hypogonadism, and absent corpus callosum (11 of 186 genes); and also in the latter group, 5 pathogenic variants. Thirteen of 20 patients carried more than one variant, and all variants from the target panels were inherited from an unaffected parent. This diversity of (potentially) pathogenic variants and inheritance from unaffected parents are suggestive of a polygenic etiology of isolated PSIS.

New candidate genes

DCHS1

Two patients (nos. 7 and 19) were compound heterozygous for variants in *DCHS1*. Protein modelling indicated disrupted interaction with the ligand FAT4A, making functional consequences likely (Fig. 1). DCHS1 and FAT4 play a role in neuronal migration (35, 36). Pathogenic mutations in *DCHS1* and *FAT4* can cause Van Maldergem syndrome, which goes along with periventricular neuronal heterotopia, indicative of altered neuronal migration, and absent corpus callosum (24). Pituitary abnormalities have not been described. *DCHS1* is expressed in

Table 4. Exome sequencing results per panel of targeted genes including number of variants for each Gene

	Case																		
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**
Pituitary Panel																				
ARNT2												1								
BMP4						1													1	
CHD4											1			1						
GLI3						1														
SIX6														1					1	
IGSF1				1																
Holoprosencephaly Panel																				
GLI2			2			1	1			3				3		1				
Hypogonadotropic Hypogonadism Panel																				
B9D1													1							
CHD7																		1		
FGF8											1									
NR0B1												1								
OTUD4								1												
PROK2													1							
TACR3																	1			
Corpus Callosum Agenesis Panel																				
ASPM											1									
CC2D2A												1								1
DHCR7				1														1		
INPP5E					1															
NDE								1												
RELN				1																
SLC12A6																				1
New Candidate Genes																				
CCDC88C																	2			
DCHS1							2												2	
KAT6A						1														
KIF14															1					
ROBO2									1											

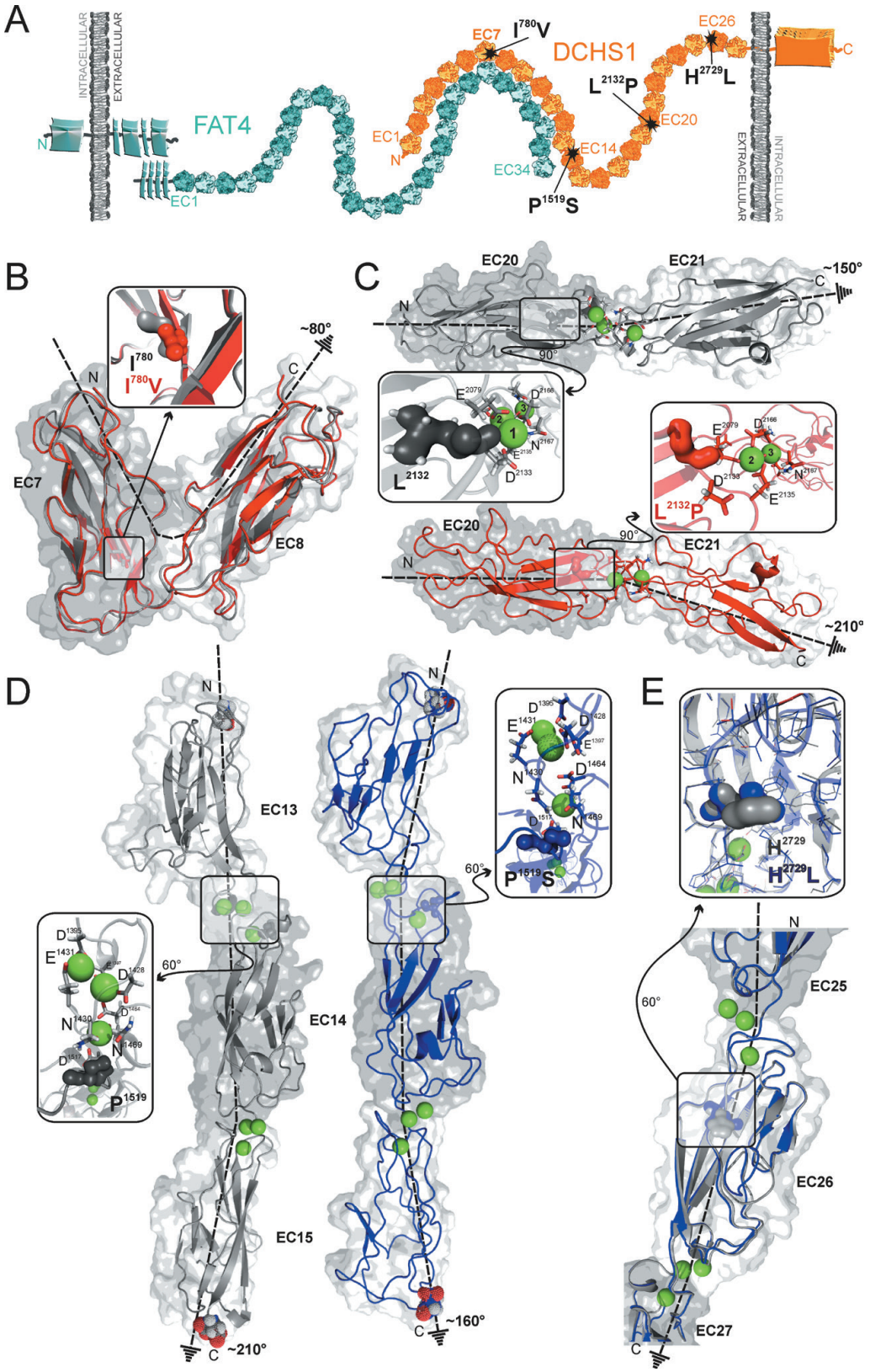

Figure 1. (A) Schematic overview of the structure and interaction of DCHS1 and FAT4, including the sites of the presently reported mutations. (B-E) Extracellular (EC) domains shown in cartoon presentation with mutation position marked and zoomed in on. The dashed arrows present orientation of extracellular domain in comparison to the neighboring domains, and the numbers present the corner of orientation. Affected

Figure 1. Continued

residues are shown in sticks or line presentation. Calcium in green spheres. (B, C) Mutations in case 7 are shown. (B) c.2338A>G, p.Ile780Val: wild-type = gray, mutation = red. Mutation is in EC7 and has a slight effect on structural stability of EC7 domain. EC6 and EC7 interact with FAT4, which may loosen the interaction. (C) c.6395T>C, p.Leu2132Pro: wild-type = gray, mutation = red. Mutation is in EC20 and has structural effect on EC20 and even more so on EC21. Both mutant extracellular domains lose structural stability because of effects on Ca binding ability. Mutation also affects orientation of the EC20, which may cause less favorable DCHS1-FAT4 interaction. (D, E) Mutations in case 19 are shown. (D) c.4555C>T, p.Pro1519Ser: wild-type = gray, mutation = blue. Mutation is in EC14 and has a structural effect on EC13, EC14, and even more so on EC15. EC15 almost unfolds; only Ca2+ still holds the structure together. Immediately after Ca concentration went down, EC15 completely unfolded. Pro1519Ser disturbed beta structure, which affected Ca-binding sites between EC14 and EC15, potentiating the structural instability of EC15. Mutation also affected orientation of all mentioned extracellular domains, consequently leading to distorted interaction between DCHS1 and FAT4. (E) c.8186A.T, p.His2729Leu: wild-type = gray, mutation = blue. Mutation is in EC26. No significant structural effect was observed.

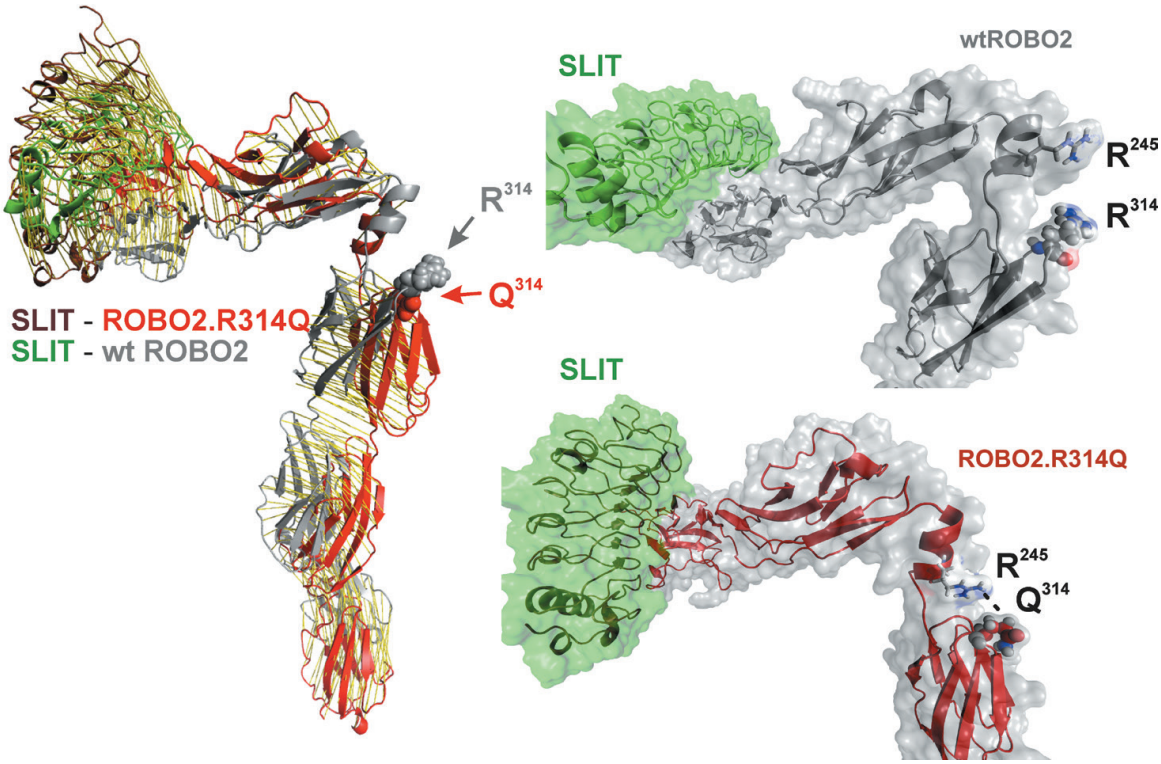

Figure 2. Schematic overview of the structure and interaction of ROBO2 and SLIT. Right panel: SLIT (green), wild-type ROBO2 = gray, mutation (c.941G.A, p.Arg314Gln) = red. The mutation leads to an extra H-bond between residues 245 and 314, causing ROBO2 to become more rigid. Left panel: superposition of wild-type ROBO2-SLIT and mutated ROBO2 (c.941G.A, p.Arg314Gln)–SLIT complex. Mutations are marked by arrows. Yellow lines indicate distance between their backbone atoms.

the developing pituitary gland in mice (37). The present two patients had isolated PSIS without other brain abnormalities at MRI. We examined the patients carefully for signs suggestive of Van Maldergem syndrome, but except for broad hands and feet, present in both patients and absent in parents, there were no specific physical characteristics. One of the patients also had a *GLI2* variant (c.1294G>A, maternally inherited), previously reported in patients with MPHD, and an ectopic posterior pituitary lobe (10, 11, 13). Because *DCHS1* is involved in neuronal migration and is expressed in the pituitary gland and because we found two patients in this series of 20 patients with isolated PSIS, the *DCHS1* gene may be considered a candidate gene for PSIS, but possibly only when variants are present in one or more other genes.

ROBO2

Patient 9 had a de novo *ROBO2* mutation (c.914G>A), which was not previously reported and was predicted to be probably damaging. Protein modelling indicated that the mutation is not in the ROBO2-SLIT interface but is in the most mobile region of the ROBO2 protein. The mutation leads to an extra H-bond and solidifies the ROBO2 protein, making functional consequences likely (Fig. 2). ROBO2 belongs to the ROBO family, part of the immunoglobulin superfamily of proteins that are highly conserved from fly to human. The encoded protein is a transmembrane receptor for the slit homolog 2 protein and functions in axon guidance across the midline of the mammalian central nervous system (38). *ROBO2* mutations are associated with vesicoureteral reflux (39). *ROBO2* isoform a is highly expressed in the developing human brain but not in the adult brain (40). The present patient did not have vesicoureteral reflux. After we had finished our study, Bashamboo et al. (41) identified *ROBO1* mutations in five cases of PSIS. Four of the five patients had ocular anomalies, which the present patient did not have. The function of ROBO2 in axon guidance across the midline, the results of protein modelling, and the recent finding of *ROBO1* mutations in PSIS make *ROBO2* an excellent candidate gene for pituitary malformations, but functional studies are needed to prove pathogenicity.

CCDC88C

Patient 17 was compound heterozygous for *CCDC88C* variants. The R1299C was predicted to be possibly damaging by Polyphen2; the E662K mutation is very rare (MAF, 0.0001), predicted to be benign by Polyphen2. This gene encodes a ubiquitously expressed coiled–coil domain–containing protein that interacts with the dishevelled protein and is a negative regulator of the Wnt signaling pathway. Autosomal recessive mutations in *CCDC88C* cause nonsyndromic hydrocephalus, associated with midline brain malformation (42). The present patient had no hydrocephalus and no midline brain malformation other than PSIS. Besides the *CCDC88C* variants, the patient also carried a *TACR3* mutation, which was not described before and was predicted to be probably damaging. Homozygous *TACR3* mutations are associated with hypogonadotropic hypogonadism. The present patient has complete anterior pituitary insuf-

ficiency, including hypogonadism. In this case, it is feasible that *TACR3* contributed to the phenotype in a polygenic model.

Functional studies will have to be performed to provide more evidence for the pathogenicity of the *CCDC88C* mutations, but the phenotype, including midline brain abnormalities, and the importance of the Wnt pathway in pituitary development make *CCDC88C* a promising candidate gene for PSIS.

KIF14

Patient 15 had a de novo *KIF14* mutation (c.3728A>G) that has not been described before and is predicted to be possibly damaging. Autosomal recessive *KIF14* mutations have been linked to a lethal fetal ciliopathy, resembling Joubert syndrome (43). Primary cilia are involved in central nervous system development and are involved in signaling pathways, such as Shh and Wnt pathways. Ciliopathies are disorders caused by defects in the primary ciliary structure and include Joubert syndrome and Bardet-Biedl syndrome.

In *KIF14* mutant mice, the development of laminated structures in the central nervous sys tem is affected, and the olfactory bulb was shown to be cytoarchitecturally disorganized (44). The olfactory placode is involved in hypothalamic-pituitary development with gonadotrophin-releasing hormone neurons migrating from the olfactory placode into the hypothalamus. Disruption of this migration is a key feature of Kallmann syndrome, consisting of hypogonadotropic hypogonadism and anosmia. Given this, mutations in *KIF14* may well influence pituitary development. Because we found other heterozygous pathogenic mutations in genes linked to ciliopathies (*INPP5E*, *CC2D2A*, and *B9D1*), genes involved in primary ciliary structure/function may be involved in the PSIS phenotype and should be studied in more detail.

KAT6A

A de novo *KAT6A* mutation (c.235C>T) leading to a premature stop codon was found in patient 6. This patient's case has been published in detail elsewhere (28). This patient had a developmental delay, with severely delayed speech, which was initially attributed to hypoglycemic brain damage due to untreated central adrenal insufficiency and GH deficiency in the first 2 years of life. In retrospect, she fulfils the criteria for KAT6A neurodevelopmental disorder and should not have been included in this study on isolated PSIS. Because subtle midline brain abnormalities were reported in KAT6A patients (cavum septum pellucidum, absent bulbous olfactorius), PSIS may be part of the phenotypic spectrum. However, because this patient also had potentially pathogenic *BMP4* and *GLI3* variants and a rare *GLI2* variant (all present in the unaffected father), a polygenic cause of the pituitary malformation is also possible.

Variants in genes associated with midline brain malformation

Hypogonadotropic hypogonadism genes: PROK2, NR0B1

A pathogenic *PROK2* frameshift mutation leading to a premature stop codon (c.163delA) was found in patient 13. The mutation was inherited from the unaffected father. Homozygous and heterozygous *PROK2* and *PROKR2* variants are associated with Kallmann syndrome and hypogonadotropic hypogonadism. Recently, *PROKR2* variants were reported in patients with MPHD. In a UK series of 422 patients with MPHD (89% with septo-optic dysplasia and 11% with holoprosencephaly or midline clefts), *PROKR2* mutations were found in 11 cases, but no mutations were found in *PROK2* (16). In a Brazilian series of 156 patients with MPHD, two patients with a *PROKR2* mutation were identified (*PROK2* was not investigated) (17). In a French series of 72 patients with PSIS, two *PROKR2* mutations were found (6).

The presently reported *PROK2* mutation was reported in a Portuguese family in which homozygous family members had hypogonadotropic hypogonadism with and without anosmia and heterozygous family members were unaffected (31). A Swiss patient with the same homozygous *PROK2* mutation had hypogonadotropic hypogonadism with anosmia; data on heterozygous carriers were not available (32). The presently reported patient and his unaffected father both also had a rare, possibly damaging mutation in *B9D1* associated with Joubert syndrome. The incomplete penetrance in the father may be due to the presence of variants in other genes or to epigenetic influences, which may fit a complex polygenic background for PSIS.

A very rare missense mutation (MAF, 0.0001%) in the X-linked *NR0B1* (c.315G>C) was found in patient 12. The mutation was inherited from the mother and predicted to be damaging. It has previously been linked to isolated mineralocorticoid deficiency and a mild form of congenital adrenal underdevelopment (30). Midline brain anomalies have not been reported before in individuals with *NR0B1* variants. The same patient also had a rare pathogenic *CC2D2A* mutation.

Absent corpus callosum: DCHR7, CC2D2A

In three patients, pathogenic mutations in genes associated with absent corpus callosum were found: *DCHR7* (c.452G>A; patients 4 + 18) and *CC2D2A* (c.3055C>T; patient 12). These genes are known to cause autosomal recessive disorders; *DCHR7* mutations can cause Smith-Lemli-Opitz syndrome, and *CC2D2A* mutations can cause Meckel syndrome and Joubert syndrome. In each patient, the mutation was inherited from an unaffected parent, indicating that being a carrier in itself is insufficient to develop PSIS.

CC2D2A is involved in ciliopathies, as is *KIF14* (described previously). In the series of genes with variants classified as having unknown clinical significance, there are three genes (*INPP5E*, *CC2D2A*, and *B9D1*) that function as ciliopathy genes. This suggests that ciliopathy genes may be involved in pituitary development.

Holoprosencephaly: GLI2 variants

GLI2 is associated with holoprosencephaly and acts in the Wnt pathway. In a review, 25 patients (16 families) were reported to have heterozygous mutations in *GLI2* and disturbed pituitary development (14). Most patients had an ectopic posterior pituitary. The suggested pattern of inheritance was autosomal dominant with incomplete penetrance and variable expression (14).

In the current study, six patients had *GLI2* variants with a MAF <0.05. Two patients had the combination of *GLI2* variants M1352V + D1520N (patients 3 + 14). This combination has previously been reported in patients with PSIS, and functional studies have demonstrated reduced transcription activity and reduced luciferase activity (10, 13, 27). Another presently reported patient had a V432M variant that has also been described in patients with PSIS (10, 11, 13). The R1382H variant found in another patient has not been described before, was inherited from an unaffected mother, is very rare (MAF, 0.0002), and is potentially pathogenic.

GLI2 variants with a MAF <0.05 were not more common in PSIS than in the general population (1000 Genomes); however, despite the small numbers, the specific combination of M1352V + D1520N variants seems to be important in PSIS because we found the combination in two of 20 patients (10%) compared with only 17 of 2504 individuals (0.68%) in the reference population (1000 Genomes). *GLI2* variants have been reported at a relatively high frequency in patients with MPHD/PSIS, and *GLI2* is likely an important factor in the polygenic background of pituitary development.

Strengths and limitations

Strengths of this study are the homogeneous group of study participants. A limitation is the inclusion of the KAT6A patient who had syndromic PSIS and not the isolated form. It emphasizes the importance of proper phenotyping in such studies (45). All studied patients had congenital central hypothyroidism, and therefore the results may not be representative for PSIS patients without central hypothyroidism. We presumed all parents were unaffected on the basis of their medical history. However, we did not perform endocrine studies or MRIs in parents. Exome sequencing by itself is limited by incomplete coverage of the exome and easily missed copy number variations, although the presently used targeted approach decreased the likelihood of missing deletions and duplications. Absolute proof for causality of combinations of variants in candidate genes can be obtained only by functional studies (46). It was beyond the aims and possibilities of the current study to perform such functional studies for each (combination of) candidate genes. Because we searched only for variants in genes in lymphocytes, mosaicisms confined to affected tissue(s) cannot be excluded. Exome sequencing and genome sequencing can detect variants in genes, but epigenetic mechanisms such as DNA methylation, histone modification, and microRNAs may play a role, warranting additional studies. Systematically investigating large series of carefully phenotyped patients and collaboration between research groups are essential in the search for genes and pathways underlying disorders with a polygenic etiology (9).

In conclusion, searching for causes and pathogenesis of a potentially polygenic disorder is complex. Here, we added four candidate genes for isolated PSIS (*DCHS1*, *ROBO2*, *CCDC88C*, and *KIF14*) and one for syndromic PSIS (*KAT6A*). In addition, we found 11 *GLI2* variants in six patients and verified the higher frequency of a combination of two *GLI2* variants in the study group compared with a reference population. We detected various (potentially) pathogenic variants in genes associated with midline brain anomalies and in genes involved in ciliary structure and function and suggest that these genes be included in future searches for a polygenic cause of PSIS and MPHD.

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8

Variants in KAT6A and Pituitary Anomalies

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To The Editor:

With great interest we have read the article by Millan et al. describing six patients with pathogenic de novo variants in *KAT6A* (1). The patients shared a common phenotype of moderate to severe neurodevelopmental delay, severe speech delay, hypotonia, and a characteristic face. We recently identified another de novo *KAT6A* mutation in a girl with a similar phenotype who also had multiple pituitary hormone deficiencies associated with malformation of the pituitary gland.

After an uncomplicated pregnancy and delivery, the girl was born at term with a normal birth weight standard deviation score (SDS) +1.0, length SDS −0.7, and head circumference SDS +1.0. In the neonatal period she developed severe feeding difficulties, failure to thrive, and was hypotonic. Subsequently, she demonstrated developmental delay (sitting independently at 18 months, walking unaided at 30 months). Speech development was severely delayed (essentially non-verbal until age 6 years). Facial characteristics included marked ptosis for which she received surgical correction, a short nose with a full nasal tip, and small mouth with dental crowding (Table 1). Echocardiography failed to demonstrate a congenital heart defect and thyroid function tests, including free T4 at the age of 2 months, were within age specific reference ranges.

Persisting feeding difficulties necessitated gastric tube feeding which improved weight. Linear growth continued to decelerate, resulting in a height SDS of −5.0 at age 2 years. Subsequent endocrine investigations showed severe growth hormone deficiency (maximum growth hormone value 1.5 mU/L after stimulation with arginine [normal response >30 mU/L]). Repeat thyroid function testing results showed central (i.e., hypothalamic-pituitary) hypothyroidism (freeT4 8.3 pmol/L [reference range 10–23]; TSH 3.3 mU/L [reference range 0.5–5.0]), and subsequent dynamic testing of the hypothalamic pituitary adrenal axis demonstrated central adrenal insufficiency (maximum cortisol value 320 nmol/L after stimulation with low dose [=1 microgram] ACTH [normal response >550 nmol/L]). MRI imaging showed a small anterior pituitary lobe, absent pituitary stalk, and ectopic posterior pituitary lobe (Figure 1).

The diagnosis multiple pituitary hormone deficiencies (MPHD) due to Pituitary Stalk Interruption Syndrome (PSIS) was made, and treatment with growth hormone, levothyroxine, and hydrocortisone led to normalization of linear growth. Currently, at age 10 years, the girl's height is normal (138 cm; SDS: −1.1). Motor development has also progressed to normal abilities, and there are no longer feeding problems. Although speech delay is only mild, there is obvious cognitive impairment with a full scale IQ test result of 71 (Wechsler test).Within the framework of an ongoing study to detect novel genetic causes of congenital pituitary function abnormalities exome sequencing was performed in the girl and both parents which demonstrated a de novo *KAT6A* mutation: c.235C>T, p.(Arg79*) (NM_006766.4) (Figure 2). This mutation is in the first coding exon (exon 2), leading to a premature stop codon. No other likely pathogenic de novo

Table 1. Frequently reported clinical characteristics in patients with KAT6A mutation

Clinical characteristics	In number of patients from 3 case series[a]	In study patient
Global developmental delay	17/17	+
Speech delay	17/17	+
Neonatal hypotonia	14/16	+
Microcephaly	8/16	-
Facial dysmorphism		
Nasal anomalies *(broad nasal bridge/bulbous tip)*	16/17	+
Thin upper lip	13/17	-
Dental anomalies *(abnormally shaped teeth/dental crowding)*	5/17	+
Eye problems		
Ptosis	5/17	+
Strabismus	7/11	-
Gastrointestinal disease		
Feeding problems/reflux	12/17	+
Congenital heart disease *(patent ductus arteriosus, atrial septum defect, ventricular septum defect)*	10/15	-
Brain MRI anomalies		
Midline brain anomalies *(cavum septum pellucidum (2), missing olfactory bulb (1))*	3/16	+
Other brain anomalies *(cystic periventricular leukomalacia (1), minimal lateral ventricle hydrocephalus(1), Hyperintense signal posterior periventricular white matter (1))*	3/16	-

[a]Millan et al., 2016; Tham et al.,2015; Arboleda et al., 2015

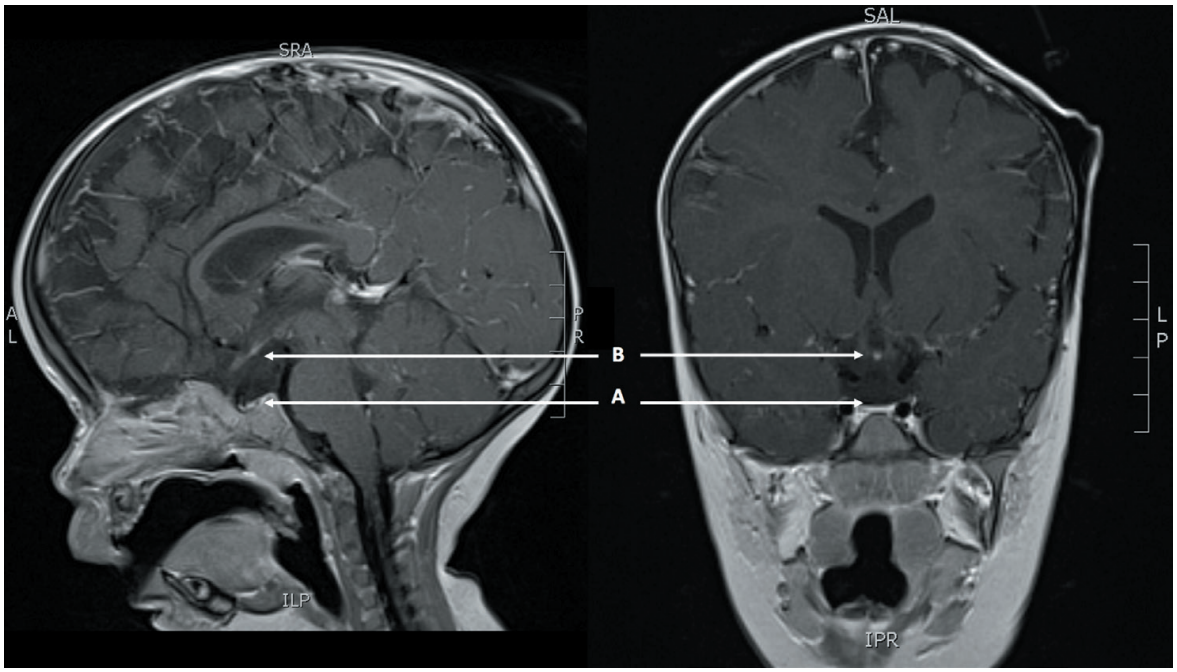

Figure 1. Sagittal and coronal MRI image of patient's brain with pituitary malformation. The pituitary stalk is absent. Note (A) small anterior pituitary lobe, and (B) ectopic posterior pituitary lobe.

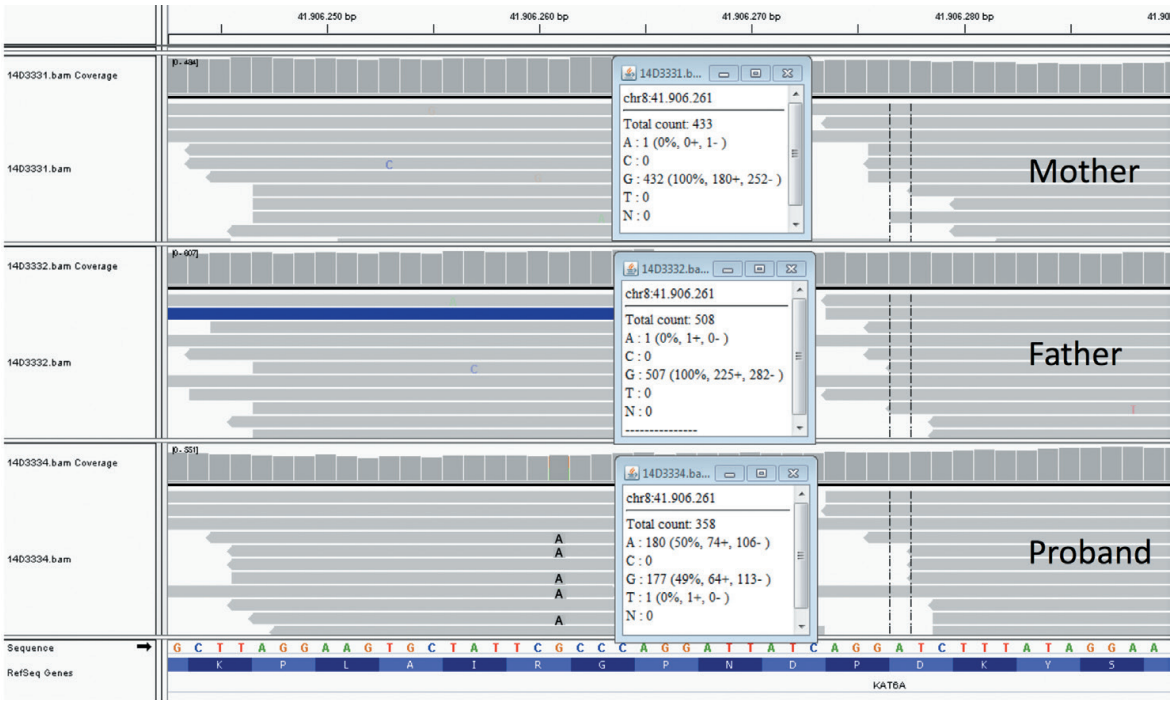

Figure 2. Screenshot showing aligned reads with MQ>30 at chromosome position chr8:41906261 (hg19) in Integrative Genomics Viewer IGV (http://software.broadinstitute.org/software/igv/). The mutation Chr8(GRCh37):g.41906261G>A is predicted to result in a premature stop codon in *KAT6A* (c.235C>T; p.[Arg79*]).

variants and homozygous or compound heterozygous variants were found. In a targeted analysis of exome data searching for rare variants (frequency <1% in the general population) in 37 genes known to be associated with pituitary development and/or function (*ARNT2*, *BMP2*, *BMP4*, *CDON*, *FGF8*, *FGF10*, *FGF18*, *FGFR1*, *GATA2*, *GLI1-3*, *GPR161*, *HESX1*, *IGSF1*, *LHX3*, *LHX4*, *NR5A1*, *OTX2*, *PAX6*, *PITX1*, *PITX2*, *POU1F1*, *PROP1*, *SHH*, *SIX1-6*, *SOX1-3*, *TBX19*, *TGIF*, *WNT5a* (2,3)) we found two potentially pathogenic variants: a deletion in *BMP4* (p.R269_L272del, not described before) and a missense mutation in *GLI3* (c.539G>A, p.R180Q, rs140772904 [dbSNP database], minor allele frequency 0.0002 [based on 1000Genomes project], predicted to be "probably damaging" by in silico prediction Polyphen2). Both variants were also present in the unaffected father. The capture used to enrich for exome sequences was Agilent SureSelect Human All Exon V5 (50M) kit. The average depth of sequencing of the proband was 164.47× and the fraction of target covered >20× was 96.55%.

PSIS is a well-known cause of congenital MPHD (5). The pathogenesis of PSIS remains usually unknown, as mutations in genes involved in embryonic pituitary development (*HESX1*, *LHX4*, *OTX2*, *SOX3*, *PROKR2*) are detected in less than 5% of patients. PSIS belongs to the spectrum of midline brain abnormalities and is often associated with other extra-pituitary midline malformations (5). Thus far 17 patients with dominantly acting mutations in *KAT6A* have been

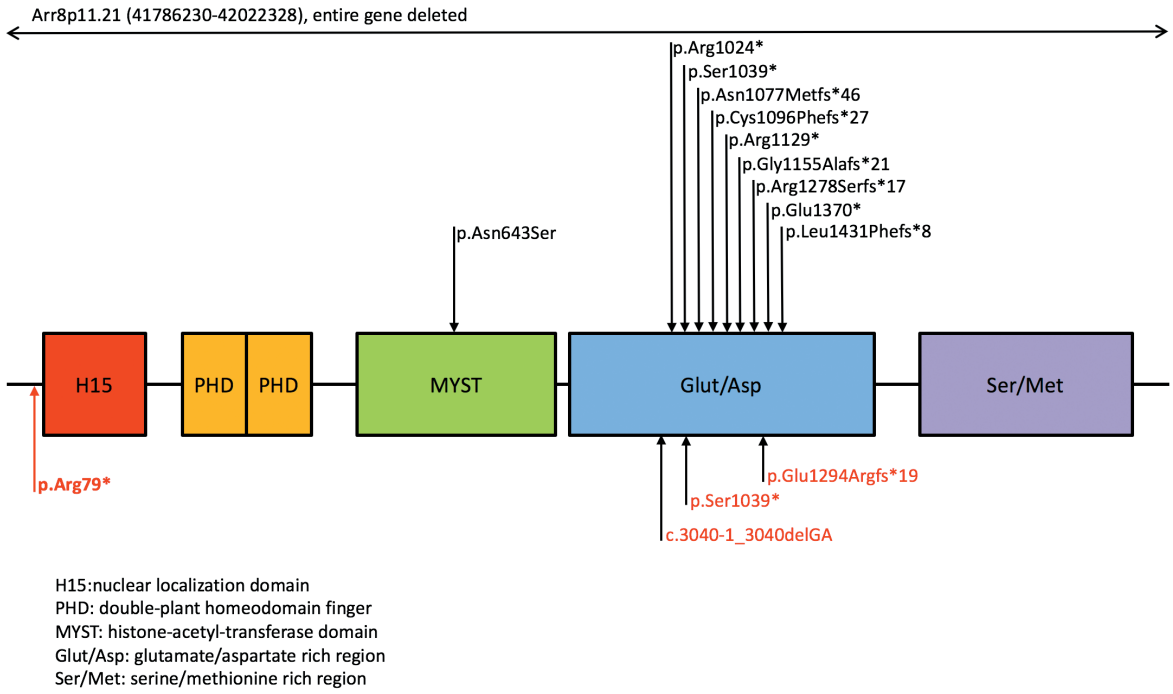

Figure 3. Cartoon showing the present (p.Arg79*) and all earlier reported pathogenic mutations in *KAT6A*, including a microdeletion with deletion of the entire gene. Mutations in patients with midline disturbances of the brain are indicated in red (p.Arg79*, c.3040-1_3040delGA, p.Ser1039*, p.Glu1294Argfs*19).

described, and in all affected individuals the phenotype consisted of neurodevelopmental and speech delay, and a typical facial morphology (1,6,7). Brain MRI studies were reported in 16 of these children, and demonstrated subtle midline brain abnormalities in three: one had a missing olfactory bulb (1), and two had a cavum of the septum pellucidum (7). Pituitary abnormalities were not described, and in none of the earlier reports endocrine deficiencies were mentioned. The *KAT6A* mutation in our patient lies in the first coding exon, just before the H15 domain while most of the reported mutations, also mainly truncating mutations, are in the acidic glutamate/aspartate rich region (Figure 3). Since all patients have a similar phenotype, including one patient with a complete deletion of the gene, the location of the truncating mutation does not seem to influence the phenotype and indicates haploinsufficiency of *KAT6A* as cause of the phenotype.

In the present patient, the PSIS may be a coincidental finding. Since there is evidence for a polygenic cause for PSIS we also cannot rule out a contribution of the *BMP4* and *GLI3* variants (Fang et al., 2016). However, since (subtle) midline defects have been reported in other *KAT6A* mutated patients, PSIS may be part of the phenotypic spectrum, and because of the obvious consequences for clinical management it may be prudent to evaluate patients for endocrine disturbances, especially in case of linear growth deceleration.

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V

Summary

General discussion and future perspectives

Dutch summary

Dankwoord

List of publications

Curriculum vitae

SUMMARY

The focus of the studies described in this thesis lies on diagnostics and pathogenesis of congenital central hypothyroidism (CH-C). The importance of, and difficulties in diagnosing CH-C are emphasized while novel strategies are developed to alleviate these difficulties. In addition, the complex polygenic etiology of CH-C within the framework of multiple pituitary hormone deficiencies (MPHD) is highlighted.

Since 1995, the Dutch neonatal congenital hypothyroidism screening program has consisted of a three-step screening approach, measuring T4 in all neonates with additional TSH determination in the lowest 20% of T4 concentrations and thyroxine-binding globulin (TBG) measurement in the lowest 5% of T4 concentrations (17,18). This screening approach has proven to be very effective in detecting CH-C, probably accounting for the world's highest CH-C prevalence of around 1 in 16,000 neonates in The Netherlands (19).

A frequently used argument against screening for CH-C is the presumed mild severity of the hypothyroidism not likely to be associated with brain damage. In the latest European Society for Pediatric Endocrinology (ESPE) consensus guidelines on screening, diagnosis and management of congenital hypothyroidism (CH), severity of CH is classified based on initial free T4 (FT4) concentrations; severe CH: FT4 < 5 pmol/l, moderate CH: FT4 5 - <10 pmol/l, mild CH: FT4 ≥ 10 pmol/l (23). In the study described in **Chapter 2** we used this classification to assess disease severity in 143 patients with CH-C and 1057 patients with CH of thyroidal origin (CH-T). Based on FT4 concentrations four children with CH-C (2.8%) had severe CH, 75 (52.4%) moderate and 64 (44.8%) mild. In the CH-T group 280 children (26.5%) had severe CH, 341 (32.3%) moderate and 436 (41.2%) mild. Our results indicate that, based on initial FT4-values, severe CH was much more prevalent in CH-T (26.5%) compared to CH-C (2.8%). However, CH-C itself should not be considered as only mild since more than half of CH-C patients had moderate CH with initial FT4 below 10 pmol/l (24). The clinical relevance of moderate CH is not questioned, and the ESPE consensus guidelines for CH advise that treatment with T4 should be started immediately when FT4 values are below norms for age to prevent brain damage due to thyroid hormone deficiency (23).

Besides the degree of hypothyroidism, the severity of CH-C is also determined by the presence of other pituitary hormone deficiencies such as growth hormone (GH) deficiency and adrenocorticotrophic hormone (ACTH) deficiency that are potentially brain damaging and life threatening, due to hypoglycemia and circulatory insufficiency. Early detection and treatment of children with CH-C is assumed to reduce morbidity and mortality, especially in cases of MPHD (18,24). However, actual follow-up data on morbidity and mortality in early or late diagnosed CH-C or MPHD are lacking. In **Chapter 3** the mortality in the Dutch cohort of CH-C patients detected by neonatal screening between 1995 and 2013 is described. Since the start of the Dutch neonatal screening program in 1981, the Netherlands Organization for Scientific Research

(TNO Leiden) registers all abnormal neonatal screening results for CH and collects data on diagnoses by sending out questionnaires to treating pediatricians at several time points. A first questionnaire is sent out in the first month after a child is referred, a second questionnaire at the age of four years. To verify the diagnosis CH-C and to trace patients for a long-term follow-up study, we sent out a third questionnaire. Subsequently, we received several notifications of deaths of CH-C patients, prompting us to collect data on mortality. The TNO database contained 141 patients classified as CH-C and detected by neonatal screening between January 1, 1995 and January 1, 2013. Total observation time was 1414 years, with a median follow up duration of 10.2 years. Two patients did not have CH-C and one MPHD patient could not be traced. Of the remaining 138 patients, 82 patients had MPHD (69.5% male) and 56 patients had isolated CH-C (82.4% male). Three patients had isolated CH-C combined with a congenital disorder of glycosylation syndrome (CDG).

In the studied cohort fifteen patients (10 MPHD and 5 isolated CH-C) had died (mortality rate 10.9%; 108.6 per 1000). Nine patients died within the first year of life, resulting in an infant mortality rate (IMR) of 65.2 per 1000. The Dutch IMR in the same period was 4.7 per 1000 live born children (Odds ratio 14.5, 95% confidence interval 7.6–29.3, $p<0.0001$). Fourteen patients died before the age of 5, corresponding with an under-5 mortality of 101.4 per 1000 live born children, compared to 5.4 per 1000 live born children in the Netherlands during the same time period (Odds ratio 21.1, 95% confidence interval 12.1-36.6, $p<0.0001$). Main causes of death were congenital brain and cardiac malformations, birth asphyxia and infections. In only one patient cause of death was attributed to pituitary hormone deficiency. Given the association between hypopituitarism and cerebral anomalies an increased mortality in this group of patients was to be expected and therefore the results of this study are not surprising. However, at the same time, by tracing all patients with CH-C ± MPHD detected by neonatal screening since 1995, we were able to show that the mortality in hypopituitarism without concurrent diagnoses is low: in our cohort only one patient died. In conclusion, we report an increased mortality in early detected CH-C patients which does not seem to be related to endocrine disease. This suggests that mortality due to pituitary insufficiency is low in an early detected and treated CH-C population. It is important to realize that CH-C is not easy to diagnose. Since patients were diagnosed by various pediatricians, misdiagnosis cannot be ruled. In the deceased patients with presumed isolated CH-C and other severe illnesses the abnormal thyroid function tests might be explained by non-thyroidal illness.

The diagnosis central hypothyroidism is usually made when a low serum free thyroxine (FT4) concentration is accompanied by a “normal”, or low TSH concentration. However, due to the presence of TSH with reduced bioactivity, TSH concentrations as measured by immunoassays are not always normal or low but may be even slightly elevated (25,26,55). This may make distinguishing CH-C from mild primary hypothyroidism a challenge. In *acquired* forms of central

hypothyroidism, TSH concentrations have been reported to range from well below the reference range up to around 11 mU/l (upper limit reference range 4.0) (54-56). Since there were no reports on TSH concentrations in large groups of patients with *congenital* central hypothyroidism we studied TSH concentrations in 120 children with CH-C using children with TBG deficiency, which does not lead to any signs or symptoms, as a control group (**Chapter 4**). In addition, we studied whether FT4 levels are helpful in distinguishing CH-C from mild CH-T. The study included 50 patients with isolated CH-C, 70 patients with MPHD and 350 controls (TBG deficiency). First measured diagnostic TSH values were significantly different between the CH-C group and the control group with a higher percentage of low and higher percentage of elevated TSH values in the CH-C group. TSH was significantly higher in MPHD compared to isolated CH-C with a highest TSH of 12.9 mU/l. When comparing FT4 concentrations in newborns with CH-C to those in a group of newborns with mild CH-T with a similar TSH (<13 mU/l), FT4 concentrations were significantly lower in the CH-C patients. In conclusion, TSH values in CH-C are more often lower as well as more often higher compared to controls. TSH concentrations were higher in CH-C within the framework of MPHD compared to isolated CH-C, probably indicating the hypothalamic origin of the hypothyroidism in most MPHD patients. CH-C patients may have TSH concentrations up to 13 mU/l. FT4 levels were lower in CH-C compared to CH-T, and may be helpful in distinguishing CH-C from mild CH-T (59). In our study, we encountered 5 patients who may have been misclassified as CH-T of which two most likely should have been classified as CH-C. This emphasizes how challenging diagnosing CH-C may be.

The thyroid function parameters TSH, FT4 and FT3 show large inter-individual differences leading to wide laboratory reference intervals, while the intra-individual variability is much smaller, suggesting that each individual has his or her own specific hypothalamic-pituitary-thyroid (HPT) axis set point (27,28). Diagnosing central hypothyroidism relies on determining whether FT4 levels are too low. In general, FT4 levels below age specific reference intervals are considered to indicate hypothyroidism. However, this does not take into account the individual's FT4 set point. Both genetic and environmental factors seem to contribute to this individual set point (29). To test the hypothesis that the fetal environment determines the postnatal thyroid hormone concentration and the T4 set point, we conducted a classical twin-study, comparing the resemblance of neonatal screening blood T4-concentrations in 1264 mono- and 2566 dizygotic twin pairs retrieved from the population-based Netherlands Twin Register (**Chapter 5)**. Maximum-likelihood estimates of variance explained by genetic and environmental influences were obtained by structural equation modelling in data from full- and pre-term twin pairs.

In full-term infants, genetic factors were found to explain 31-40% of the variation in postnatal T4 concentrations, and environmental factors (unique and shared) were responsible for 60-69% of the variation. So, while genetics do influence postnatal T4 concentrations, the environment seems to play an important role. Since blood T4 concentrations were measured on average on

the 5th day of life, the fetal environment is the most likely candidate for the shared environmental influences on postnatal T4 concentrations. Genetic influences on the T4 set point diminished with declining gestational age, especially in girls. This may be due to major environmental influences, like immaturity and non-thyroidal illness in very preterm infants (32).

Given that both genetic and environmental factors are important in HPT axis set point determination and that, after the fetal period, the HPT axis continues to mature postnatally, we hypothesized that thyroxine treatment in the first years of life, resulting in a clearly higher than normal plasma FT4 concentration, may cause an HPT axis set point change that persists later on in life. In a follow-up study of a randomized controlled trial (RCT) comparing thyroxine treatment vs placebo during the first two years of life in a group of children with Down syndrome, we were able to analyze thyroid function almost nine years later (**Chapter 6**). We included 123 children with Down syndrome 8.7 years after the end of the RCT and performed thyroid function tests and thyroid ultrasound. We analyzed TSH and FT4 concentrations in the subgroup of 71 children who were currently not on thyroid medication and had no evidence of auto-immune thyroiditis. FT4 was significantly higher in the thyroxine treated group compared with the placebo group (14.1 vs. 13.0 pmol/L; P=0.02) while TSH concentrations were the same.

There was an increase in anti-TPO positivity, from 1% at age 12 months, to 6 % at age 24 months and 25% at age 10.7 years with a greater percentage of children with anti-TPO positivity in the placebo group (32%) compared with the thyroxine treated group (18.5%) (P=0.12).

Thyroid volume at age 10.7 years (mean 3.4 ml; range 0.5-7.5 ml) was significantly lower (P<0.01) compared with reference values (5.5 ml; range 3-9 ml) and was similar in the thyroxine and placebo group. In conclusion, thyroxine treatment during the first two years of life led to a mild increase in FT4 almost 9 years later on and this observation may point to an interesting new mechanism influencing the maturing HPT axis set point. Furthermore, there was a trend towards less development of thyroid autoimmunity in the thyroxine treatment group, suggesting a protective effect of the early thyroxine treatment. Lastly, thyroid volume was low possibly reflecting Down specific thyroid hypoplasia (38).

Given the difficulties in diagnosing central hypothyroidism based on thyroid hormone parameters alone, genetic confirmation would be of great value. Unfortunately, genetic causes are only found in a minority of CH-C case (4,39). The majority of CH-C cases is not isolated and occurs within the framework of multiple pituitary hormone deficiencies (MPHD). Most patients with MPHD exhibit a pituitary malformation characterized by an absent or thin pituitary stalk, a hypoplastic anterior pituitary lobe and an ectopic posterior pituitary lobe. This malformation is known as pituitary stalk interruption syndrome (PSIS) (47). PSIS often occurs in isolation but may be accompanied by additional midline brain abnormalities (syndromic PSIS).

A number of transcription factors are involved in the initial formation of the pituitary gland and the following cellular differentiation. However, mutations in genes encoding these tran-

scription factors have been found in only less than 5% of PSIS cases, and mostly in syndromic forms (48). Various genetic studies show an association between PSIS and genes involved in holoprosencephaly, which is characterized by severely disturbed midline brain development, suggesting that PSIS may represent a mild form of this disorder (60). Especially *GLI2* variants, associated with holoprosencephaly, have been reported in a relatively high frequency in isolated PSIS (61-64). Furthermore, genes involved in hypogonadotropic hypogonadism (*PROKR2, FGF8, FGFR1*) have been associated with MPHD (65-68). However, clinical expression is variable, including unaffected mutation carrying relatives suggesting involvement of additional genetic or environmental factors. Most studies searching for genetic causes of MPHD have been done in heterogeneous patient populations including patients with other midline brain malformations, and most studies have focused on pathogenic mutations in specific genes, assuming a Mendelian inheritance (69). As Mendelian forms of PSIS are detected only rarely, a polygenic and multifactorial etiology for PSIS should be considered as well (48).

To provide further evidence for a non-Mendelian, polygenic etiology of PSIS, we performed whole exome sequencing (WES) in 20 patients with isolated PSIS and their unaffected parents (**Chapter 7**). In addition to searching for (potentially) pathogenic *de novo* and biallelic variants, we performed a targeted search in a large panel of genes associated with midline brain development. We included genes with a known association with pituitary formation and also genes associated with other midline brain malformations: holoprosencephaly, hypogonadotropic hypogonadism and absent corpus callosum. For each patient, all pathogenic variants and variants of unknown clinical significance (potentially pathogenic) were listed. Since rare *GLI2* variants have been frequently reported in PSIS, we searched for (potentially) pathogenic *GLI2* and also for all relatively rare *GLI2* variants (reported frequency <5% in the general population). Although rare *GLI2* variants have been reported in association with PSIS, the frequency of (combinations of) these rare variants in the general population has not been investigated. To verify whether these rare *GLI2* variants are indeed more common in PSIS, we compared the frequency of these variants in our study group to a reference population. We found four new candidate genes for isolated PSIS (*DCHS1*, *ROBO2*, *CCDC88C, KIF14)* and one for syndromic PSIS *(KAT6A*). Eleven *GLI2* variants were present in six patients. A higher frequency of a combination of two *GLI2* variants (M1352V+D1520N) was found in the study group compared to a reference population (10% vs 0.68%). Various (potentially) pathogenic variants were identified in genes associated with midline brain anomalies, including holoprosencephaly, hypogonadotropic hypogonadism and absent corpus callosum, and in genes involved in ciliary structure and function (70).

In the abovementioned exome sequencing study, we identified a child with a pathogenic *KAT6A* gene mutation (case report described in **Chapter 8**). This patient had developmental delay and severely delayed speech which had been attributed to hypoglycemic brain damage due to untreated ACTH deficiency and growth hormone deficiency in the first two years of life. In retrospect, the patient fulfilled all the criteria for KAT6A neurodevelopmental disorder

and should not have been included in the exome sequencing study on isolated PSIS (50-52). In this patient, the PSIS may be a coincidental finding. However, since (subtle) midline defects have been reported in other *KAT6A* mutated patients, PSIS may be part of the phenotypic spectrum (71).

GENERAL DISCUSSION AND FUTURE PERSPECTIVES

Worldwide most neonatal screening programs focus on detecting congenital hypothyroidism of thyroidal origin (CH-T) and do not detect congenital hypothyroidism of central origin (CH-C) (16). A frequently used argument against screening is the presumed mild hypothyroid character not likely to cause brain damage. In this thesis we showed that the severity of CH-C should not be underestimated (24). Congenital central hypothyroidism cannot be considered only mild since more than half of patients have moderate to severe hypothyroidism with initial FT4 below 10 pmol/l. These results make a strong argument for adapting existing neonatal CH screening programs to enable detection of both CH-T and CH-C. The importance of this message was emphasized by the inclusion of the abstract addressing this issue in the Yearbook of Pediatric Endocrinology 2015 of the European Society of Pediatric Endocrinology as one of the highlights of publications in the field of Pediatric Endocrinology that year (72). Of course, adapting existing screening programs is not easily done and will have financial consequences, depending on local logistic circumstances. In the Netherlands, the introduction of the T4/TBG ratio into a program using a primary T4 with supplemental TSH approach generated an extra cost of $11,206 per additional case detected and was considered to be acceptable (17).

The most important argument to screen for CH-C should be that early detection and treatment of central hypothyroidism and associated pituitary deficiencies actually improves mortality and morbidity. Studies comparing mortality and morbidity of CH-C in screened and unscreened populations are lacking. We studied the mortality in the Dutch cohort of screened and early treated CH-C and found an increased mortality rate that was mainly due to severe congenital malformations and infections. In only one case death was due to acute adrenal insufficiency in a child receiving treatment for MPHD. These results indicate that the mortality due to endocrine deficiencies is low in an early treated population. Unfortunately, data on mortality in unscreened populations is lacking. As mentioned earlier the reported prevalence of CH-C in the Netherlands is one of the world's highest probably as a result of an effective neonatal screening program. Since the prevalence of CH-T does not differ much between countries with screening programs it is reasonable to expect the prevalence of CH-C also to be similar worldwide. This suggests that most cases of CH-C in unscreened populations remain undetected. Perhaps early death in children with MPHD due to neonatal hypoglycemia or acute adrenal insufficiency might partly explain the lower prevalence. It may be particularly difficult to get a proper estimate of mortality in unscreened populations due to misdiagnosis. For example, in cases of acute adrenal insufficiency in an unrecognized MPHD patient, death may be falsely attributed to other causes such as sepsis-like syndrome or sudden infant death syndrome.

Data on morbidity and developmental outcome in early versus late treated CH-C are lacking. We only found two reports on outcome in late diagnosed CH-C (73,74). Of the first study only the abstract was available reporting an IQ of below 90 in 9 out of 33 patients with idiopathic central hypothyroidism based on a clinical diagnosis (73). In the second study developmental delay was

seen in 19 out of 34 (56%) children with CH-C with an average age at diagnosis of 16.9 months (74). Almost all these children had multiple pituitary hormone deficiencies including ACTH and GH deficiency. Currently, we are conducting a nation-wide follow-up study on morbidity and developmental outcome in early detected CH-C patients. All patients with CH-C (both isolated forms and within the framework of MPHD) detected by neonatal screening since 1995 are invited to our department at the Academic Medical Center, Amsterdam, to undergo neurodevelopmental tests. In this study, we are using healthy siblings as a control group. To answer the question whether screening is superior to clinical case detection in regards to outcome, the results of this study will need to be compared to groups of patients with late diagnosed CH-C. This implies international collaboration comparing screened versus unscreened populations.

We would like to make a plea for adapting existing neonatal screening programs in order to include detection of CH-C. However, even after a child presents with an abnormal neonatal screening result indicating CH-C, confirming the diagnosis CH-C may be challenging. As we showed in chapter 3, distinguishing CH-C from mild CH-T can be quite difficult and in our study several patients appeared to have been misclassified (59). Mildly elevated TSH (up to 13 mU/l), may not only indicate mild CH-T but also CH-C. Therefore, mildly elevated TSH levels should be carefully interpreted. If FT4 levels are below the reference range, CH-C must be considered. Additional analysis (e.g. thyroid imaging, pituitary function testing) and careful monitoring of thyroid function tests in the first months of life may provide evidence for a more certain diagnosis.

The diagnosis of CH-C depends on determining when free T4 levels are too low. This is complicated by the lack of neonatal reference values for thyroid function tests. Even with the availability of proper reference intervals, ideally, the individual's HPT axis set point should to be taken into account. Knowing a patient's unique HPT axis set point would not only help in identifying when thyroid hormone levels are too low but also provide an individual treatment target. Up to 10% of patients treated for thyroid disease report disabling complaints of hypo- or hyperthyroidism despite having thyroid hormone levels within population-based reference intervals (75). Being able to predict an individual's HPT axis set point is an important step towards personalized medicine.

Both genetic and environmental factors are involved in determining this set point (28,29). By comparing neonatal T4 screening results in a classical twin study we showed that the fetal environment is important in determining the individual T4 set point (32). We hypothesize that the mechanism underlying T4 set point determination lies in epigenetic modifications of genes involved in the HPT axis development during fetal life. This set point continues to mature during the first years of life and we showed that an intervention with thyroxine treatment during the first two years leads to a small but significant set point change measurable nine years later on. Various genetic studies have revealed several candidate genes that may be involved in T4 set point determination (76-78). However, the contribution of each of these genes to the variability in thyroid hormone concentrations is small. The observation that set point may be influenced

by environmental factors suggests that studies on determinants of T4 set point should not only focus on genetic variants but also on epigenetic factors such as DNA methylation, histone modification and microRNAs.

In the last chapter of this thesis we focused on searching for a genetic cause of pituitary stalk interruption syndrome (PSIS). In our study, using exome sequencing, we identified various new candidate genes and various potentially pathogenic variants in genes associated with midline brain anomalies (70). Our study provides further evidence for a polygenic background of PSIS, including the involvement of various genes associated with midline brain anomalies such as holoprosencephaly (especially *GLI2* gene), hypogonadotropic hypogonadism and absent corpus callosum. Functional studies are necessary to provide more evidence for the role of each individual new candidate gene in pituitary development. Besides the involvement of multiple gene variants, epigenetic mechanisms such as DNA methylation, histone modification and microRNAs may also play a role, and exploring these factors will require additional studies.

The case report of the patient with KAT6A neurodevelopmental syndrome illustrates the importance of careful phenotyping. We intended to include only isolated cases of PSIS in the exome sequencing study but in retrospect this patient fulfilled all the criteria for KAT6A neurodevelopmental syndrome and indeed had a pathogenic *KAT6A* mutation (50-52,71). This is the first report of KAT6A neurodevelopmental syndrome with a pituitary abnormality. Since other midline abnormalities have been described in KAT6A syndrome, PSIS may be part of the phenotypic spectrum. Because of the obvious consequences for clinical management it may be prudent to evaluate patients with KAT6A syndrome for endocrine disturbances, especially in case of linear growth deceleration.

Systematically investigating large series of carefully phenotyped patients, and collaboration between research groups are essential elements in the search for genes and pathways underlying disorders with such a polygenic etiology.

In this thesis, we emphasize the importance of diagnosing CH-C. Despite the use of a T4-based screening program, confirming the diagnosis CH-C in case of an abnormal screening result is not always straightforward. Low T4 and FT4 concentrations in a newborn with severe illness may be due to non-thyroidal illness. Distinguishing mild CH-C from normal and distinguishing CH-C from mild CH-T may be difficult. As shown in chapter 4, in case of a low FT4 accompanied by a TSH up to 13 mIU/l the diagnosis CH-C seems more likely than mild CH-T (59). Determining whether FT4 is low is essential for the diagnosis CH-C. Knowing an individual's FT4 set point would be helpful and although knowledge on factors determining FT4 set point is growing, we are still far from using FT4 set point in clinical practice. In addition to TSH and FT4 other thyroid hormone parameters should be taken into consideration. For example, elevated concentrations of reverse T3 will help to identify non-thyroidal illness. The value of the thyrotropin-releasing hormone (TRH) stimulation test has been questioned although an exaggerated high TSH response (so-called tertiary response) does seem to be associated with CH-C within the framework of MPHD

(79,80). A blunted nocturnal TSH surge may indicate central hypothyroidism but this is of little value in an infant with a developing day-night rhythm (81). Ultimately specific tissue parameters would be helpful in distinguishing a hypothyroid state from a euthyroid state. Lipid-profiles and sex hormone binding globulin (SHBG) have long been known to be correlated with thyroid function but have limited clinical value because they are also influenced by non-thyroidal disturbances (82,83). In recent years various studies have analyzed proteins and metabolites in an attempt to associate specific molecular patterns with thyroid hormone function. These so-called proteomes and metabolomes consist of molecules involved in various systems such as energy expenditure, defense mechanisms against oxidative stress, glucose metabolism and pro-thrombotic and pro-inflammatory state (84-86). In future, these patterns may prove to be very valuable in diagnosing hypo- and hyperthyroid states and also in evaluating treatment practices.

The lack of proper neonatal reference values for FT4 in the first weeks of life complicates diagnosing mild cases of CH-C. Within the first 72 hours after birth full-term infants display TSH surge, secondary to cold-induced TRH secretion (87). This TSH surge leads to FT4 concentrations peaking at day three of life, and slowly declining thereafter. As a consequence, thyroid hormone concentrations vary from day-to-day. This is important to realize when interpreting FT4 concentrations within the first few weeks. In an attempt to create day-to-day specific reference values our research group is performing a meta-analysis of thyroid function tests. In addition, we are performing a prospective study in which we are collecting venous blood samples in healthy newborns around the 4th day of life (simultaneously with the newborn screening) and at the age of 14 days. Preliminary data indicate a lower limit of the FT4 reference range at 14 days of around 14 pmol/l. Since referral for a screening result suspect of CH-C is usually at age 14 days these data are very important for clinical practice in the Netherlands. In the Netherlands, the most used FT4 assays have an adult reference range of 10-22 pmol/l. In the current Dutch CH-C guidelines, the lower limit of the reference range in neonates is considered to be 12 pmol/l (88). Based on our data infants with a FT4 concentrations up to 14 pmol/l may have CH-C, especially in combination with TSH concentrations below 13 mIU/l. These children should be evaluated for potential additional pituitary deficiencies. In a child with FT4 concentrations between 12 and 14 pmol/l and the absence of signs or symptoms indicating CH-C it seems wise to perform serial FT4 measurements. Additional pituitary function testing, genetic testing and pituitary imaging may aid in confirming the diagnosis. When FT4 persists to be around or below the lower limit of 12 pmol/l treatment with thyroxine should be considered. In cases of isolated CH-C without genetic confirmation, treatment may be stopped at the age of 3 years for reevaluation.

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NEDERLANDSE SAMENVATTING

De hypothalamus-hypofyse-schildklier-as zorgt voor een optimaal schildklierhormoongehalte in het bloed. In de hypothalamus wordt “thyrotropin-releasing-hormone” (TRH) geproduceerd, onder andere in neurosecretoire neuronen in de nucleus paraventricularis die projecteren naar de median eminence. Dit TRH stimuleert de hypofyse tot de aanmaak en afgifte van “thyroid-stimulating-hormone” (TSH). TSH stimuleert vervolgens de schildklier tot de aanmaak en afgifte van schildklierhormoon (thyroxine en triiodothyronine; afgekort T4, resp. T3). Via een negatief terugkoppelingsmechanisme wordt de productie van TRH en TSH geremd door schildklierhormoon. Hiermee wordt het schildklierhormoongehalte in het bloed binnen nauwe grenzen gehouden.

In geval van een tekort aan schildklierhormoon door een probleem op het niveau van de schildklier (= primaire hypothyreoïdie) zullen de verlaagde schildklierhormoonwaardes zorgen voor een toegenomen afgifte van het hypothalame TRH en hypofysaire TSH. Het verhoogde TSH gehalte in het bloed kan worden gebruikt om de diagnose primaire hypothyreoïdie te stellen. In het geval van centrale hypothyreoïdie is de oorzaak van de verlaagde schildklierhormoon productie juist een probleem op het niveau van de hypothalamus of hypofyse. Het TSH gehalte zal dan niet verhoogd zijn en het meten van TSH helpt dan dus niet bij het stellen van de diagnose. De diagnose centrale hypothyreoïdie is daarom gebaseerd op het herkennen van situaties waarin de schildklierhormoonwaardes zelf te laag zijn. Bij een verworven vorm van centrale hypothyreoïdie kan de medische voorgeschiedenis, zoals bijvoorbeeld hypofyse chirurgie, hersenbestraling of een neurotrauma een aanwijzing vormen en helpen bij de diagnose. Aangezien een medische voorgeschiedenis ontbreekt bij pasgeborenen is het diagnosticeren van congenitale (= aangeboren) centrale hypothyreoïdie (CH-C) vaak een uitdaging.

De studies in dit proefschrift richten zich op de diagnostiek en pathogenese van CH-C. Het belang van het stellen van de diagnose alsook de moeilijkheden bij het stellen van de diagnose CH-C worden belicht. Tevens wordt de complexe genetische achtergrond van CH-C gecombineerd met multipele hypofyse hormoon uitval bestudeerd.

Omdat schildklierhormoon van essentieel belang is voor normale groei en hersenontwikkeling kan een aangeboren tekort aan dit hormoon leiden tot ernstige ontwikkelingsachterstand. Sinds eind jaren 70 van de vorige eeuw worden in de meeste Westerse landen alle kinderen kort na de geboorte gescreend op een aangeboren schildklierhormoontekort. Dit gebeurt door middel van de neonatale hielprik. Het is aangetoond dat in de eerste levensweken gestarte behandeling met schildklierhormoon leidt tot een vrijwel normale groei en ontwikkeling. In de meeste landen wordt TSH gebruikt om te screenen op een aangeboren tekort aan schildklierhormoon (= congenitale hypothyreoïdie; afgekort CH). Hiermee worden kinderen met een aandoening van de schildklier zelf (congenitale primaire hypothyreoïdie; afgekort CH-T) effectief opgespoord. Kinderen met CH-C zullen hiermee echter niet opgespoord worden. In

Nederland wordt sinds 1995 een unieke drie-staps screeningsmethode gebruikt waarin eerst T4 gemeten wordt, gevolgd door een TSH meting bij de kinderen met de 20% laagste T4 gehaltes en een meting van het schildklierhormoonbindend eiwit (TBG) bij kinderen met de laagste 5% T4 gehaltes. De Nederlandse methode om te screenen op congenitale hypothyreoïdie is bewezen effectief in het opsporen van zowel CH-T als CH-C.

Een vaak gebruikt argument om niet op CH-C te screenen is de aanname dat de mate van schildklierhormoontekort bij centrale hypothyreoïdie minder ernstig is dan bij primaire hypothyreoïdie en minder risico geeft op hersenschade. Door de European Society for Pediatric Endocrinology (ESPE) wordt de ernst van hypothyreoïdie geclassificeerd op basis van initiële vrij T4 concentraties; ernstige CH (vrij T4 < 5 pmol/l), matig ernstige CH (vrij T4 5-10 pmol/l) en milde CH (vrij T4 ≥ 10 pmol/l).

In **hoofdstuk 2** onderzochten we de ernst van CH-C in 143 kinderen met CH-C en 1057 kinderen met CH-T opgespoord in de Nederlandse CH screening. Op basis van de vrij T4 concentraties bij diagnose werd in de CH-C groep 2.8% geclassificeerd als ernstige CH, 52.4% als matig ernstige CH en 44.8% als milde CH. In de CH-T groep werd 26.5% geclassificeerd als ernstige CH, 32.3% als matig ernstige CH en 41.2% als milde CH. Deze resultaten laten zien dat, op basis van initiële vrijT4 concentraties, ernstige CH veel vaker voorkomt bij CH-T dan bij CH-C. CH-C mag echter niet slechts als mild beschouwd worden aangezien meer dan de helft van de patiënten matig ernstige CH heeft met een initieel vrij T4 kleiner dan 10 pmol/l. Bij matig ernstige CH-C wordt niet getwijfeld aan de noodzaak van behandeling en wordt in de ESPE richtlijn geadviseerd direct met behandeling te starten om hersenschade door schildklierhormoon te voorkomen. De resultaten van onze studie benadrukken het belang van screening op CH-C.

De meerderheid van patiënten met CH-C heeft uitval van meer dan één hypofyse hormoon (= multiple pituitary hormone deficiencies; afgekort MPHD). In het bijzonder groeihormoontekort en tekort aan cortisol kunnen leiden tot levensgevaarlijke hypoglykemieën en circulatoire insufficiëntie. De belangrijkste reden om kinderen met CH-C vroeg op te sporen en te behandelen berust op de aanname dat vroeg gestarte behandeling morbiditeit en mortaliteit vermindert. Er zijn echter geen gegevens van morbiditeit en mortaliteit bekend. In **hoofdstuk 3** hebben we de mortaliteit onderzocht in het cohort CH-C patiënten dat tussen 1995 en 2013 met de neonatale screening opgespoord werd. Sinds de start van het Nederlandse neonatale CH screeningsprogramma worden alle afwijkende uitslagen geregistreerd door TNO in Leiden. In de TNO database stonden 141 patiënten geregistreerd met de diagnose CH-C, gedetecteerd tussen 1995 en 2013. Om alle patiënten te traceren werd een vragenlijst gestuurd aan de behandelend (kinder-) artsen. Bij twee patiënten bleken niet de diagnose CH-C te hebben en één patiënt konden we niet traceren. Van de overige 138 CH-C patiënten (82 MPHD en 56 geïsoleerde CH-C) waren 15 patiënten overleden (10 MPHD en 5 geïsoleerde CH-C; mortaliteit van 10.9%). Negen kinderen waren overleden in het eerste levensjaar (zuigelingensterfte van 6.5%). Dit was veel hoger dan de totale Nederlandse zuigelingensterfte in diezelfde periode van 0.47%. De doodsoorzaken waren voornamelijk ernstige congenitale hersenafwijkingen, congenitale

hartafwijkingen en congenitale infecties. Slechts één kind was zeer waarschijnlijk overleden vanwege een acuut tekort aan cortisol. Concluderend rapporteren wij een verhoogde mortaliteit in vroeg gedetecteerde CH-C patiënten die niet primair het gevolg was van hypofysaire hormoon tekorten. De mortaliteit ten gevolge van hypofyse insufficiëntie lijkt in een vroeg gedetecteerde en behandelde populatie laag te zijn. De mortaliteit in niet gescreende populaties is onbekend.

Momenteel zijn wij in het AMC een follow-up studie gestart naar morbiditeit en cognitieve ontwikkeling in vroeg behandelde CH-C patiënten. Om de vraag te beantwoorden of vroege detectie door middel van neonatale screening leidt tot een betere uitkomst zullen de resultaten van onze follow-up studie vergeleken moeten worden met dezelfde uitkomstmaten in een later gediagnostiseerde groep CH-C patiënten. Hiervoor zal internationale samenwerking noodzakelijk zijn.

Wij houden een pleidooi voor het aanpassen van neonatale screenings programma's, zodat naast CH-T ook CH-C opgespoord wordt. Toch blijkt het bevestigen van de diagnose CH-C, zelfs na een afwijkende neonatale screeningsuitslag, niet altijd gemakkelijk. De diagnose centrale hypothyreoïdie is gebaseerd op lage vrij T4 concentraties gepaard met een normale of lage TSH concentratie. In centrale hypothyreoïdie kunnen TSH concentraties echter ook licht verhoogd zijn. Er is dan sprake van een afwijkend TSH molecuul met een verminderde bioactiviteit. Zulke licht verhoogde TSH gehaltes maken het lastig om CH-C te onderscheiden van milde CH-T. In **hoofdstuk 4** onderzochten we de hoogte van de TSH concentraties in CH-C. In vergelijking met een controlegroep met normale schildklierfunctie was het TSH bij CH-C patiënten vaker laag of hoog (U-vormig verband). Patiënten met MPHD hadden hogere TSH concentraties vergeleken met geïsoleerde CH-C, met een hoogst gemeten TSH concentratie van 12.9 mU/l. Om te onderzoeken of vrij T4 concentraties helpen in het onderscheiden van CH-C en milde CH-T, vergeleken we de vrij T4 concentraties in de CH-C groep met die van milde CH-T met een vergelijkbaar TSH (< 13 mU/l). In de CH-C groep waren vrij T4 waardes significant lager dan in de milde CH-T groep. Vrij T4 kan dus helpen in het onderscheid maken tussen CH-C en milde CH-T. In onze studie kwamen we vijf patiënten tegen die de diagnose CH-T hadden gekregen maar bij wie de bloeduitslagen bij nadere analyse meer passen bij CH-C. Dit laat zien hoe lastig het stellen van de diagnose CH-C kan zijn.

Schildklierfunctie parameters (TSH, T4, T3) laten een grote inter-individuele variatie zien terwijl de intra-individuele variatie veel kleiner is. Dit wijst op een individueel "setpoint" voor schildklierhormoonwaardes. Verschillende studies hebben getoond dat zowel genetische als omgevingsfactoren betrokken zijn bij het bepalen van dit individuele setpoint. In **hoofdstuk 5** worden de resultaten beschreven van een tweelingenstudie waarin neonatale T4 screenings uitslagen vergeleken werden in 1264 mono- en 2566 dizygote tweelingparen. Terwijl genetische factoren 31-40% van de variatie in schildklierhormoonwaardes verklaarden, werd 60-69% verklaard door omgevingsfactoren. Aangezien we schildklierwaardes uit de neonatale fase

hebben onderzocht wordt de omgevingsfactor meest waarschijnlijk bepaald door het foetale milieu. Mogelijk is het mechanisme van T4 setpoint determinatie gelegen in epigenetische modificatie van genen betrokken in de ontwikkeling van de hypothalamus-hypofyse-schildklier-as tijdens het foetale leven.

In **hoofdstuk 6** laten we zien dat het T4 setpoint ook postnataal beïnvloed kan worden. In een follow-up studie van een gerandomiseerde gecontroleerde trial waarin bij een groep kinderen met Down syndroom gedurende de eerste twee levensjaren schildklierhormoonbehandeling werd vergeleken met placebobehandeling, onderzochten we het effect van deze behandeling op de schildklierhormoonconcentraties bijna 9 jaar later. In de behandelde groep bleek de vrij T4 concentratie bescheiden maar significant hoger dan in de onbehandelde groep (1.1 pmol/L). Kennelijk heeft schildklierhormoonbehandeling in de eerste levensjaren het ontwikkelen van het T4 setpoint beïnvloed.

Aangezien het stellen van de diagnose CH-C lastig is, zou genetische bevestiging van de aandoening van grote waarde zijn. Helaas wordt slechts in een klein deel van CH-C patiënten een genetische oorzaak gevonden. De meeste patiënten met CH-C hebben multipele hypofysehormoon uitval met als meest voorkomende oorzaak een aanlegstoornis van de hypofyse, het zogenaamde “Pituitary Stalk Interruption Syndrome” (PSIS). Dit bestaat uit een te kleine of afwezige hypofyse voorkwab, een onderbroken of afwezige hypofysesteel en een ectopisch gelegen hypofyse achterkwab.

In **hoofdstuk 7** worden de resultaten beschreven van een exome sequencing studie waarin gezocht werd naar een polygene verklaring voor PSIS. In een groep van 20 patiënten met PSIS werden vier nieuwe kandidaat genen (*DCHS1*, *ROBO2*, *CCDC88C*, *KIF14*) gevonden voor geïsoleerde PSIS en één voor een syndromale vorm van PSIS (*KAT6A*). Verder werden meerdere (mogelijk) pathogene varianten gevonden in genen die betrokken zijn bij midline hersenaanleg stoornissen zoals holoprosencephalie, hypogonadotroop hypogonadisme en corpus callosum agenesie. Met name (mogelijk) pathogene *GLI2* varianten werden frequent gevonden (11 varianten bij 6 patiënten). Een hogere frequentie van de combinatie van twee *GLI2* varianten (M1352V+D1520N) werd aangetoond vergeleken met een referentie populatie (10% vs. 0.68%). De casus beschreven in **hoofdstuk 8** benadrukt het belang van nauwkeurige fenotypering in geval van genetische studies. In onze exome sequencing studie was het de bedoeling alleen patiënten met geïsoleerde PSIS te includeren. Bij de beschreven patiënt werd een pathogene *KAT6A* gen mutatie gevonden en dit meisje bleek achteraf te voldoen aan de criteria voor het KAT6A syndroom, dat o.a. gepaard gaat met ontwikkelingsachterstand. Hypofyse afwijkingen zijn bij patiënten met KAT6A syndroom nog niet beschreven. Aangezien subtiele midline hersenafwijkingen wel beschreven zijn lijkt het raadzaam om patiënten met KAT6A syndroom te screenen op endocriene deficiënties.

Concluderend is CH-C een matig-ernstige vorm van CH waarvoor vroege diagnose en behandeling van belang is om hersenschade te voorkomen. Ook aanvullende potentieel levensbedreigende hypofyse hormoonuitval maken tijdige diagnose belangrijk. In Nederland lijkt de verhoogde mortaliteit onder vroeg behandelde kinderen met CH-C niet geassocieerd te zijn met endocriene ziekte.

Het stellen van de diagnose CH-C wordt bemoeilijkt doordat niet op TSH concentraties gevaren kan worden maar alleen op te lage vrij T4 concentraties. Bovendien kan TSH zelfs mild verhoogd zijn en daarmee het onderscheid tussen CH-C en milde CH-T lastig maken. Bij de evaluatie van vrij T4 concentraties moet rekening gehouden worden met het individuele T4 setpoint. Prenataal lijkt het foetale milieu een belangrijke rol te spelen bij het ontwikkelen van dit setpoint. Daarnaast zijn er aanwijzingen dat het setpoint ook postnataal beïnvloed kan worden.

PSIS lijkt een polygene etiologie te hebben. In een exome sequencing studie werden meerdere nieuwe kandidaat genen aangetoond en lijken genen betrokken bij midline hersenaanleg stoornissen van belang. De zoektocht naar genen betrokken bij aandoeningen met een polygene etiologie is complex en vergt systematische analyse van nauwkeurig gefenotypeerde patiënten. Samenwerking tussen onderzoeksgroepen en het delen van data zijn hierbij noodzakelijk.

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CURRICULUM VITAE

Nitash Soonawala was born on 28th July 1974 in Leiderdorp. She grew-up in Zoeterwoude-Dorp where she received her primary schooling. She attended secondary school at the Stedelijk Gymnasium in Leiden and graduated in 1992. She studied Medicine at the University of Leiden and after passing the doctoral exam cum laude in 1996, she spent 6 months at the Institute of Neurology, Queen Square London, UK, where under supervision of Professor Niall Quinn she was involved in several research projects. After returning from London she completed her medical training in Leiden in 1999 (artsexamen, cum laude). Although she returned from London intending to specialize in Neurology she fell in love with Pediatrics during her internship at Leiden University Medical Center. In 1999, she started her training in Pediatrics at Emma Children's Hospital, Academic Medical Center, Amsterdam, under supervision of Professor Hugo Heymans. This was followed in 2005 by a fellowship in Pediatric Endocrinology at the same Center under supervision of Dr. Thomas Vulsma and Dr. Paul van Trotsenburg. From 2009 onwards she has been working as a Pediatric Endocrinologist at Emma Children's Hospital with a special interest in pediatric thyroid disease and specifically congenital hypothyroidism.

In 2014 the first steps were made in setting up a follow-up study to investigate the neuro-developmental outcome of children with early detected and treated congenital central hypothyroidism. The studies in this thesis were generated during the preparatory phase of the follow-up study. Nitash continues to work at the Department of Pediatric Endocrinology in Amsterdam combining her clinical work with research in the field of pediatric thyroid disease.

Nitash lives in Amstelveen together with her husband Sander Zwaveling and their two daughters, Annabelle and Elise.