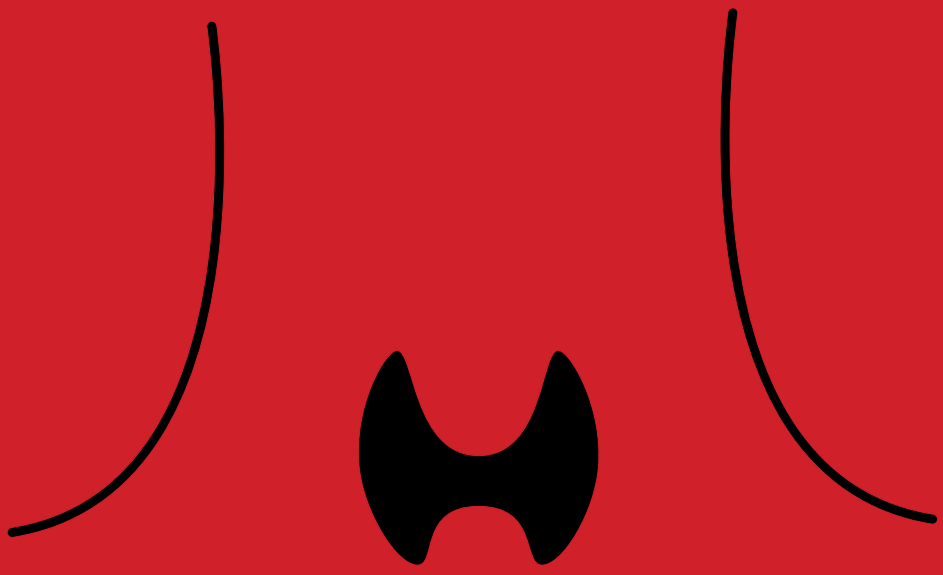
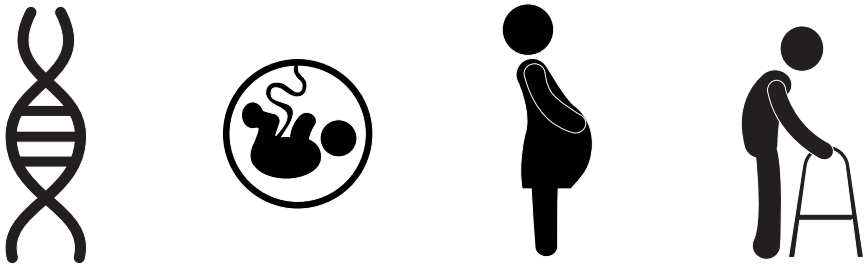
Causes and consequences of thyroid dysfunction throughout life

A population-based and genetic approach





Marco Medici

Causes and Consequences of Thyroid Dysfunction throughout Life: a population-based and genetic approach

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Causes and Consequences of Thyroid Dysfunction throughout Life: a population-based and genetic approach

Oorzaken en gevolgen van schildklier dysfunctie gedurende het leven: een populatie gebaseerde en genetische aanpak

Proefschrift

ter verkrijging van de graad van doctor aan de Erasmus Universiteit Rotterdam op gezag van de rector magnificus

Prof.dr. H.A.P. Pols

en volgens besluit van het College voor Promoties.
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Promotor	Prof.dr.ir. T.J. Visser
Overige leden	Prof.dr. A.G. Uitterlinden
	Prof.dr. E. Fliers
	Dr. A.R. Cappola
Copromotor	Dr. R.P. Peeters

VOOR MIJN OUDERS

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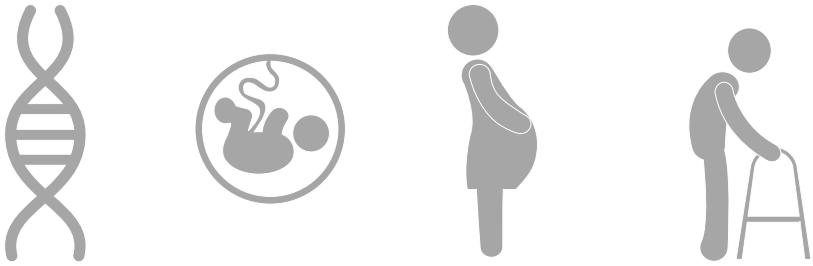
Genetic determination of the hypothalamic-pituitary-thyroid axis: where do we stand?

M. Medici, W.E. Visser, T.J. Visser, R.P. Peeters
Submitted

Thyroid function in pregnancy: What is normal?

M. Medici*, T.I.M. Korevaar*, W.E. Visser, T.J. Visser, R.P. Peeters
**Both authors contributed equally to this work.*
Submitted



THYROID HORMONE SYNTHESIS

Adequate thyroid hormone (TH) levels are essential for normal growth and differentiation, for the regulation of energy metabolism, and for the physiological function of virtually all human tissues. This is illustrated by the well-known effects of hypo- and hyperthyroidism. In addition, more recent studies show that also minor variation in serum TH levels, even within the normal range, can have important effects on clinical endpoints, such as bone mineral density (1), atrial fibrillation (2), metabolic syndrome (3) and cardiovascular mortality (4, 5).

The production of TH is regulated by the classic hypothalamus-pituitary-thyroid (HPT) axis. Hypothalamic thyrotropin releasing hormone (TRH) stimulates TSH secretion from the anterior pituitary. TSH binds to the TSH receptor (TSHR), a G-protein coupled receptor essential for the development and function of the thyroid gland. TSHR is mainly coupled

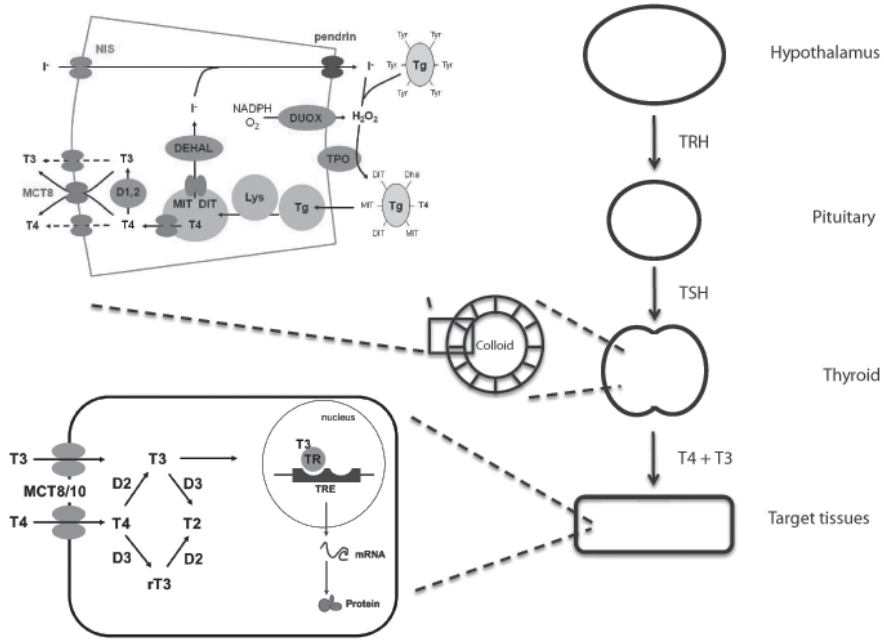


Figure 1: Simplified overview of the hypothalamus-pituitary-thyroid axis, thyroid hormone synthesis and thyroid hormone action in target tissues.
TRH, thyrotropin releasing hormone; TSH, thyroid stimulating hormone; T4, thyroxine; T3, 3,3,5-triiodothyronine; NIS, sodium/iodide symporter; Tg, thyroglobulin; DUOX, dual oxidase; TPO, thyroid peroxidase; Lys, lysosome; MIT, monoiodotyrosine; DIT, diiodotyrosine; D1, type 1 deiodinase; D2, type 2 deiodinase; MCT8, monocarboxylate transporter 8; MCT10, monocarboxylate transporter 10; TR, thyroid hormone receptor; TRE, thyroid hormone response element

to Gs at physiological TSH levels, and therefore the cAMP pathway mediates most of its effects. The synthesis of TRH and TSH subunit genes is inhibited at the transcriptional level by TH, which also inhibits posttranslational modification and release of TSH (6).

The synthesis of TH involves multiple steps, of which a simplified overview is given in Figure 1. Ingested iodine, which is present in the circulation as iodide, is actively transported across the basolateral membrane of the thyroid follicular cells by the sodium/iodide symporter (NIS). The efflux into the follicular lumen is facilitated by the SLC26A4 transporter (Pendrin). Thyroglobulin (Tg) is a glycoprotein whose tyrosine residues are a substrate for iodination and TH formation. Thyroid peroxidase (TPO) plays an important role in this iodination, as it reduces H_2O_2 and binds the iodines to the tyrosine residues. Iodinated tyrosines within Tg are subsequently coupled to form iodothyronines, for which also H_2O_2 is required as a co-substrate. Dual oxidase 2 (DUOX2) and its maturation factor (DUOXA2) are responsible for generating H_2O_2. Subsequently, thyroxine (T4) is formed by coupling of two diiodotyrosines (DIT), and 3,3,5-tri-iodothyronine (T3) is formed by coupling of one monoiodotyrosine (MIT) and one DIT. T4 and T3 are released into the circulation by transporters, including monocarboxylate transporter 8 (MCT8). Iodine is recycled by dehalogenation of iodothyronines by iodotyrosine deiodinase (IYD, also known as DEHAL1).

80% of the circulating T3 is produced outside the thyroid by peripheral conversion of the prohormone T4 by the iodothyronine deiodinases type 1 and 2 (D1 and D2). D1 is present in liver and kidney, whereas D2 is mainly present in brain, brown adipose tissue, skeletal muscle and the heart. The type 3 iodothyronine deiodinase (D3) is mainly responsible for the intracellular degradation of T4 and T3 to reverse T3 (rT3) and T2, and is present in brain, skin, and placenta (7).

The cellular uptake of T4 and T3 is mediated by a number of membrane transporters. OATP1C1 is an organic anion transporter family member, which is mainly expressed in brain capillaries, whereas MCT8 and MCT10 are expressed in various tissues (8, 9). T3 is considered to be the major bioactive TH, which exerts its effects by binding to the intracellular TH receptors alpha and beta (TRα and TRβ). Both receptors have a wide expression pattern, with a predominance of TRα1 in brain, heart and bone and a predominance of TRβ1 in liver, kidney, and thyroid, and TRβ2 in retina, cochlea, and pituitary (10).

THYROID FUNCTION DURING PREGNANCY

During pregnancy, profound changes in thyroid physiology occur. Maternal supply of thyroid hormone (TH) to the fetus and degradation of TH by placental D3 necessitate an increased production of TH (11, 12). This requires an intact thyroid gland and adequate availability of dietary iodine, and is in part mediated by the pregnancy hormone human

chorionic gonadotrophin (hCG), which is a weak agonist of the TSH receptor (13-16). As a consequence, serum FT4 levels increase and TSH levels decrease during the first trimester of pregnancy compared to the non-pregnant state, and reference ranges for TSH and FT4 are different in the pregnant state (17, 18). For this reason, the guidelines of the Endocrine Society and American Thyroid Association recommend to calculate trimester-specific reference ranges per center (19, 20). If these calculated ranges are not available in the laboratory, TSH reference ranges of 0.1–2.5 mU/liter for the first trimester and of 0.2–3.0 mU/liter for the second trimester are recommended (19, 20).

It has been known for long that thyroid dysfunction during pregnancy is associated with maternal and child complications (12, 21-24). Overt hypothyroidism and hyperthyroidism during pregnancy increase the risk of pregnancy loss, premature deliveries, and (pre)eclampsia. In addition, overt hypothyroidism and hypothyroxinemia have been associated with neurodevelopmental delay of the child, and overt hyperthyroidism with an increased risk of low birthweight children. More recently, also subclinical hypothyroidism has been associated with most of these complications (21, 24). In recent years it has become increasingly clear that in the non-pregnant state even variation in thyroid function within the normal range is associated with detrimental health outcomes (5). It is therefore remarkable to note that little is known about the effects of variation in normal-range thyroid function during pregnancy on the risks of maternal and child complications.

GENETIC DETERMINANTS OF THE HPT AXIS

In healthy persons, serum thyroid parameters show substantial inter-individual variability, whereas the intra-individual variability lies within a narrow range (Figure 2A) (25). This suggests that every person has a unique HPT axis set-point which is mainly determined by genetic factors, in addition to environmental factors such as iodine intake and smoking (26, 27). Indeed, this concept is supported by two classical twin studies from Denmark and the UK which found an estimated heritability of serum TSH and FT4 levels of 39-65% (Figure 2B) (28, 29). In another study investigating serum thyroid parameters in a Mexican–American population, heritability estimates ranged from 26-64% (30). Various studies have shown that persons on thyroxine replacement therapy have a decreased well-being, despite having serum TH parameters within the normal range, suggesting that the achieved serum TH parameters may not match the patient's physiological setpoint (31, 32).

For these reasons, many candidate gene and linkage studies have been performed over the years to decipher the genetic basis of thyroid function and dysfunction. Besides the rare monogenic causes of congenital hypo- or hyperthyroidism (discussed in Chap-

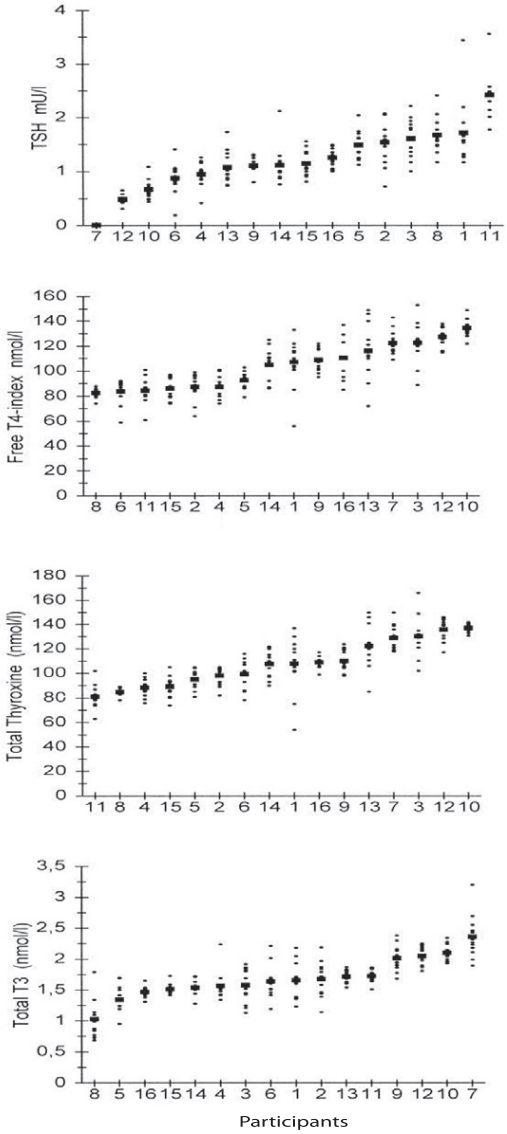


Figure 2A: Adapted from Andersen *et al*., J Clin Endocrinol Metab 2002 (26). Serum TSH, T3, T4, and FT4 levels in 16 healthy subjects taken monthly for 12 months. Each dot represents a monthly measurement and horizontal bars indicate individual parametric means. Laboratory reference ranges are TSH, 0.3–5.0 mU/L; T_3, 1.2–2.7 nmol/L; T_4, 60–140 nmol/L; and FT4 index, 70–140 nmol/L. This study showed substantial inter-individual variability in serum thyroid parameters, whereas the intra-individual variability lies within a narrow range.

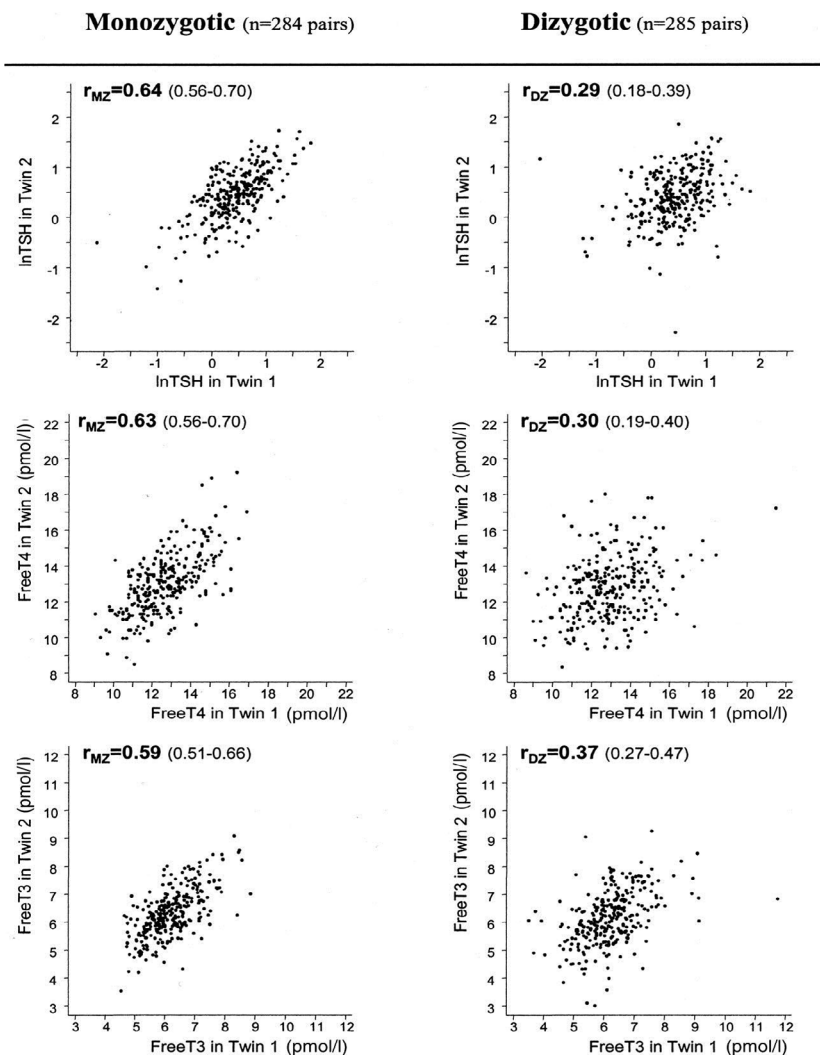


Figure 2B: Adapted from Hansen *et al*., J Clin Endocrinol Metab 2004 (29). Scatterplots and correlations between twins of serum TSH, FT4 and FT3 levels according to zygosity. Correlations were higher in monozygotic twins compared to dizygotic twins, supporting an important role for genetic factors in the HPT-axis. Heritability estimates were 64%, 65% and 64% for TSH, FT4 and FT3, respectively.

ter 14), consistent associations have only been reported for a limited number of genes, including *D1*, *TSHR* and *MCT8*. However, advances in genotyping techniques have made it possible to perform genome-wide association studies (GWAS). In this hypothesis-free approach, 100,000 to 500,000 variants are genotyped across the whole genome, and tested against the phenotype of interest. A stringent p-value threshold of $P < 5x10^{-8}$ is used to prevent false-positive results due to multiple testing. As can be expected for 'common' variants with an allele frequency > 1-5%, effect sizes are small and therefore large populations and often meta-analyses of populations are needed to reach sufficient statistical power.

OUTLINE OF THE THESIS

The studies in this thesis consist of three major parts. In the first part of this thesis (Chapters 2-6), we studied determinants and effects of thyroid function during pregnancy in the Dutch population-based Generation R Study. Given the substantial differences in reported serum thyroid reference ranges between pregnancy cohorts, we calculated trimester specific population-based reference ranges, and compared these with other populations, as well as with the fixed serum TSH reference ranges recommended by international guidelines (19, 20) (Chapter 2). As the Generation R Study is a multi-ethnic cohort, we were able to additionally study the effects of ethnicity on these serum thyroid reference ranges (Chapter 3).

Furthermore, most of the studies on the associations between maternal iodine status and maternal and child thyroid function have been performed in iodine-deficient regions. As little is known about these relations in iodine-sufficient regions, we investigated these relations in the iodine-sufficient Generation R Study (Chapter 4). Finally, we studied the effects of maternal thyroid function during pregnancy on the risk of maternal hypertensive disorders of pregnancy (Chapter 6), as well as the effects on child birth weight (Chapter 5).

In the second part of this thesis (Chapters 7-9), we searched for new genetic determinants of thyroid function and thyroid autoimmunity. We did so by performing a large-scale candidate gene analysis of 68 TH pathway genes with serum TSH and FT4 levels (Chapter 7). As the identified variants only explained a minor part of the total variation in thyroid function, a consortium was started in order to perform large-scale GWAS. This resulted in a GWAS on serum TSH and FT4 levels (Chapter 8). We furthermore performed a GWAS on thyroid autoimmunity (TPO-antibodies), in which the hits were also related to the risk of various forms of clinical thyroid disease (Chapter 9).

The third part of the thesis (Chapters 10-12) includes 2 studies on the effects of genetic variation in the *THRA* locus on human bone and brain (Chapters 10 and 11).

TRα is the predominant TR in these tissues, and various TRα mutant mouse models have an abnormal bone and brain phenotype. Despite this, little is known about the role of common genetic variation in the *THRA* locus in human bone and brain. We therefore studied the effects of common genetic variation in this locus on bone mineral density, bone geometry and fracture risk (Chapter 10), as well as on MRI-derived brain volumes (Chapter 11).

It has been known for long that both hypo- and hyperthyroidism are associated with an increased risk of depression, but the effects of normal-range thyroid function on the risk of depression remain to be determined. Chapter 12 describes a study in which we investigated these effects in a population-based cohort study of elderly subjects.

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Chapter 2

Maternal early pregnancy and newborn thyroid hormone parameters: the Generation R study.

M. Medici, Y.B. de Rijke, R.P. Peeters, W. Visser, S.M. de Muinck Keizer-Schrama, V.V. Jaddoe, A. Hofman, H. Hooijkaas, E.A. Steegers, H. Tiemeier, J.J. Bongers-Schokking, T.J. Visser

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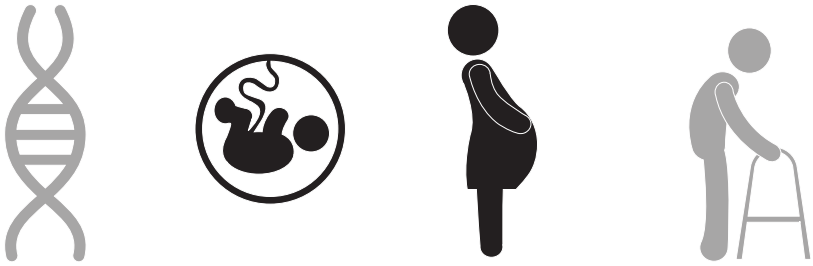

ABSTRACT

Context: Abnormal maternal thyroid parameters are associated with adverse pregnancy outcomes, with consequences for both mother and child. Although various studies have studied maternal thyroid parameters during the first half of pregnancy, little is known about their relations with thyroid parameters of the child.

Objective: To study maternal thyroid parameters during the first half of pregnancy, as well as their relations with cord thyroid parameters.

Design, Setting and Participants: Serum TSH, FT4, T4 and TPO-antibody (TPOAb) levels were determined once between gestational wk 9-18 in 5474 pregnant women from the population-based Generation R study. Cord serum TSH and FT4 levels were determined in 3036 newborns.

Results: Between gestational wk 9-18, the maternal TSH reference range (2.5^{th}-97.5^{th} percentile) was 0.03-4.04 mU/L. Gestational age was positively correlated with maternal TSH (r=0.06, $P=6.3x10^{-5}$) and total T4 (r=0.21, $P=1.4x10^{-44}$), and negatively with FT4 (r=-0.27, $P=7.3x10^{-76}$) and TPOAb-positivity (r=-0.04, $P=0.01$). TPOAb-positivity was associated with more subclinical (20.1% vs 2.4%, $P=1.5x10^{-39}$) and overt hypothyroidism (3.3% vs 0.1%, $P=1.4x10^{-10}$). Maternal and cord TSH were positively associated ($\beta=0.47\pm0.15$, $P=1.3x10^{-5}$), as well as maternal and cord FT4 ($\beta=0.11\pm0.02$, $P=4.5x10^{-6}$).

Conclusions: In this large cohort study, we confirm correlations of maternal thyroid parameters with gestational age during the first half of pregnancy, and show a substantially increased risk of (subclinical) hypothyroidism in TPOAb-positive mothers. A substantial part of the mothers had a TSH level above 2.5 mU/L. Maternal and cord thyroid parameters were positively correlated, the exact biological basis of which remains to be determined.

INTRODUCTION

Maternal thyroid dysfunction during pregnancy is associated with various pregnancy complications and adverse peri- and neonatal outcomes, such as preeclampsia, miscarriage, fetal death, preterm delivery and impaired neurodevelopment (1-3). To effectively identify and treat patients with thyroid disorders during pregnancy, it is of importance to have a proper understanding of the physiological changes in thyroid hormone (TH) levels during pregnancy. Changes in TH levels are most pronounced in the first half of pregnancy (4). A few large studies have studied maternal thyroid parameters in the first half of pregnancy extensively (5-8). However, most studies were based on a limited sample size, or did not measure thyroid peroxidase antibodies (TPOabs) or FT4 levels (9-14).

Sufficient supply of TH to fetal tissues is important for proper fetal development (15). This is illustrated by the harmful effects of iodine deficiency during pregnancy, which can lead to insufficient supply of TH to the developing fetal brain, resulting in mental and motor retardation of the child (16). A number of studies have analyzed the effects of maternal thyroid status during pregnancy and mental and motor development of the child (17-21). However, limited data are available on the relation between maternal TH levels during pregnancy and fetal TH levels.

For these reasons, we studied maternal thyroid parameters in 5186 mothers in the first half of pregnancy, and studied their relations with cord thyroid parameters in 3036 of their newborns.

MATERIALS AND METHODS

Design

This study was embedded in the Generation R Study, a population-based cohort from early fetal life onwards in Rotterdam, the Netherlands, which has been described in detail previously (22). Mothers with a delivery date between April 2002 and January 2006 were enrolled in the study. The study was approved by the Medical Ethics Committee of the Erasmus Medical Center, Rotterdam. Written informed consent was obtained from all adult participants.

Population for analysis

Data on serum TSH, FT4 and T4 levels were complete for 5474 pregnant women. Women with known thyroid disease or thyroid (interfering) medication usage (n = 80) were excluded. Twin pregnancies (n = 63) and pregnancies after fertility treatment (n = 64) were also excluded. In total, 5186 women were included in one or more analyses. Cord serum TSH and FT4 levels were available in 3036 of their newborns.

Thyroid parameters

Maternal serum samples were obtained in early pregnancy (mean = 13.3 wk; SD = 1.7), and cord serum samples were obtained at birth (mean = 39.9 wk; SD = 1.9). Maximally 3 h after sampling, plain tubes were centrifuged and serum was stored at –80 C. TSH, FT4 and T4 were determined in maternal serum samples, and TSH and FT4 levels were determined in cord samples using chemiluminescence assays (Vitros ECI, Ortho Clinical Diagnostics, Rochester, NY). The intra- and interassay coefficients of variation were < 4.1% for TSH, < 5.4% for FT4 and < 6.4% for T4.

Maternal TPOAb was measured using the Phadia 250 immunoassay (Phadia AB, Uppsala, Sweden) and regarded as positive when > 60 IU/mL.

Covariates

Information about maternal age, prenatal smoking, socioeconomic status (SES) and ethnicity was obtained by questionnaires during pregnancy. Maternal prenatal smoking was classified as no smoking, smoking until pregnancy, and continued smoking during pregnancy. SES was defined by educational level, net household income, and employment status (22).

Statistical analysis

Reference ranges for maternal TSH, FT4 and T4, and cord TSH and FT4 levels were defined as the range between the 2.5th and 97.5th percentiles, after exclusion of women with known thyroid disease, thyroid (interfering) medication usage, twin pregnancies, and pregnancies after fertility treatment. In addition, TPOAb positive women were excluded. Differences between ethnic groups in maternal TSH, FT4, T4, and TPOAb positivity were studied using AN(C)OVA and logistic regression. For the cord TSH and FT4 reference ranges, we additionally excluded premature births, defined as delivery at a gestational age less than 37 weeks.

To achieve normal distribution, TSH was transformed by the natural logarithm. Pearson correlation coefficients, AN(C)OVA and linear regression were used to study the relation between gestational age and maternal TSH, FT4 and T4 levels in mothers with normal range TSH, FT4 and T4 levels. Pearson correlation coefficients, AN(C)OVA and logistic regression were used to study the relation between gestational age and TPOAb status. Pearson correlation coefficients and linear regression analysis were used to study the relation between maternal TSH and FT4 levels in the normal range. Associations between TPOAb status and subclinical hypothyroidism, overt hypothyroidism, TSH and FT4 levels were studied using logistic regression and AN(C)OVA.

Pearson correlation coefficients and linear regression were used to study the relation between maternal and cord TSH and FT4 levels in mother-child pairs with normal range

maternal and cord TSH and FT4 levels. Analyses were adjusted for gestational age at birth and gestational age at maternal venous puncture.

All analyses were additionally adjusted for maternal age, prenatal smoking, SES and ethnicity.

RESULTS

Baseline characteristics of the study population are shown in Table 1.

Table 1. Population characteristics

Characteristic (n = 5186)	Mean (SD)
Maternal age (yr)	29.6 (5.1)
Maternal ethnicity (% western)*	63.9%
Socioeconomic status	
Low	10.2%
Middle	46.4%
High	43.4%
Maternal smoking during pregnancy	15.5%
Gestational age at delivery (weeks)	39.9 (1.9)

*51.8% Dutch, 12.0% Surinam/Antillean, 8.4% Turkish, 5.9% Moroccan, 12.2% Other Western, 9.7% Other non-Western.

Reference ranges maternal and cord thyroid parameters

Based on the 2.5th and 97.5th percentiles, maternal reference ranges were 0.03 - 4.04 mU/L for TSH, 10.4 – 22.0 pmol/L for FT4, and 96.0 – 219.0 nmol/L for T4. Subdivided into the first and second trimesters, these ranges were 0.01 – 4.00 mU/L and 0.05 – 4.05 mU/L for TSH, 10.86 – 24.00 pmol/L and 10.28 – 21.50 pmol/L for FT4, and 89.9 – 210.0 nmol/L and 97.8 – 221.0 nmol/L for T4. Fig.1 shows the distribution of TSH measurements within the reference range in the first and second trimester. TSH levels of ≤ 2.5 mU/L for the first trimester and ≤ 3.0 mU/L for the second trimester are regarded as target TSH levels in the treatment of hypothyroidism (23). In the first trimester, 8.6 % of the women with normal range TSH levels had a TSH level > 2.5 mU/L. In the second trimester, 4.9 % of the women with normal range TSH levels had a TSH level > 3.0 mU/L (Fig.1).

Maternal subclinical hypothyroidism was defined as a normal FT4 (10.4 – 22.0 pmol/L) with a high TSH (> 4.04 mU/L). Maternal overt hypothyroidism was defined as a high TSH (> 4.04 mU/L) with a low FT4 (< 10.4 pmol/L).

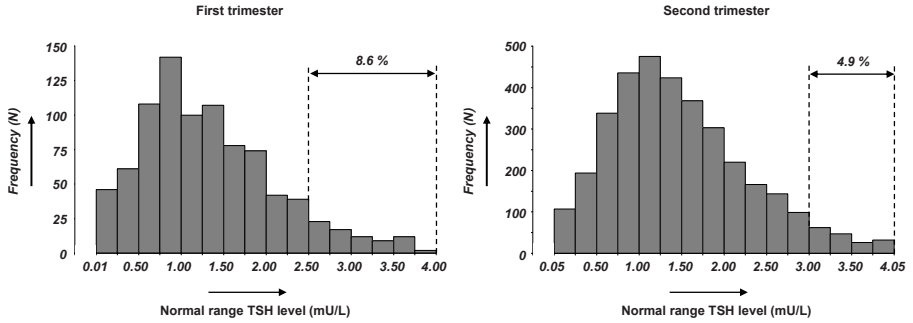

Fig. 1 Distribution of normal range serum TSH levels in the first and second trimester, after exclusion of women with TPOAb positivity, known thyroid disease, thyroid (interfering) medication usage, twin pregnancies, and pregnancies after fertility treatment. In the first trimester, 8.6 % of the women with normal range TSH levels had a TSH level > 2.50 mU/L. In the second trimester, 4.9 % of the women with normal range TSH levels had a TSH level > 3.00 mU/L.

Table S1 shows the thyroid parameters in the different ethnic groups. No differences in FT4 levels between ethnic groups were found. Compared to the Dutch, all ethnic groups had higher T4 levels. Compared to the Dutch, the Surinam/Antillean, Moroccan, and other non-Western (including Cape-Verdians and non-Western Americans, Asians and Africans) ethnic groups had lower TSH levels, and the Turkish had a higher prevalence of TPOAb positivity. Associations remained similar after additional correction for maternal age, smoking, and SES (data not shown).

Cord reference ranges were 3.41 – 33.80 mU/L for TSH, and 15.3 – 28.1 pmol/L for FT4.

Maternal thyroid parameters

Between week 9 and 18 of gestation, gestational age was positively correlated with maternal TSH levels (r = 0.06, $P = 6.3 \times 10^{-5}$), negatively correlated with FT4 levels (r = -0.27, $P = 7.3 \times 10^{-76}$), positively correlated with T4 levels (r = 0.21, $P = 1.4 \times 10^{-44}$), and negatively correlated with TPOAb positivity (r = -0.04, $P = 0.01$). The breakdown of the changes in these thyroid parameters per 2-week period is presented in Table 2. Associations remained similar after adjusting for maternal age, smoking, SES and ethnicity (data not shown).

Maternal TSH and FT4 levels were negatively correlated (r = -0.14; $P = 3.9 \times 10^{-21}$), with a 1 pmol/L increase in FT4 leading to a 0.05 (0.01) mU/L (mean (SE)) decrease in TSH levels ($P = 3.4 \times 10^{-28}$). A similar association was observed after exclusion of TPOAb positive mothers (β = -0.05 (0.01) mU/L, $P = 2.0 \times 10^{-24}$).

TPOAb positivity was associated with higher maternal TSH levels, lower FT4 levels, and a 8-fold and 26-fold higher risk of subclinical hypothyroidism and overt hypothyroidism, respectively (Fig.2).

Table 2. Maternal TSH, FT4, T4 levels and TPOAb status by gestational weeks 9 - 18

Gestational age (weeks)	TSH (mU/L, Median (IQR))	FT4 (pmol/L, Mean (SE))	T4 (nmol/L, Mean (SE))	TPOAb positivity (%, Mean (SE))
9-10 (n = 115*)	1.16 (0.70, 1.93)	16.37 (0.21)	136.9 (2.4)	4.7 (2.0)
11-12 (n = 758*)	1.29 (0.83, 1.91)	15.59 (0.08)	140.5 (0.9)	5.2 (0.8)
13-14 (n= 1916*)	1.32 (0.85, 2.00)	15.21 (0.05)	146.2 (0.6)	5.1 (0.5)
15-16 (n = 1057*)	1.38 (0.95, 1.95)	14.35 (0.07)	151.7 (0.8)	3.3 (0.6)
17-18 (n = 589*)	1.45 (0.98, 2.09)	13.81 (0.09)	155.9 (1.1)	3.1 (0.9)
	$P = 1.2 \times 10^{-4}$	$P = 2.8 \times 10^{-76}$	$P = 4.8 \times 10^{-44}$	$P = 0.01$

Median serum TSH, and mean serum FT4, T4, and TPOAb positivity by gestational weeks 9–18. All analyses were corrected for maternal age. The p-value of the linear regression over gestational weeks 9–18 is shown in the last row.

* Indicated are the number of subjects in the gestational age groups for the TSH, FT4 and T4 level analyses. For the TPOAb positivity analyses, these numbers were 106, 726, 1836, 1028, and 504, for weeks 9-10, 11-12, 13-14, 15-16 and 17-18, respectively.

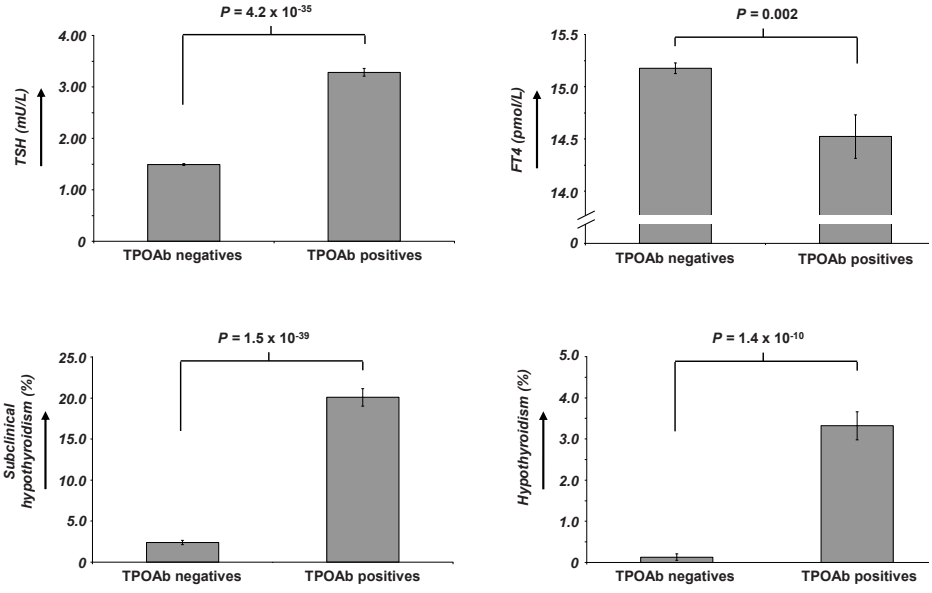

Fig. 2 Maternal TPOAb status vs TSH, FT4 levels, and (subclinical) hypothyroidism. Analyses were based on 4,494 TPOAb negatives and 266 TPOAb positives. All analyses corrected for maternal age and gestational age at venous puncture.

Correlations maternal and cord thyroid parameters

As shown in Table 3, maternal and cord TSH levels were positively correlated ($r = 0.08$, $P = 2.7 \times 10^{-5}$; $\beta = 0.47$ (0.15) mU/L, $P = 1.3 \times 10^{-5}$), as well as maternal and cord FT4 levels (*r*

= 0.09, $P = 1.5 \times 10^{-6}$; $\beta = 0.11$ (0.02) pmol/L, $P = 4.5 \times 10^{-6}$). Associations remained similar after exclusion of TPOAb positive mothers and additional correction for smoking, SES and ethnicity (TSH: $\beta = 0.36$ (0.16) mU/L, $P = 0.001$; FT4: $\beta = 0.12$ (0.02) pmol/L, $P = 3.0 \times 10^{-6}$).

Table 3. Correlations between maternal and cord thyroid parameters

	Correlation		Effect	
Thyroid Parameter	r	*P*	β (SE)	*P*
TSH (n = 2,424; mU/L)	0.08	2.7×10^{-5}	0.47 (0.15)	1.3×10^{-5}
FT4 (n = 2,424; pmol/L)	0.09	1.5×10^{-6}	0.11 (0.02)	4.5×10^{-6}

Correlations between maternal and cord TSH levels, and maternal and cord FT4 levels. Analyses were performed in mother-child pairs with normal range maternal and cord TSH and FT4 levels. Additionally, the effect of 1 mU/L increase in maternal TSH on cord TSH level, and the effect of 1 pmol/L increase in maternal FT4 on cord FT4 level are shown. Analyses were corrected for maternal age, gestational age at maternal venous puncture and gestational age at birth.

DISCUSSION

In this study, we examined maternal thyroid parameters between week 9 and 18 of gestation, together with their relations with cord thyroid parameters. It has long been recognized that pregnancy affects thyroid physiology, thereby leading to changes in thyroid parameters (2, 4). As not only overt thyroid disease, but also more subtle differences in TH levels can lead to a wide range of complications in the mother and newborn (23), various studies have studied the physiological changes in maternal thyroid parameters during pregnancy. Studies have shown an inverse correlation between TSH and FT4 levels, due to the thyrotropic properties of hCG. After an initial increase in the first 10 weeks of pregnancy, hCG levels subsequently decrease, leading to a decrease in FT4 and increase in TSH levels (4). In addition, high estrogen levels lead to a rise in TBG levels, thereby increasing total T4 levels (4). A few large studies have studied maternal thyroid parameters in early pregnancy extensively (5-8). However, many studies that analyzed maternal thyroid parameters during early pregnancy were based on a limited sample size, or did not measure TPOAbs or total T4 (9-14). In the present study, we replicate previously reported findings in a large population-based cohort, taking the effects of various potential interfering (e.g., TPOAb status or thyroid (interfering) medication use) and confounding (e.g., smoking) factors into account.

We calculated trimester-specific thyroid parameter reference ranges for our population, as various population-specific characteristics (e.g., iodine status and ethnicity) can influence thyroid parameters (24-26). The current guidelines of the American Thyroid

Association for the diagnosis and management of thyroid disease during pregnancy and postpartum recommends to use TSH reference ranges of 0.1 – 2.5 mU/L (first trimester) and 0.2 - 3.0 mU/L (second trimester), if reference ranges are not available in the laboratory (23). In addition, these reference ranges are also recommended to be used as target TSH levels for the titration of thyroxine substitution in overt and subclinical hypothyroidism during pregnancy (23). It is remarkable to note that in the Netherlands, which is an iodine-sufficient country (27), a substantial part of the TPOAb-negative women with normal range TSH levels have a TSH level above 2.5 mU/L and 3.0 mU/L, in the first and second trimester, respectively (Fig.1). This underlines the importance of using population-specific thyroid parameter reference ranges.

Benhadi et al. previously showed lower TSH levels in Moroccan, Turkish and Surinam pregnant women, when compared to the Dutch. This is in line with our results, which show a somewhat lower TSH level in the Surinam/Antillean and Moroccan groups (Table S1). A slightly lower TSH level was also found in the Turkish group, but did not reach significance. We observed a higher prevalence of TPOAb positivity in the Turkish group, which was also the case in the study by Benhadi et al., where it did not reach statistical significance. We additionally show that, compared to the Dutch, all other ethnic groups have higher T4 levels, whereas there were no differences in FT4 levels. The exact origin of these differences in thyroid parameters between pregnant ethnic groups should be clarified in future studies, taking differences in dietary patterns into account.

Our data indicate that between week 9 and 18 of gestation, gestational age is negatively correlated with TPOAb positivity, which is consistent with the immunosuppressive effect of pregnancy (28). Although hypothyroidism has been associated with TPOAb positivity in various populations, few of these studies were carried out during pregnancy. Most studies included a limited number of TPOAb positive mothers, or only reported the effects on the average TSH and FT4 levels of the studied groups, without reporting the actual risk of (subclinical) hypothyroidism (14, 29). Recently, Wang et al. showed an increased prevalence of hypothyroidism in Chinese TPOAb positive mothers (30). This is in line with the results from our study, in which we show a substantially increased risk of both subclinical and overt hypothyroidism in TPOAb positive mothers (Fig.2).

In the last two decades, various studies have shown an association between abnormal maternal TH parameters during pregnancy and abnormal mental and motor development of the child (17-21). In this context, it is remarkable to note that there are limited data available on the relations between maternal TH parameters during pregnancy and fetal TH levels. Most studies have focused on mothers with overt (31-33) or subclinical thyroid disorders (34). A few studies have analyzed these associations in mothers without known thyroid abnormalities, but did not find any associations (35, 36). However, sample sizes were either limited (35) or neonatal TH parameters were determined 2 days after birth, a moment at which associations are likely to be influenced by the neonatal

TSH surge (36). Hume et al. studied maternal, fetal and cord TH parameters, but did not correlate maternal TH parameters during pregnancy with cord TH parameters (37). Recently, Shields et al. were the first to show a positive correlation between maternal FT4 levels at 28 wk gestation and cord FT4 levels in 616 mother-child pairs, but did not find a correlation between maternal and cord TSH levels (38). Based on these data, the authors explain the positive correlation between maternal and cord FT4 levels by the maternal transfer of T4 across the placenta during late pregnancy. In this study, we show in 2563 mother-child pairs a positive correlation between maternal FT4 levels in early pregnancy and cord FT4 levels, together with a positive correlation between maternal and cord TSH levels. Bajoria et al. have shown that TSH is sparingly transferred by the placenta (39). Taken together, our data show that maternal TH parameters in early pregnancy are associated with cord TH parameters. The exact mechanism by which this correlation is driven should be clarified in future studies, taking shared factors between mother and newborn into account, such as genetics and nutrition (e.g., iodine intake).

In conclusion, we studied maternal TH parameters between week 9 and 18 of gestation, together with their relations with cord TH parameters. We observed a positive correlation between maternal and cord thyroid parameters. In addition, we show that a substantial part of the women in this healthy, TPOAb negative population has a TSH level above 2.5 mU/L and 3.0 mU/L, in the first and second trimester, respectively. Finally, we found a substantial increased risk of (subclinical) hypothyroidism in TPOAb positive mothers, and confirm previously reported relations between maternal TH parameters in the first half of pregnancy.

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Supplemental table S1. Maternal TSH, FT4, T4 levels and TPOAb status by ethnicity

Ethnicity	TSH (mU/L, Median (IQR))	FT4 (pmol/L, Mean (SE))	T4 (nmol/L, Mean (SE))	TPOAb positivity (%, Mean (SE))
Dutch (n = 2477)	1.42 (0.89, 2.12)	15.11 (0.07)	143.7 (0.6)	5.4 (0.5)
Surinam/Antillean (n = 568)	1.16 (0.75, 1.82)**	15.40 (0.15)	156.0 (1.3)**	4.5 (1.0)
Turkish (n = 400)	1.39 (0.86, 2.15)	15.11 (0.18)	161.2 (1.6)**	9.5 (1.2)*
Moroccan (n = 282)	1.14 (0.72, 1.83)**	14.91 (0.21)	155.2 (1.9)**	5.7 (1.4)
Other Western (n = 583)	1.40 (0.86, 2.10)	15.22 (0.14)	147.3 (1.3)*	6.6 (1.0)
Other non-Western (n = 464)	1.30 (0.82, 1.83)**	15.11 (0.16)	150.7 (1.5)**	4.4 (1.1)

Median serum TSH, and mean serum FT4, T4, and TPOAb positivity by ethnicity. All analyses were corrected for gestational age at venous puncture. For the different ethnic groups, the number of subjects are indicated between brackets. For the TPOAb positivity analyses, these numbers were 2364 (Dutch), 538 (Surinam/Antillean), 377 (Turkish), 265 (Moroccan), 553 (Other Western), and 428 (Other non-Western).
*$P < 0.01$ ** $P < 0.001$

Chapter 3

Ethnic differences in maternal thyroid parameters during pregnancy: the Generation R study.

T.I.M. Korevaar*, M. Medici*, Y.B. de Rijke, W. Visser, S.M. de Muinck Keizer-Schrama, V.W. Jaddoe, A. Hofman, H.A. Ross, W.E. Visser, H. Hooijkaas, E.A. Steegers, H. Tiemeier, J.J. Bongers-Schokking, T.J. Visser, R.P. Peeters

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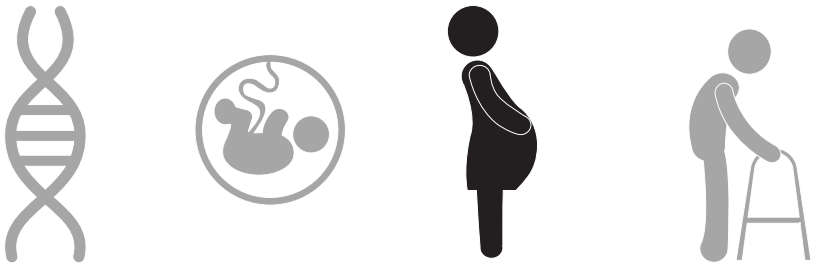
**Both authors contributed equally to this work.*



ABSTRACT

Context: Abnormal maternal thyroid function during pregnancy is associated with various complications. International guidelines advocate the use of population-based trimester-specific reference ranges for thyroid function tests. When unavailable, an upper TSH limit of 2.5 for the first,- and 3.0 mU/L for the second and third trimesters is recommended. Although inter-individual differences in thyroid function tests can partially be explained by ethnicity, data on the influence of ethnicity on TSH and (F)T4 reference ranges during pregnancy are sparse.

Design: Serum TSH, FT4, T4, and TPOAb levels were determined during early pregnancy in 3944 women from the Generation R study, Rotterdam, the Netherlands.

Results: The study population consisted of 2765 Dutch, 308 Moroccan, 421 Turkish and 450 Surinamese women. Mean TSH was higher in Dutch and Turkish women than in Moroccan or Surinamese women (1.50-1.48 *vs.* 1.29-1.33 mU/L;*P*<0.01). Although no differences in FT4 were seen, T4 was lowest in Dutch women (142 *vs.* 150-156 nmol/L;*P*<0.01). Turkish women had the highest frequency of TPOAb positivity (9.3% *vs*. 5.0-5.8%;*P*<0.05) and of elevated TSH levels in the second trimester (11.0% *vs*. 3.8-7.3%;*P*<0.01). A comparison of disease prevalence between a population-based versus an ethnicity-specific reference range changed the diagnosis for 18% of women who were initially diagnosed as having an abnormal thyroid function test.

Conclusions: We show ethnic differences in serum TSH, T4 and TPOAb positivity and found significant diagnostic discrepancies depending on whether population or ethnicity-specific reference ranges were used to diagnose thyroid disease.

INTRODUCTION

Abnormal maternal thyroid function during pregnancy is associated with various maternal and child complications such as preeclampsia, miscarriage, preterm delivery and impaired neurodevelopment of the child (1-3). Recent guidelines by the Endocrine Society and the American Thyroid Association (ATA) advocate the use of population-based trimester-specific reference ranges to diagnose thyroid dysfunction in pregnant women. When trimester-specific reference ranges are not available in the laboratory, upper TSH limits of 2.5 mU/L during the first,- and 3.0 mU/L during the second and third trimesters are recommended (4, 5). These recommendations are mainly based on large studies conducted in divergent populations from America, Europe, China and India which can nowadays be considered multi-ethnic as a result of increased migration (6-11). A number of studies have shown that inter-individual differences in thyroid hormone levels may, at least partially, be explained by ethnic background (12-14). So even within trimester-specific reference ranges, differences may exist as a result of multi-ethnicity.

Only a few studies analyzed the effect of ethnicity on thyroid function tests during pregnancy. A small study in 589 pregnant women demonstrated that African-American women have lower TSH values than Caucasian women (15). Subsequently, La'ulu *et al.* reported that reference range values for thyroid parameters may differ between Asian, white, black and Hispanic Americans (16, 17). Benhadi *et al*. demonstrated significantly lower mean TSH levels in pregnant Dutch women compared to Turkish, Moroccan and Surinamese pregnant women (18). In contrast, a study by Pearce *et al.* showed that ethnicity was not a contributing factor to either TSH, FT4 or T4 in pregnancy (19), but this may have been due to the number of groups compared and a relatively small sample size.

These ethnic differences between different pregnant populations underline the importance of calculating population-specific reference ranges during pregnancy, and suggest that it may not be optimal to apply the same upper limit for TSH during pregnancy for various populations world-wide. Since most populations nowadays are considered multi-ethnic, we investigated the consequences of calculating ethnicity-specific reference ranges for the diagnosis of thyroid disease in a large, multi-ethnic population of pregnant women from Rotterdam, the Netherlands. To exclude an interfering role for iodine deficiency in specific ethnic groups, we also analyzed urinary iodine levels in a subset of pregnant women.

MATERIALS AND METHODS

Design

This study was embedded in the Generation R Study, a population-based cohort from early fetal life onwards in the multi-ethnic city of Rotterdam, The Netherlands, which has been described in detail previously (20). Written informed consent was obtained from all adult participants.

Population for analyses

Data on TSH, FT4 and T4 were available for 4192, and TPOAbs for 3928 Dutch, Moroccan, Turkish and Surinamese pregnant women. Women with twin pregnancies (N=128), pre-existing thyroid disease (N=35), thyroid (interfering) medication usage (N=32) or fertility treatment (N=53) were excluded. Data on ethnicity and ethnic origin were derived by questionnaires. Ethnicity was determined by country of origin which was defined according to the classification of Statistics Netherlands (20), ethnic origin was determined by common ancestry. The final population comprised 3944 women which were included in one or more analyses.

Thyroid parameters

Maternal serum samples were obtained in early pregnancy (mean 13.4 weeks; SD 2.0). Plain tubes were centrifuged and serum was stored at -80 C. TSH, FT4 and T4 were determined in maternal serum samples using chemiluminescence assays (Vitros ECI; Ortho Clinical Diagnostics, Rochester, NY). The intra- and interassay coefficients of variation were <5.4% for FT4 at a range of 14.3-25.0 pmol/L, and <6.4% for T4 at a range of 94-151 nmol/L. For TSH specifically the intra- and interassay coefficients of variation were <4.1% at a range of 3.97-22.70 mU/L, performance characteristics and comparison to other assays have been described previously (21).

During pregnancy, profound changes in thyroid physiology occur (4, 5). Maternal supply of TH to the fetoplacental unit necessitates an increased TH production, requiring an intact thyroid gland and an adequate availability of dietary iodine. This process is in part mediated by the pregnancy hormone human chorionic gonadotrophin (hCG), which is a weak agonist of the TSH receptor and so stimulates the maternal thyroid to produce more TH (22, 23). As a consequence, reference ranges during pregnancy are different compared to a non-pregnant state (4, 5). Therefore reference ranges for TSH, FT4 and T4 were calculated for this specific population (7). Maternal TPOAbs were measured using the Phadia 250 immunoassay (Phadia AB, Uppsala, Sweden) and regarded as positive when greater than 60 IU/ml (24).

Iodine measurements

Urinary iodine concentrations were determined in a random subset of 793 women during early pregnancy (mean = 12.9 weeks; SD = 1.8). Urinary iodine was measured through the ceric-arsenite reaction following destruction by means of ammonium persulphate, which has been described previously (25).

Covariates

Information on maternal age, smoking status and socio-economic status (SES) were obtained by questionnaires during pregnancy. Maternal smoking status was classified as no smoking, smoking until known pregnancy, and continued smoking during pregnancy. SES was defined by educational level, net household income, and employment status. Weight and length were measured at intake (same time as blood sample collection) and were used to calculate body mass index (BMI) (26).

Statistical analyses

Descriptive characteristics were compared using ANOVA, logistic regression analyses, and the Wilcoxon–Mann–Whitney U test. Total and subgroup reference ranges for maternal TSH, FT4 and T4 were defined as the range between the 2.5th and 97.5th percentiles. Additionally, TPOAb-positive women were excluded. To achieve normal distribution, TSH was logarithmically transformed. Mean TSH, FT4 and T4 levels, and TPOAb positivity were compared using ANOVA and logistic regression and additionally adjusted for maternal age, gestational age at sampling, SES, smoking, parity and BMI. The percentage of women with a TSH >2.5 mU/L in the first trimester or >3.0 mU/L in the second and third trimesters per ethnic group were compared using logistic regression analyses. The prevalences of (subclinical) hyperthyroidism, (subclinical) hypothyroidism and hypothyroxinemia in the four ethnic groups were calculated using both total-population and ethnicity-specific reference ranges.

Hyperthyroidism was defined as a low (<2.5th percentile) TSH with a high (>97.5th percentile) FT4; subclinical hyperthyroidism as a low TSH with a normal (2.5th – 97.5th percentile) FT4; hypothyroidism as a high TSH with a low FT4; subclinical hypothyroidism as a high TSH with a normal FT4 and hypothyroxinemia as a low FT4 with a normal TSH.

For pregnant populations, the WHO regards median urinary iodine levels of <150 µg/L as insufficient, 150 – 249 µg/L as adequate, 250 – 499 above requirements and >500 µg/L as excessive (27). Median urinary iodine levels were compared between the ethnic groups using the Wilcoxon–Mann–Whitney U test. The percentage of women with urinary iodine levels <150 µg/L and >500 µg/L were compared using chi square tests and logistic regression analyses.

RESULTS

The study population consisted of 3944 women of which 70.1% were Dutch, 7.8% were Moroccan, 10.7% were Turkish and 11.4% were Surinamese. Descriptive characteristics of the studied ethnic groups are shown in Table 1. Dutch women were older, had a lower gestational age at blood sampling, had fewer pregnancies, a higher SES and lower body mass index (BMI). The Turkish women had the highest smoking prevalence.

Table 1. Descriptive statistics.

	Total population	Dutch	Moroccan	Turkish	Surinamese	*P*-value
Women included (N (%))	3944 (100)	2765 (70.1)	308 (7.8)	421 (10.7)	450 (11.4)	
Age in years (mean (SD))	30.0 (4.9)	31.1 (4.3)	28.0 (5.4)*	26.7 (4.6)*	27.6 (5.6)*	<0.01
Gestational age at sampling						
(mean (SD))	13.4 (2.0)	13.2 (1.9)	14.3 (2.1)*	13.8 (2.1)*	13.6 (2.1)*	<0.01
Parity (N (%))						
0	2271 (57.7)	1681 (60.9)	119 (38.8)*	208 (49.4)*	263 (58.4)	<0.01
1	1195 (30.4)	826 (29.9)	104 (33.9)	131 (31.1)	134 (29.8)	0.54
>1	469 (11.9)	251 (9.1)	84 (27.4)*	82 (19.5)*	52 (11.6)*	<0.01
Smoking during pregnancy (N (%))						
Yes	632 (17.5)	410 (16.2)	17 (6.0)*	129 (33.9)*	76 (18.1)	<0.01
Stopped	336 (9.3)	246 (9.7)	5 (1.8)*	32 (8.4)	53 (12.6)	<0.01
Non-smokers	2645 (73.2)	1875 (74.1)	259 (92.2)*	220 (57.7)*	291 (69.3)*	<0.01
Socio-economic status (N (%))						
Low	345 (8.9)	100 (3.6)	84 (29.0)*	121 (30.1)*	40 (9.0)*	<0.01
Middle	1745 (44.9)	1028 (37.4)	166 (57.2)*	216 (53.7)*	335 (75.3)*	<0.01
High	1798 (46.2)	1623 (59.0)	40 (13.8)*	65 (16.2)*	70 (15.7)*	<0.01
Body mass index (mean (SD))	24.5 (4.4)	24.1 (4.0)	26.1 (4.6)*	25.7 (5.0)*	24.8 (5.0)*	<0.01
TSH (median; mU/L)	1.35	1.41	1.14*	1.39	1.13*	<0.01
FT4 (median; pmol/L)	14.9	14.9	14.4*	14.5*	14.9	0.02
T4 (median; nmol/L)	144	140	151*	157*	153*	<0.01
TPOAb positivity (N (%))	224 (6.1)	151 (5.8)	14 (5.0)	36 (9.3)*	23 (5.6)	0.05

* Significant (*P*<0.05*)* compared to Dutch group.
P-values for maternal age, gestational age at sampling and BMI were calculated using ANOVA. *P*-values for parity, smoking, socio-economic status and TPOAb positivity were calculated using logistic regression. *P*-values for median thyroid hormone levels were calculated using the Wilcoxon–Mann–Whitney U test.

Ethnic differences in serum TSH, (F)T4, and TPOAb positivity

With regard to unadjusted thyroid parameters, TSH values were significantly higher in Dutch and Turkish women than in Moroccan and Surinamese women (1.41-1.39 *vs.* 1.14-1.13 mU/L; *P*<0.01). Although unadjusted FT4 levels were significantly lower in Moroccan and Turkish women (14.4-14.5 *vs*. 14.9 pmol/L; *P*=0.02), significance was lost after correction for gestational age at sampling and exclusion of TPOAb-positive women (Table 2). T4 levels in the Dutch women were lower than those in all other ethnic groups (140 nmol/L *vs.* 151-157 nmol/L; *P*<0.01. Turkish women were more frequently TPOAb-positive compared to the Dutch women (9.3% *vs*. 5.8%; *P*<0.01). As shown in Table 2, differences in TSH and T4 between ethnic groups remained significant after exclusion of TPOAb-positive women, and after additional adjustment for maternal age, gestational age at sampling, parity, smoking, SES and BMI. Figure 1 shows the distribution of serum TSH levels in the total population and in the different ethnic subgroups separately.

Table 2. Ethnicity specific mean TSH, FT4, and T4 levels and reference ranges during pregnancy.

	Total population	Dutch	Moroccan	Turkish	Surinamese	*P*-value[a]	Adjusted *P*-value[b]
Adjusted mean TSH (mU/L)	1.40	1.50	1.29*	1.48	1.33*	<0.01	<0.01
Reference range	0.06 – 4.51	0.12 – 4.72	0.004 – 3.99	0.04 – 4.50	0.002 – 3.85		
(TPOAb-positives excluded)	(0.06 – 4.08)	(0.11 – 4.18)	(0.004 – 3.56)	(0.03 – 4.26)	(0.002 – 3.80)		
Adjusted mean FT4 (pmol/L)	15.1	15.1	14.9	15.1	15.2	0.30	0.12
Reference range	10.4 – 21.9	10.6 – 21.8	9.9 – 21.2	9.8 – 22.5	10.2 – 23.2		
(TPOAb-positives excluded)	(10.6 – 21.9)	(10.8 – 21.8)	(9.9 – 21.0)	(9.8 – 22.3)	(10.3 – 23.9)		
Adjusted mean T4 (nmol/L)	150	142	150*	156*	152*	<0.01	<0.01
Reference range	96 – 219	95 – 204	98 – 233	105 – 242	93 – 238		
(TPOAb-positives excluded)	(96 – 219)	(96 – 204)	(97 – 231)	(104 – 238)	(93 – 246)		

* Significant (*P*<0.05*)* compared to Dutch group.

[a] Adjusted for gestational age at sampling.

[b] Adjusted for gestational age at sampling, maternal age, SES, smoking, parity and BMI.

Mean values were calculated as the mean for the 2.5th - 97.5th percentiles of TSH, TT4 or FT4 after exclusion of TPOAb positive women and after correction for gestational age. Adjusted *P*-values were additionally corrected for maternal age, parity, smoking, and socio-economic status.

Reference ranges were defined as the 2.5th – 97.5th percentiles of respective group after exclusion of twin pregnancies, pre-existing thyroid disease, thyroid (interfering) medication usage or fertility treatment, additionally TPOAb positive women were excluded.

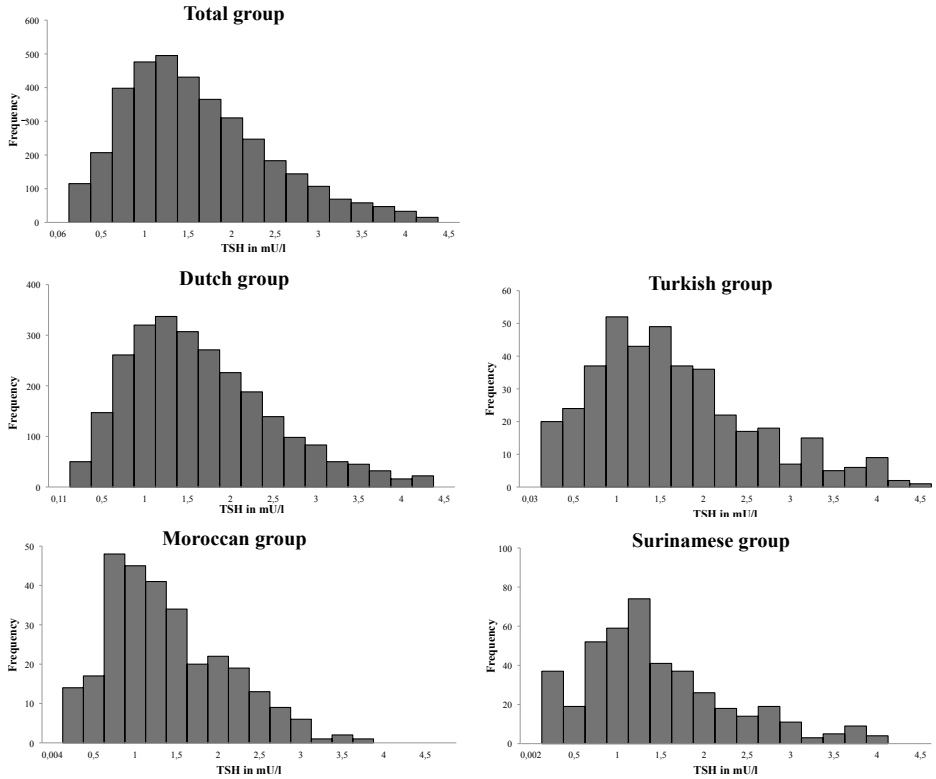


Figure 1 Histograms showing the distribution of normal range maternal TSH for the total group and the separate ethnic groups. Normal ranges for maternal TSH were defined as the 2.5th – 97.5th percentiles of respective group after exclusion of twin pregnancies, pre-existing thyroid disease, thyroid (interfering) medication usage, fertility treatment and TPOAb positive women.

For completeness, women from Morocco and Surinam were subsequently classified according to ethnic origin, since inhabitants of these countries belong to two or more large ethnic groups. Moroccan women were classified as Berber, Arabic or unspecified origin whereas Surinamese women were classified as Creoles, Hindustani or other origin. Analyses showed that, in addition to ethnicity (defined by country of origin), thyroid parameters may also differ according to ethnic origin (defined by common ancestry). Besides common geography, cultural habits and recent heritage, subdivision according to ethnic origin may reflect genetic similarities more thoroughly (see supplemental Table 1).

Ethnic differences in the risk of elevated TSH levels

Current Endocrine Society and ATA guidelines recommend to use an upper limit of TSH of 2.5 mU/L in the first trimester and of 3.0 mU/L in the second and third trimesters when population-based trimester-specific reference ranges are not available. Table 3

displays the number of women with elevated trimester-specific TSH levels according to these cut-off values. Turkish women had a significantly higher frequency of elevated TSH values in the second trimester than the Dutch women (13.6% *vs*. 9.5%; *P*=0.02) whereas Moroccan and Surinamese women displayed a significantly lower frequency compared to the Dutch women (5.0-5.8% *vs*. 9.5%; *P*=0.02). This effect remained significant after the exclusion of TPOAb-positive women. Moroccan women had a borderline significantly lower frequency of elevated TSH levels (*P*=0.05).

Table 3 Percentage of women with a TSH level >2.5 mU/L in the first and >3.0 mU/L in the second trimester.

		Total population	Dutch	Moroccan	Turkish	Surinamese	*P*-value
TSH >2.5 mU/L 1st trimester	**N (%)**	122 (14.8)	96 (15.5)	1 (2.7)	8 (10.8)	17 (17.9)	0.17
TPOAb positive women excluded	**N (%)**	88 (12.0)	70 (12.6)	1 (3.0)	6 (9.5)	11 (13.6)	0.43
TSH >3.0 mU/L 2nd trimester	**N (%)**	278 (9.2)	199 (9.5)	13 (5.0)*	46 (13.6)*	20 (5.8)*	<0.01
TPOAb positive women excluded	**N (%)**	192 (7.1)	138 (7.3)	9 (3.8)	32 (11.0)*	13 (4.2)*	<0.01

* Significant (*P*<0.05*)* compared to Dutch group.
P-values were calculated using logistic regression.

Diagnostic consequences of the use of ethnicity-specific reference ranges

Subsequently, we studied whether the diagnosis of (subclinical) thyroid disease in these different ethnic groups was influenced by the use of reference ranges based on the total population (total population reference range, TPRR), or based on each ethnic group separately (ethnicity specific reference ranges, ESRR). In total, of all 279 women who were diagnosed as having an abnormal thyroid function test when a TPRR was used, 51 women (18%) were re-classified when ESRR were used; 44 changed to a normal thyroid function test and 7 changed to a different disease entity. *Vice versa*, of all 3665 women who had a normal thyroid function test using TPRR, 45 (1.2%) had an abnormal thyroid function test when using ESRR. Table 4 shows the diagnostic changes per disease entity for the total group.

Iodine status in ethnic subgroups

To exclude that the differences between different ethnic groups in our study were due to iodine deficiency in specific populations, urinary iodine levels were measured in a random selection of the total population. As is illustrated in Table 5, all ethnic groups were iodine sufficient according to the WHO criteria (27), with median urinary iodine levels between 201 and 305 µg/L. These results remained similar after adjustment for urinary

creatinine (data not shown). Compared to the Dutch women, median iodine levels were significantly higher in Moroccan, Turkish and Surinamese women (201 *vs.* 235-305 µg/L) while Dutch women more often presented with urinary iodine levels <150 µg/L and less frequently with urinary iodine levels >500 µg/L.

Table 4 Number of pregnant women diagnosed with (subclinical) thyroid disease when using the total population- (TPRR) or ethnicity specific (ESRR) reference ranges in the total group.

	Number of subjects N (%)			
Diagnosis	**TPRR**	**ESRR**	**Change – out**	**Change – in**
Hypothyroidism	12 (0.3)	9 (0.2)	4 (36)	0 (0)
Subclinical hypothyroidism	86 (2.2)	88 (2.2)	11 (13)	11 (0.4)
Hypothyroxinemia	85 (2.2)	88 (2.2)	17 (22)	17 (0.6)
Hyperthyroidism	36 (0.9)	35 (0.9)	5 (15)	4 (0.1)
Subclinical hyperthyroidism	60 (1.5)	60 (1.5)	14 (21)	13 (0.4)
Total	279 (7.1)	280 (7.1)	51 (18)	45 (1.2)

"Change - out" is the number of pregnant women who were originally diagnosed with an abnormal thyroid function test using TPRR, but who became euthyroid or were diagnosed with a different disease entity when ESRR were used. "Change - in" is the number of pregnant women who were euthyroid when the TPRR was used, but were classified within the respective disease entity when the ESRR was used. Disease entities were diagnosed according to the reference ranges including TPOAb positive women as displayed in Table 2.

Table 5 Urinary iodine levels in the 4 ethnic subgroups.

	Total (N=793)	**Dutch (N=545)**	**Moroccan (N=76)**	**Turkish (N=90)**	**Surinamese (N=82)**	***P*-value**
Median urinary iodine (µg/L, (inter quartile range))	224 (127 – 358)	201 (109 – 329)	305* (201 – 506)	269* (178 – 368)	235* (148 – 417)	<0.01
Urinary iodine <150 µg/L (%)	239 (30.1)	193 (35.4)	11 (14.5)*	15 (16.7)*	20 (24.4)*	<0.01
Urinary iodine >500 µg/L (%)	94 (11.9)	48 (8.8)	19 (25.0)*	14 (15.6)*	13 (15.9)	<0.01

* Significant (*P*<0.05*)* compared to Dutch group.

DISCUSSION

Ethnic differences are currently not taken into account for the diagnosis of thyroid disease during pregnancy. In the current study we demonstrate that ethnic differences, even within one geographical area, may influence the diagnosis of thyroid disease. The use of ESRR instead of TPRR changed the diagnosis for 18% of women who were initially diagnosed as having an abnormal thyroid function test.

Differences in TSH between pregnant women from different ethnic groups have been shown in a few other studies (15-18). Studies in relatively small populations from different parts of the United States have shown ethnic TSH differences, without corresponding effects on FT4 (15-17, 19). A study amongst 589 pregnant women found that African-American women had a median TSH value of 1.1 mU/L compared to 1.5 mU/L in Caucasian women (15). A subsequent study amongst 2568 pregnant women in the first trimester of pregnancy found that black women had a median TSH value of 0.82 mU/L whereas white women had a median TSH level of 1.02 mU/L (16). The same authors showed similar differences in median TSH between black and white women during the second trimester (0.97 and 1.21 mU/L, respectively). Benhadi *et al.* found that Dutch women had a higher mean TSH value than Moroccan, Surinamese and Turkish women (1.19 *vs.* 0.87, 0.91 and 0.96 mU/L respectively). Even though the study of Benhadi *et al.* was conducted in a similar population, TSH values in our study were slightly higher overall, which may be explained by different assays used to determine TSH and FT4 levels. Additional adjustment for SES in our study combined with possible differences in population iodine status, which was not assessed in the study by Benhadi *et al*., may also underlie these findings. Similar study differences may also explain why TSH levels in our study are not different between Dutch and Turkish women, despite the larger sample size in our study.

Although no significant differences in FT4 levels were observed between the different ethnic groups, T4 levels were ethnicity dependent. Data on ethnic T4 differences are sparse. A previous study in a relatively small first trimester pregnancy population by Pearce *et al*. (N=668) showed that ethnicity was not a factor significantly contributing to T4 levels (19). However, Aoki *et al*. (N=4392) showed that T4 levels were higher in Mexican Americans compared to non-Hispanic black and non-Hispanic white Americans, but this was studied in a predominantly non-pregnant population (28). In the current study, we found that pregnant Dutch women had significantly lower T4 levels than all other ethnic groups. The discrepancy between FT4 and T4 levels might reflect ethnic differences in binding proteins such as thyroid hormone binding globulin (TBG), transthyretin (TTR) and albumin.

In our study 224 (6.1%) women were TPOAb-positive, which is similar to what has been shown previously in other international studies and in a different pregnant populations in the Netherlands (18, 22, 29). Ethnic variety of TPOAb positivity has been shown in large American studies amongst men and non-pregnant women (13), as well as in pregnant women (16, 17). However, other studies on pregnant populations failed to replicate these results (15, 18, 19). Turkish women in our study had the highest prevalence of TPOAb positivity. Interestingly, Turkish women in our cohort were also more likely to smoke. Since smoking has been shown to reduce the chance of TPOAb positivity (30), it may well be that the reported increased risk of TPOAb positivity in Turkish women in this

population is even an underestimation. TPOAb positivity during pregnancy is associated with an increased risk of postpartum thyroiditis, miscarriage and fetal death (31, 32). Whether Turkish women in the Netherlands are more susceptible to these pregnancy adversities remains to be investigated in future studies.

To investigate if part of the ethnic differences could be explained by differences in iodine intake, we analyzed urinary iodine excretion in a random sample of this population. As iodine intake is highly variable within populations, even iodine sufficient populations such as the United States of America and the Netherlands can contain subgroups with iodine deficiency or excess. Nevertheless, all four ethnic groups were iodine sufficient according to the WHO criteria (27). Compared to the other groups, the Dutch group more frequently exhibited a low urinary iodine (<150 µg/L) and less frequently a high urinary iodine (>500µg/L). However, since all populations were iodine sufficient, it is unlikely that these differences may have caused the differences in serum thyroid function tests. Furthermore, additional adjustment for urinary iodine excretion in the subset of 793 women that had this data available did not alter ethnic group differences or mean thyroid hormone levels.

In the absence of trimester-specific population-based reference ranges, TSH limits of 2.5 mU/L in the first, and 3.0 mU/L in the second trimester are recommended as trimester-specific upper limits (4, 5). Even in TPOAb-negative women, a TSH level above these cut-offs has been related to increased pregnancy loss (33), but ethnic differences high TSH levels according to these limits have not yet been investigated. Our results demonstrate ethnic differences in both the first and second trimester, with Turkish women having a higher risk of an elevated TSH than Dutch women, regardless of TPOAb status. We show that the Dutch and Surinamese women less frequently had elevated TSH levels whereas the Moroccan and Turkish women more frequently had high TSH levels in the second compared to the first trimester. Since this cannot be attributed to large ethnic differences in TSH distributions as is shown in Figure 1, the current study does not provide an explanation for this phenomenon. We also demonstrate that the use of ESRR results in a change of diagnosis for 18% of women who are diagnosed as having an abnormal thyroid function test during pregnancy using a local, population-based TPRR. An equal number of women (N=45) classified as euthyroid on the basis of TPRR were found to have an abnormal thyroid function based on ESRR.

In theory, ethnic differences in TSH during pregnancy may be explained by genetic differences in thyroid hormone pathway genes (34-37), since ~65 % of the inter-individual variation in TSH levels has been estimated to be due to genetic factors (38). Furthermore, ethnicity is a wide concept which is most often socially defined by nationality and culture. Alternatively, environmental factors such as diet or racial disparity of maternal hCG levels may be involved as well (39-41). Subtle ethnic differences in hCG have been

shown in other contexts (39, 40), but did not explain ethnic TSH differences in a study by Walker *et al.* (15).

Ideally, each laboratory would calculate both trimester and ethnicity-specific reference ranges for serum TSH. Since ethnic differences within one population from one geographical area already resulted in such a significant misclassification of thyroid disease in our hospital, it is likely that the use of fixed trimester-specific cut-offs (*i.e.* 2.5 mU/L in the first, and 3.0 mU/L in the second and third trimester) throughout the world will result in an even larger number of misclassified patients. It is therefore important to incorporate at least regional trimester-specific reference ranges, if no trimester-specific reference ranges are available in the laboratory.

To date, this is the largest and most detailed study evaluating ethnic differences in thyroid parameters during pregnancy. Moreover, no other study has yet investigated the diagnostic effects of the use of ESRR. A limitation of this study may be the size of some subgroups, especially the size of the Moroccan subgroup (N=308) was relatively small. In addition, we were unable to evaluate disease prevalence per trimester, since most of the samples were obtained in the second trimester. However, ethnic group comparisons are unlikely to be affected as the three largest groups were equally distributed over the first and second trimester. We were unable to fully exclude the effects of thyroglobulin antibodies, however, such antibodies are less common than TPOAbs and in the majority of cases coincide with TPOAb positivity (42). Finally, even though our data indicate that there are no differences in iodine status amongst the four subgroups, iodine and creatinine data were only available in a random sample of pregnant women.

In conclusion, we have shown that TPOAb status, TSH levels and T4 levels differ significantly according to ethnicity in pregnant women living in an iodine sufficient area. The use of ethnicity-specific reference ranges instead of a total-population reference range changed the diagnosis for 18% of women who were initially diagnosed as having an abnormal thyroid function test. In order to diagnose and treat pregnant women with (subclinical) thyroid disease correctly, the establishment of reliable reference ranges is of paramount importance. It is likely that ethnic differences similar to the ones shown in this study are present in other populations, but there is currently not enough evidence to incorporate ethnicity specific reference ranges in daily practice. Therefore, further investigations on racial differences in thyroid hormone parameters and their diagnostic and clinical consequences in different regions of the world are warranted.

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Supplemental table 1 Thyroid parameter differences during pregnancy according to ethnic origin.

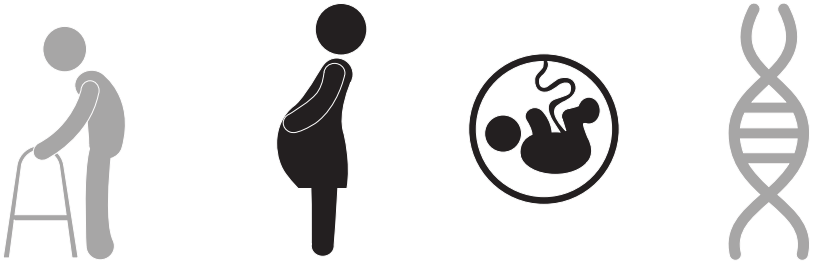
	Total population	Dutch	Moroccan			Turkish	Surinamese			Adjusted *P*-value[a]
Ethnic origin (N)			**Arabic** (N=99)	**Berber** (N=159)	**Unspecified** (N=50)		**Creoles** (N=151)	**Hindustani** (N=190)	**Other** (N=109)	
Adjusted mean TSH (mU/L)	1.40	1.50	1.26*	1.34	1.18	1.48	1.23*	1.45	1.28*	<0.01
Reference range	0.06 – 4.08	0.11 – 4.18	0.003 – 3.91	0.18 – 3.69	0.001 – 3.84	0.04 – 4.26	0.01 – 4.10	0.0002 – 4.01	0.001 – 3.42	
Adjusted mean FT4 (pmol/L)	15.1	15.1	15.3	14.7	15.0	15.1	14.8	15.7*	15.3	0.01
Reference range	10.6 – 21.9	10.8 – 21.8	10.8 – 23.8	8.7 – 20.6	10.4 – 30.0	9.8 – 22.3	9.9 – 25.9	10.7 – 26.2	10.1 – 22.8	
Adjusted mean T4 (nmol/L)	150	142	154*	148	148	156*	14	160*	150	<0.01
Reference range	96 – 219	96 – 204	106 – 232	90 - 221	89 - 267	104 – 238	92 - 243	103 – 254	87 – 222	
TPOAb positivity (N (%))	224 (6.1)	151 (5.8)	4 (4.5)	8 (5.4)	2 (4.3)	36 (9.3)*	5 (3.6)	14 (8.0)	4 (4.0)	0.14
TSH >2.5 mU/L 1st trimester (N (%))	122 (14.8)	96 (15.5)	0 (0)	1 (6.2)	0 (0)	8 (10.8)	5 (14.3)	10 (27.8)	2 (8.3)	0.42
TPOAb positive women excluded	88 (12.0)	70 (12.6)	0 (0)	1 (7.1)	0 (0)	6 (9.5)	4 (12.9)	5 (19.4)	1 (5.3)	0.88
TSH >3.0 mU/L 2nd trimester (N (%))	278 (9.2)	199 (9.5)	4 (4.9)	7 (5.1)	2 (4.8)	46 (13.6)*	4 (3.7)*	12 (8.0)	4 (4.8)	<0.01
TPOAb positive women excluded	192 (7.1)	138 (7.3)	2 (2.8)	5 (4.0)	2 (5.4)	32 (11.0)*	3 (2.9)	8 (6.1)	2 (2.6)	0.03

* Significant (*P*<0.05) compared to Dutch group.

[a] Adjusted for gestational age at sampling, maternal age, SES, smoking, parity and BMI.

Mean values were calculated as the mean for the 2.5th - 97.5th percentiles of TSH, TT4 or FT4 after exclusion of TPOAb positive women and after correction for gestational age. Adjusted *P*-values were additionally corrected for maternal age, parity, smoking, socio-economic status and BMI.

Reference ranges were defined as the 2.5th – 97.5th percentiles of respective group after exclusion of twin pregnancies, pre-existing thyroid disease, thyroid (interfering) medication usage or fertility treatment, additionally TPOAb positive women were excluded.



Chapter 4

Women with high early pregnancy urinary iodine levels have an increased risk of hyperthyroid newborns: the population-based Generation R Study.

M. Medici, A. Ghassabian, W. Visser, S.M. de Muinck Keizer-Schrama, V.W. Jaddoe, W.E. Visser, H. Hooijkaas, A. Hofman, E.A. Steegers, J.J. Bongers-Schokking, H.A. Ross, H. Tiemeier, T.J. Visser, Y.B. de Rijke, R.P. Peeters

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ABSTRACT

Objective: Iodine deficiency during pregnancy results in thyroid dysfunction and has been associated with adverse obstetric and fetal effects, leading to worldwide salt iodisation programs. As nowadays 69% of the world's population lives in iodine-sufficient regions, we investigated the effects of variation in iodine status on maternal and fetal thyroid (dys)function in an iodine-sufficient population.

Design, Participants and Measurements: Urinary iodine, serum TSH, free T4 (FT4) and TPO-antibody levels were determined in early pregnancy (13.3 (1.9) wk; mean (SD)) in 1098 women from the population-based Generation R Study. Newborn cord serum TSH and FT4 levels were determined at birth.

Results: The median urinary iodine level was 222.5 µg/L, indicating an iodine-sufficient population. 30.8% and 11.5% had urinary iodine levels <150 and >500 µg/L, respectively. When comparing mothers with urinary iodine levels <150 vs ≥150 µg/L, and >500 vs ≤500 µg/L, there were no differences in the risk of maternal increased or decreased TSH, hypothyroxinemia, or hyperthyroidism. Mothers with urinary iodine levels >500 µg/L had a higher risk of a newborn with decreased cord TSH levels (5.6±1.4 (mean±SE) vs 2.1±0.5 %, $P = 0.04$), as well as a higher risk of a hyperthyroid newborn (3.1±0.9 vs 0.6±0.3 %, $P = 0.02$). These mothers had newborns with higher cord FT4 levels (21.7±0.3 vs 21.0±0.1 pmol/L, $P = 0.04$).
Maternal urinary iodine levels <150 µg/L were not associated with newborn thyroid dysfunction.

Conclusions: In an iodine-sufficient population, higher maternal urinary iodine levels are associated with an increased risk of a hyperthyroid newborn.

INTRODUCTION

Iodine is a trace element, which is essential for the synthesis of thyroid hormone (TH). Both iodine deficiency and excess can lead to thyroid dysfunction [1, 2]. Iodine requirements increase during pregnancy due to increased maternal urinary iodide excretion, TH production and placental transfer and metabolism of TH [3-5]. Iodine deficiency in pregnancy is associated with poor obstetric outcomes, such as spontaneous abortion, prematurity and stillbirth. Furthermore, iodine deficiency in pregnancy is related to a wide range of adverse fetal effects as well, such as congenital anomalies, decreased intelligence, and neurological cretinism, which includes spasticity, deaf mutism, and mental retardation [6-10]. Therefore, the World Health Organization (WHO) recommends a higher iodine intake during pregnancy [3, 7, 11, 12]. Limited data are available on the effects of iodine excess during pregnancy, but it has been shown that in Asian populations excessive intake of iodine-rich water and food (e.g., seaweed) can lead to maternal subclinial hypothyroidism and newborn hypothyroidism [13, 14].

Most of the studies on the effects of iodine status on pregnancy and child development have been performed in iodine-deficient populations [3, 4, 6-10]. As iodine intake is highly variable within populations, even iodine-sufficient populations can contain subgroups with iodine deficiency or excess. In this context it is remarkable to note that limited data on the effects of variation in iodine status on maternal and fetal TH levels are available from iodine-sufficient pregnant populations.

For these reasons, we studied the effects of early pregnancy iodine status on mean maternal and newborn TSH and free T4 (FT4) levels, as well as on the risk of maternal and newborn hypothyroidism, hypothyroxinemia and hyperthyroidism in an iodine-sufficient population.

MATERIALS AND METHODS

Design

This study was embedded in the Generation R Study, a population-based prospective cohort study from early fetal life onwards in Rotterdam, the Netherlands, which has been described in detail previously [15, 16]. Mothers with a delivery date between April 2002 and January 2006 were enrolled in the study. The study was approved by the Medical Ethics Committee of the Erasmus Medical Center, Rotterdam. Written informed consent was obtained from all adult participants.

Population for analysis

TSH, FT4 and thyroid peroxidase antibody (TPOAb) levels were determined in 5326 pregnant women. Due to financial constraints, urinary iodine levels were determined in a random subset of these women (n = 1154). Women with known thyroid disease, thyroid medication or thyroid interfering medication (such as amiodarone) were excluded (n = 14). Twin pregnancies (n = 27) and pregnancies after fertility treatment (n = 15) were also excluded. In total, 1098 women were included in one or more analyses. Cord serum TSH and FT4 levels were determined in 1068 of their newborns.

Thyroid parameter measurements

Maternal serum samples were obtained in early pregnancy (mean (SD): 13.3(1.9) wk), and cord serum samples were obtained at birth (40.1(1.5) wk). TSH and FT4 were determined in maternal and cord samples using chemiluminescence assays (Vitros ECI; Ortho Clinical Diagnostics, Rochester, NY). The intra- and interassay coefficients of variation were <4.1% for TSH and <5.4% for FT4. Maternal TPOAbs were measured using the Phadia 250 immunoassay (Phadia AB, Uppsala, Sweden) and regarded as positive when > 60 IU/ml.

Iodine measurements

Maternal serum and urinary samples were obtained at the same time (mean (SD): 13.3(1.9) wk). Urinary iodine concentrations were determined in a random subset of 1154 women in which thyroid parameters were determined. Urinary iodine was measured through the ceri-arsenite reaction following destruction by means of ammoniumpersulphate. After brief centrifugation, sodium arsenite solution (0.1 mol/L in 1 mol/L of sulphuric acid) was added. Subsequently, ceriammonium sulphate was added and colour was allowed to develop at 250 C during 60 minutes. Optical density was assessed at 405 nm. At a level of 1.7 µmol/L iodine the within-assay CV was 5.1% and the between-assay CV was 14.3%. Of note, none of the urine samples were tested by test strips before iodine concentrations were determined [17, 18].

To assess the iodine status of a population, the WHO recommends the use of the median (not the mean) urinary iodine concentration in the population, as urinary iodine concentrations are influenced by recent iodine intake [7]. For pregnant populations, the WHO regards median urinary iodine levels of < 150 µg/ as insufficient, 150 – 249 µg/L as adequate, and > 500 µg/L as excessive [7].

Covariates

Information about maternal age, socioeconomic status (SES), ethnicity, thyroid disease, thyroid (interfering) medication usage and first trimester vomiting was obtained by questionnaires during pregnancy. SES was defined by educational level, net household income, and employment status [15]. Information on fertility treatment was obtained from

midwifes and obstetricians. At enrolment, maternal height and weight were measured to calculate body mass index (BMI, kg/m^2). Ultrasound measurements were used to establish gestational age in early pregnancy (planned at gestational age 12 wks) [15].

Statistical analysis

The Endocrine society and American Thyroid Association guidelines recommend the use of population-specific serum thyroid hormone reference ranges during pregnancy, as various studies have shown substantial differences between populations in thyroid hormone reference ranges during pregnancy [19, 20]. We therefore calculated reference ranges for maternal and cord TSH and FT4 levels in our own population. These were defined as the range between the 2.5th and 97.5th percentiles, after exclusion of women with known thyroid disease, thyroid (interfering) medication usage, twin pregnancies, and pregnancies after fertility treatment. Maternal reference ranges were 0.02 - 4.10 mU/L for TSH, and 10.3 – 22.0 pmol/L for FT4, and cord reference ranges were 3.10 – 33.00 mU/L for TSH, and 15.4 – 30.1 pmol/L for FT4. For mothers and newborns, increased TSH (including both subclinical and overt hypothyroidism) was defined as a TSH above the reference range. Similarly, hypothyroidism was defined as a high TSH with a low FT4, and hypothyroxinemia as a low FT4 with a normal TSH. Decreased TSH (including both subclinical and overt hyperthyroidism) was defined as a low TSH, and hyperthyroidism as a low TSH with a high FT4.

Median maternal urinary iodine levels were calculated, and subgroups were identified with low (<150 µg/L) and high (>500 µg/L) urinary iodine levels. We studied the relations between urinary iodine <150 µg/L vs ≥150 µg/L groups and the risk of increased maternal TSH levels and hypothyroxinemia. The associations between urinary iodine >500 µg/L vs ≤500 µg/L groups and the risk of increased maternal TSH levels and hypothyroxinemia were also studied, as higher urinary iodine levels have previously been associated with maternal subclinical hypothyroidism [14]. We additionally investigated the relations between urinary iodine >500 µg/L vs ≤500 µg/L groups and the risk of decreased maternal TSH levels and hyperthyroidism. Analyses were performed using logistic regression. Maternal TSH and FT4 levels in these groups were compared using AN(C)OVA. To achieve normal distribution, TSH was transformed by the natural logarithm. Analyses were adjusted for gestational age at urine / serum sampling, and repeated in TPOAb-negative mothers only, additionally adjusting for maternal age, SES, ethnicity, BMI, and vomiting.

We furthermore studied the relations between urinary iodine <150 µg/L vs ≥150 µg/L groups and the risk of increased cord TSH levels and newborn hypothyroxinemia. The associations between urinary iodine >500 µg/L vs ≤500 µg/L groups and the risk of increased cord TSH levels and hypothyroxinemia were also studied, as high maternal iodine intake has previously been associated with newborn hyperthyrotropinemia [13]. As mean cord FT4 levels were found to be higher in mothers with urinary iodine >500

µg/L, we additionally studied the relations between urinary iodine >500 µg/L vs ≤500 µg/L groups and the risk of decreased cord TSH levels and newborn hyperthyroidism. Cord TSH and FT4 levels in these groups were compared using AN(C)OVA. Analyses were adjusted for gestational age at urine / serum sampling, and gestational age at birth. Analyses were repeated in TPOAb-negative mothers only, adjusting for maternal age, SES, ethnicity, BMI, and vomiting, as well as for maternal TSH and FT4 levels.

RESULTS

Characteristics of the study population are shown in Table 1. There were 28 mothers with an increased TSH, 1 hypothyroid mother, 26 hypothyroxinemic mothers, 27 mothers with a decreased TSH, and 12 hyperthyroid mothers. There were 28 newborns with an increased TSH, 1 hypothyroid newborn, 26 hypothyroxinemic newborns, 27 newborns with a decreased TSH, and 10 hyperthyroid newborns.

Table 1. Population characteristics of 1098 pregnant women from the Generation R Study

Characteristic (n = 1098)	Mean (SD)
Maternal age (years)	29.9 (5.0)
Maternal ethnicity (% western) [a]	65.6%
Socioeconomic status	
Low	10.2%
Middle	42.9%
High	46.9%
Maternal vomiting (%) [b]	50.0%
Maternal BMI (kg/m^2)	24.3 (4.4)
Gestational age at maternal blood / urine sampling (weeks)	13.3 (1.9)
Maternal TSH (mU/L, median (IQR))	1.29 (0.81,1.96)
Maternal FT4 (pmol/L)	14.9 (3.4)
Maternal TPOAb-positivity (%)	6.1%
Gestational age at delivery (weeks)	40.1 (1.5)
Cord TSH (mU/L, median (IQR)) [c]	9.42 (6.38,14.30)
Cord FT4 (pmol/L) [c]	21.1 (3.7)

Abbreviations: BMI, body mass index (calculated as weight in kilograms divided by height in meters squared); IQR, interquartile range; FT4, free T4; TPOAb, thyroid peroxidase antibody; [a] 51.9% Dutch, 10.1% Surinam/Antillean, 8.6% Turkish, 7.2% Moroccan, 13.7% Other Western, 8.5% Other non-Western. [b] Ranging from less than once a week to daily vomiting; 20.0% reported to vomit more than once a week, and 9.1% on a daily basis. [c] Cord TSH and FT4 levels were available in 1068 newborns.

Maternal urinary iodine distribution

The maternal urinary iodine distribution for the current study is shown in Fig.1. Urinary iodine levels ranged from 9.3 to 1743.5 µg/L, with a median level of 222.5 µg/L. This population is therefore regarded iodine-sufficient [7]. 30.8% of the mothers had a urinary iodine level < 150 µg/L, and 11.5% had a urinary iodine level > 500 µg/L.

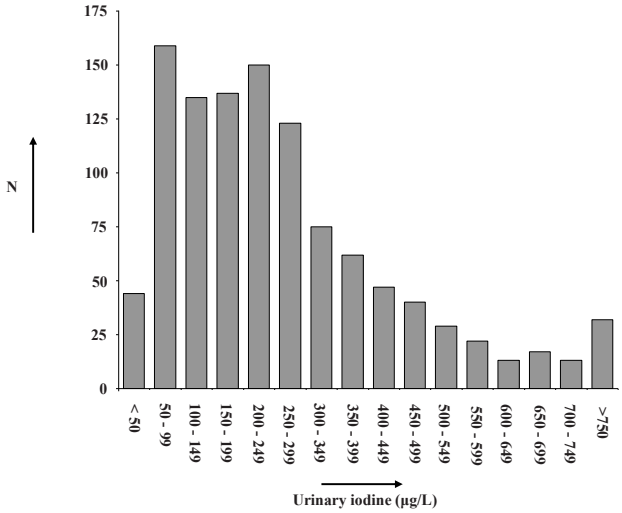

Fig. 1 Early pregnancy urinary iodine levels in 1098 pregnant women. Levels ranged from 9.3 – 1743.5 µg/L, with a median level of 222.5 µg/L. 30.8% had an urinary iodine level < 150 µg/L, and 11.5% had an urinary iodine level > 500 µg/L.

Low and high maternal urinary iodine levels vs maternal thyroid status

As shown in Table 2, mothers with urinary iodine levels < 150 µg/L did not differ in their risk of having increased TSH levels or hypothyroxinemia, compared to mothers with urinary iodine levels ≥ 150 µg/L. No differences in maternal TSH (1.48±0.06 vs 1.52±0.04 mU/L, $P = 0.56$) or FT4 (15.0±0.2 vs 14.9±0.1 pmol/L, $P = 0.93$) levels were found either.

Mothers with urinary iodine levels > 500 µg/L did not differ in their risk of having increased TSH levels (2.6±0.5 vs 2.4±1.4 %, $P = 0.95$) or hypothyroxinemia (2.3±0.5 vs 3.2±1.3 %, $P = 0.43$). As shown in Table 2, these mothers had a higher risk of decreased TSH levels, but this effect failed to reach statistical significance ($P = 0.08$). There were no differences in the risk of maternal hyperthyroidism, neither after exclusion of TPOAb-positive mothers and adjustment for confounders (Table 2). Nor were there differences in mean maternal TSH (1.45±0.09 vs 1.52±0.03 mU/L, $P = 0.17$) or FT4 (14.9±0.3 vs 15.0±0.1 pmol/L, $P = 0.81$) levels.

Table 2. Maternal early pregnancy urinary iodine levels vs the risk of maternal thyroid dysfunction.

	Maternal urinary iodine level (µg/L)					Maternal urinary iodine level (µg/L)			
Increased maternal TSH levels	< 150 (n = 338)	≥ 150 (n = 759)	OR (95% CI)	*P*	**Maternal hypothyroxinemia**	< 150 (n = 338)	≥ 150 (n = 759)	OR (95% CI)	*P*
Model 1 [a] (% (SE))	1.5 (0.9)	3.0 (0.6)	0.47 (0.18-1.25)	0.13	Model 1 [a] (% (SE))	1.7 (0.8)	2.7 (0.5)	0.58 (0.22-1.57)	0.28
Model 2 [b] (% (SE))	1.1 (0.8)	2.2 (0.5)	0.53 (0.17-1.63)	0.27	Model 2 [b] (% (SE))	2.1 (0.8)	2.4 (0.6)	0.78 (0.27-2.23)	0.65
	Maternal urinary iodine level (µg/L)					Maternal urinary iodine level (µg/L)			
Decreased maternal TSH levels	≤ 500 (n = 971)	> 500 (n = 126)	OR (95% CI)	*P*	**Maternal hyperthyroidism**	≤ 500 (n = 971)	> 500 (n = 126)	OR (95% CI)	*P*
Model 1 [a] (% (SE))	2.2 (0.5)	4.8 (1.4)	2.29 (0.91-5.80)	0.08	Model 1 [a] (% (SE))	1.1 (0.3)	0.8 (0.9)	0.73 (0.09-5.68)	0.76
Model 2 [c] (% (SE))	2.1 (0.5)	4.8 (1.4)	2.43 (0.91-6.51)	0.08	Model 2 [c] (% (SE))	1.1 (0.3)	0.9 (1.0)	0.98 (0.12-8.31)	0.99

Abbreviations: BMI, body mass index (calculated as weight in kilograms divided by height in meters squared).

Increased and decreased maternal TSH levels were defined as serum TSH > 4.10 and serum TSH < 0.02 mU/L, respectively. Maternal hypothyroxinemia was defined as serum TSH 0.02 – 4.10 mU/L and FT4 < 10.3 pmol/L. Maternal hyperthyroidism was defined as serum TSH < 0.02 mU/L and FT4 > 22.0 pmol/L.

[a] Adjusted for gestational age at urine/plasma sampling.

[b] Thyroid peroxidase antibody (TPOAb) positives excluded, adjusted for maternal age, socioeconomic status (SES), ethnicity, BMI, and vomiting (performed in 322 mothers with urinary iodine levels < 150 µg/L, and 708 mothers with urinary iodine levels ≥ 150 µg/L).

[c] TPOAb-positives excluded, adjusted for maternal age, SES, ethnicity, BMI, and vomiting (performed in 914 mothers with urinary iodine levels ≤ 500 µg/L, and 116 mothers with urinary iodine levels > 500 µg/L).

Low and high maternal urinary iodine levels vs newborn thyroid status

As shown in Table 3, mothers with urinary iodine levels < 150 µg/L did not differ in their risk of having newborns with increased TSH levels or hypothyroxinemia, compared to mothers with urinary iodine levels ≥ 150 µg/L. No differences in cord TSH (11.83±0.43 vs 11.44±0.28 mU/L, $P = 0.17$) or FT4 (21.0±0.2 vs 21.1±0.1 pmol/L, $P = 0.77$) levels were found either.

Mothers with urinary iodine levels > 500 µg/L also did not differ in their risk of having newborns with increased TSH levels (2.9±0.5 vs 0.7±1.4 %, $P = 0.20$) or hypothyroxinemia (2.7±0.5 vs 0.8±1.4 %, $P = 0.24$). However, these mothers had newborns with higher cord FT4 levels (21.7±0.3 vs 21.0±0.1 pmol/L, $P = 0.04$), which remained significant after exclusion of TPOAb-positive mothers and adjustment for confounders (21.8±0.3 vs 21.0±0.1 pmol/L, $P = 0.04$), as well as after additional adjustment for maternal TSH and FT4 levels (21.8±0.3 vs 21.0±0.1 pmol/L, $P = 0.03$). We therefore also studied the relations with newborn decreased TSH levels and hyperthyroidism. Table 3 shows that these women with urinary iodine levels > 500 µg/L had a higher risk of a newborn with decreased cord TSH levels, as well as an increased risk of a hyperthyroid newborn (Table 3). These effects remained significant after exclusion of TPOAb-positive mothers and adjustment for confounders, as well as after additional adjustment for maternal TSH and FT4 levels. Exclusion of women with low urinary iodine levels (< 150 µg/L) from these analyses, thereby comparing women with urinary iodine levels > 500 with 150-500 µg/L, resulted in a similar increased risk of a newborn with decreased cord TSH levels (5.6±1.5 vs 2.3±0.7 %), which failed to reach statistical significance ($P = 0.06$), whereas the increased risk of a hyperthyroid newborn remained significant (3.1±0.8 vs 0.3±0.4 %, $P = 0.01$).

No interactions between gestational age at birth and maternal urinary iodine levels on the risk of a newborn with decreased TSH levels or hyperthyroidism were detected ((gestational age * urinary iodine) interaction term *P*-values of 0.22 and 0.62, respectively).

DISCUSSION

In the current study, we investigated in an iodine-sufficient population the relation of maternal iodine status and abnormal maternal and cord thyroid function tests, and are the first to show that mothers with higher iodine levels have an increased risk of hyperthyroid newborns.

The WHO estimates that approximately 69% of the world's population lives in iodine-sufficient regions [7]. However, limited data from iodine-sufficient populations are available on the effects of low maternal iodine status on maternal and newborn thyroid function. Azizi et al. showed in 123 pregnant women from an iodine-sufficient popula-

Table 3. Maternal early pregnancy urinary iodine levels vs the risk of newborn thyroid dysfunction.

	Maternal urinary iodine level (µg/L)					Maternal urinary iodine level (µg/L)			
Increased newborn TSH levels	< 150 (n = 326)	≥ 150 (n = 741)	OR (95% CI)	*P*	**Newborn hypothyroxinemia**	< 150 (n = 326)	≥ 150 (n = 741)	OR (95% CI)	*P*
Model 1 [a] (%, mean (SE))	3.6 (0.9)	2.2 (0.6)	1.72 (0.80-3.69)	0.17	Model 1 [a] (%, mean (SE))	3.4 (0.9)	2.0 (0.6)	1.76 (0.79-3.89)	0.17
Model 2 [b] (%, mean (SE))	3.5 (0.9)	2.0 (0.6)	1.68 (0.74-3.79)	0.21	Model 2 [b] (%, mean (SE))	3.5 (0.9)	2.1 (0.6)	1.71 (0.73-4.03)	0.22
Model 3 [c] (%, mean (SE))	3.6 (0.9)	2.1 (0.6)	1.69 (0.75-3.84)	0.21	Model 3 [c] (%, mean (SE))	3.6 (1.0)	2.2 (0.6)	1.79 (0.76-4.25)	0.19
	Maternal urinary iodine level (µg/L)					Maternal urinary iodine level (µg/L)			
Decreased newborn TSH levels	≤ 500 (n = 941)	> 500 (n = 126)	OR (95% CI)	*P*	**Newborn hyperthyroidism**	≤ 500 (n = 941)	> 500 (n = 126)	OR (95% CI)	*P*
Model 1 [a] (%, mean (SE))	2.1 (0.5)	5.6 (1.4)	2.58 (1.07-6.25)	0.04	Model 1 [a] (%, mean (SE))	0.6 (0.3)	3.1 (0.9)	4.87 (1.35-17.57)	0.02
Model 2 [b] (%, mean (SE))	2.1 (0.5)	5.8 (1.5)	2.71 (1.07-6.87)	0.04	Model 2 [b] (%, mean (SE))	0.6 (0.3)	3.5 (0.9)	6.76 (1.58-28.90)	0.01
Model 3 [c] (%, mean (SE))	2.1 (0.5)	5.9 (1.5)	2.66 (1.04-6.78)	0.04	Model 3 [c] (%, mean (SE))	0.6 (0.3)	3.6 (0.9)	9.24 (1.76-48.44)	0.01

Abbreviations: BMI, body mass index (calculated as weight in kilograms divided by height in meters squared).

Increased and decreased newborn TSH levels were defined as serum TSH > 33.00 and serum TSH < 3.10 mU/L, respectively. Newborn hypothyroxinemia was defined as serum TSH 3.10 – 33.00 mU/L and FT4 < 15.4 pmol/L. Newborn hyperthyroidism was defined as serum TSH < 3.10 mU/L and FT4 > 30.1 pmol/L.

[a] Adjusted for gestational age at urine/plasma sampling and gestational age at birth.

[b] Thyroid peroxidase antibody (TPOAb) positives excluded, adjusted for maternal age, socioeconomic status (SES), ethnicity, BMI, and vomiting (performed in 311 and 691 mothers with urinary iodine levels < 150 and ≥ 150 µg/L, respectively , and 116 and 886 mothers with urinary iodine levels > 500 and ≤ 500 µg/L, respectively).

[c] TPOAb-positives excluded, adjusted for maternal age, SES, ethnicity, BMI, and vomiting, as well as for maternal serum TSH and FT4 levels (performed in 306 and 684 mothers with urinary iodine levels < 150 and ≥ 150 µg/L, respectively , and 113 and 877 mothers with urinary iodine levels > 500 and ≤ 500 µg/L, respectively).

tion that newborn TSH, FT4, T4 and T3 levels did not differ between mothers with urinary iodine levels < 150 µg/L and ≥ 150 µg/L [21]. This is in line with the results from the current study, in which we show that mothers from an iodine-sufficient population with urinary iodine levels < 150 µg/L and ≥ 150 µg/L do not differ in maternal or newborn cord TSH and FT4 levels. We additionally show that there are no differences in the risk of increased TSH and hypothyroxinemia in maternal serum or cord blood.

However, when comparing mothers with urinary iodine levels > 500 µg/L and ≤ 500 µg/L, mothers with urinary iodine levels > 500 µg/L had a 2.6 times increased risk of a newborn with decreased cord TSH levels, and a 4.9 times increased risk of a hyperthyroid newborn. These effects remained significant after taking account of a number of potentially interfering factors, including maternal TPOAb status, age, SES, ethnicity, BMI, and vomiting, as well as maternal serum TSH and FT4 levels. When comparing women with urinary iodine levels > 500 with 150-500 µg/L, a similar increased risk of a newborn with decreased cord TSH levels was observed. However, this effect failed to reach statistical significance ($P = 0.06$), which could be due to a lack of statistical power due to the exclusion of the large group of women with urinary iodine levels < 150 µg/L (i.e., 30.8% of the total population). After exclusion of this group, a 9.6 times increased risk of a hyperthyroid newborn remained significant. Various studies have shown that fetal hyperthyroidism is associated with fetal loss, intrauterine growth restriction, and premature birth [22]. Fetal hyperthyroidism is also associated with a wide range of neonatal complications, such as heart failure, cardiac arrhythmia, poor weight gain, and thrombocytopenia [22, 23].

We did not observe any differences in mean maternal TSH and FT4 levels between mothers with urinary iodine levels > 500 µg/L and ≤ 500 µg/L. There was a trend towards a higher risk of maternal decreased TSH in mothers with urinary iodine levels > 500 µg/L, but this effect failed to reach statistical significance, and neither were there differences in the risk of maternal hyperthyroidism. It has been shown that higher urinary iodine levels in Chinese and Japanese pregnant women are associated with an increased risk of maternal subclinical hypothyroidism [14, 24]. Furthermore, Japanese women consuming large quantities of iodine-rich seaweed have been reported to have an increased risk of newborns with transient hypothyroidism or persistent hyperthyrotropinemia [13]. We did not find an effect of higher maternal urinary iodine levels on the risk of maternal or newborn increased TSH levels or hypothyroxinemia in the current study. This may be explained by the fact that the maximum urinary iodine levels in these Chinese and Japanese populations were much higher compared to the current study, leading to hypothyroidism as a result of failure to escape from the acute Wolff-Chaikoff effect [14, 25, 26].

A number of potential mechanisms could explain the observed association between high maternal early pregnancy iodine status and the increased risk of a hyperthyroid newborn. The fact that no effects of high maternal iodine status on maternal thyroid status were detected, and associations with the risk of newborn hyperthyroidism re-

mained significant after adjustment for maternal thyroid status, suggests that especially the fetal thyroid is not able to deal with the high iodine status. Studies have shown that, postnatally, the thyroid gland is able to deal with variation in iodide supply by adjusting iodide uptake via regulation of the expression of the sodium-iodide symporter (NIS) [27, 28]. However, limited data are available about the regulation of iodine uptake in the fetal thyroid gland. To study if maturity of the fetal thyroid could be a factor, we additionally studied the interaction between gestational age at birth and high maternal iodine levels on the risk of newborn decreased cord TSH levels or hyperthyroidism. Although we did not find a significant effect, it would be interesting to further explore this relation in large cohorts of premature pregnancies.

An alternative explanation of the increased risk of hyperthyroid newborns in mothers with a high iodine status could be that mothers with (subclinical) Graves' disease, which may develop earlier when iodine is high [29], were overrepresented in this group. Mothers with Graves' disease have an increased risk of hyperthyroid newborns due to transplacental passage of thyroid stimulating immunoglobulins [30]. However, the fact that no associations with maternal serum TSH and FT4 levels were found, nor with the risk of maternal hyperthyroidism, makes this explanation highly unlikely.

Taken together, the exact mechanism underlying the observed association between high maternal early pregnancy iodine status and the increased risk of hyperthyroid newborns therefore remains to be clarified in future studies, taking fetal thyroid gland compensatory mechanisms for abnormal iodide supply into account.

Various studies have shown substantial differences between populations in the prevalence of TPOAb-positivity during pregnancy [31-33]. In the current study, we found a prevalence of 6.1 %, which is low compared to other populations [32, 33]. Although a similar low frequency (i.e. 5.7%) has previously been found in another large multi-ethnic pregnant population from the Netherlands [31], the exact reasons of these differences in TPOAb-positivity prevalences remain to be clarified.

We are aware of a number of potential limitations of the current study. Maternal urinary iodine levels were only measured once. As individual iodine status can be influenced by recent food intake, multiple urinary iodine measurements will give a better estimation of the average iodine status of an individual [34,35]. Given the relatively large size of our population, we assume that the groups with higher and lower iodine levels will on average have a higher and lower iodine status too. However, this intra-individual variation in iodine levels may have led to an underestimation of the observed effect sizes. Furthermore, we show that a higher maternal iodine status is associated with an increased risk of biochemical newborn hyperthyroidism, but did not study the effects on detrimental pregnancy and postnatal outcomes associated with newborn hyperthyroidism. The current study was underpowered to do so, given the relatively low prevalence of newborn hyperthyroidism or decreased TSH levels. However, various other studies

have convincingly shown an increased risk of a wide range of pregnancy and neonatal complications in newborn hyperthyroidism [22, 23]. Finally, no data were available on potential exposure to iodinated radiographic contrast.

Since 1990, international and national authorities have taken concerted action to eliminate iodine deficiency disorders using salt iodisation as the main strategy [1, 3, 5, 7, 9]. The Netherlands also has a long history of iodine fortification programs, the most important of which include iodised bread salt and table salt [36,37]. The focus of most iodine studies in pregnant women has been on the detrimental effects of maternal iodine deficiency during pregnancy [6-10]. Given the worldwide implementation of iodine fortification programs, it is remarkable to note that limited data are available about the effects of high maternal iodine levels during pregnancy and newborn thyroid status. To our knowledge, this is the first study in an iodine-sufficient population on the risk of newborn thyroid dysfunction in mothers with a higher iodine status. The current study was performed in a pregnant population, whose iodine status is regarded adequate, not excessive, by the internationally recognized WHO criteria [7]. Despite this, we identified a substantial subgroup (i.e., 11.5% of the general pregnant population) with higher urinary iodine levels, which had a considerable increased risk of a hyperthyroid newborn. This group consisted of more non-Westerns (46.0±4.2 vs 31.5±1.5 %, $P = 0.001$), and less subjects with a high SES (35.4±4.7 vs 48.2±1.7 %, $P = 0.01$). However, the observed effects remained significant after correction for these factors. A potential source of the high iodine status could be the intake of iodine-containing supplements. For the Dutch mothers we had data available on whether supplements were taken during pregnancy. The intake of iodine-containing supplements was compared between mothers with urinary iodine levels > 500 µg/L and ≤ 500 µg/L, but no differences were found (16.9±5.8 vs 20.9±1.8 %, $P = 0.51$). However, no data were available on the frequency and number of tablets that were taken. Therefore, the origin of the higher iodine levels should be clarified in future studies, taking the role of dietary patterns into account. Irrespective of the exact cause of these higher iodine levels, our results suggest that, in iodine-sufficient populations, it may be of interest to closer monitor this large group of pregnant women with a higher iodine status as well.

In conclusion, we show that in an iodine-sufficient population, mothers with higher iodine levels have an increased risk of hyperthyroid newborns. These data provide insight into the effects of variation in maternal early pregnancy iodine status on maternal and fetal thyroid status. Furthermore, these data should prompt further studies on the identification and closer monitoring of this subgroup of mothers with a higher iodine status during early pregnancy.

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Chapter 5

Maternal thyroid hormone parameters during early pregnancy and birth weight: the Generation R Study.

M. Medici, S. Timmermans, W. Visser, S.M. de Muinck Keizer-Schrama, V.W. Jaddoe, A. Hofman, H. Hooijkaas, Y.B. de Rijke, H. Tiemeier, J.J. Bongers-Schokking, T.J. Visser, R.P. Peeters, E.A. Steegers.

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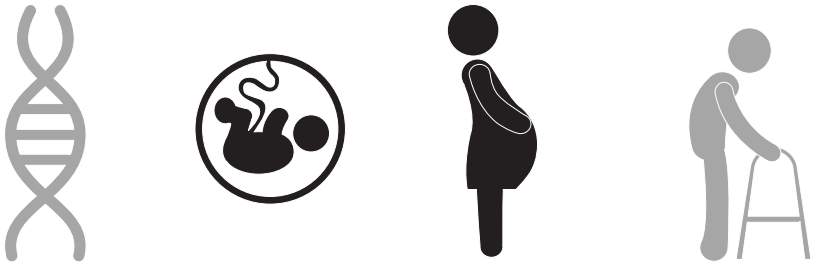

ABSTRACT

Context: Maternal hyperthyroidism during pregnancy is associated with an increased risk of low birth weight, predisposing to neonatal morbidity and mortality. However, the effects of variation in maternal serum thyroid parameters within the normal range on birth weight are largely unknown.

Objective: To study the effects of early pregnancy maternal serum thyroid parameters within the normal range on birth weight, as well as the relation between umbilical cord thyroid parameters and birth weight.

Design, Setting and Participants: In early pregnancy, serum TSH, FT4 (free T4) and TPO-antibody levels were determined in 4464 pregnant women. Cord serum TSH and FT4 levels were determined in 2724 newborns. Small size for gestational age at birth (SGA) was defined as a gestational age adjusted birth weight below the 2.5th percentile. The associations between normal range maternal and cord thyroid parameters, birth weight and SGA were studied using regression analyses.

Results: In mothers with normal range FT4 and TSH levels, higher maternal FT4 levels were associated with lower birth weight (β = -15.4 (3.6) g/pmol/L [mean (SE)], P=1.6 x 10^{-5}), as well as with an increased risk of SGA newborns (OR (95% CI) = 1.09 (1.01-1.17), P=0.03). Birth weight was positively associated with both cord TSH (β = 4.1 (1.4) g/mU/L, P=0.007) and FT4 levels (β = 23.0 (3.2) g/pmol/L, P=9.2x10^{-13}).

Conclusions: We show that maternal high-normal FT4 levels in early pregnancy are associated with lower birth weight, and an increased risk of SGA newborns. Additionally, birth weight is positively associated with cord TSH and FT4 levels. These data demonstrate that even mild variation in thyroid function within the normal range can have important fetal consequences.

INTRODUCTION

Abnormal maternal thyroid function during pregnancy is associated with a wide range of adverse fetal and neonatal outcomes, including intrauterine fetal death, impaired neurodevelopment and low birth weight (1-3). Low birth weight can either be due to intrauterine growth retardation (SGA; small size for gestational age at birth) or prematurity. SGA is associated with an increased risk of perinatal mortality and other complications such as low Apgar scores and seizures (4). For decades it has been known that SGA is also associated with the occurrence of various diseases in later life, such as coronary heart disease, type 2 diabetes, and hypertension (5). SGA has also been associated with a wide range of other diseases, including renal failure, osteoporosis, male reproductive problems, and depression (6-10).

Various studies have investigated the effects of abnormal maternal thyroid function during pregnancy on birth weight (11-15). A few of these large studies have shown a substantially increased risk of low birth weight in children born to hyperthyroid mothers (11-13). However, little is known about the effects of variation in maternal serum thyroid parameters within the normal range on birth weight.

Various maternal autoimmune diseases such as systemic lupus erythematosus, antiphospholipid syndrome and rheumatoid arthritis have been associated with a lower birth weight (16-19). As thyroid peroxidase antibody (TPOAb) positivity is a common finding in pregnant women, it is remarkable that limited data are available on the relation between maternal TPOAb status and birth weight.

For these reasons, we investigated the effects of early pregnancy maternal serum thyroid parameters within the normal range on birth weight in 4464 mother-child pairs from a population-based cohort study, as well as the effects of maternal TPOAb status on birth weight. In addition, the associations between cord thyroid parameters and birth weight were studied. We hypothesized that, also in the normal range, higher FT4 (free T4) and/or lower TSH levels would be associated with a lower birth weight. As maternal autoimmune diseases have been associated with a lower birth weight (16-19), we additionally corrected the TPOAb status and birth weight analyses for maternal thyroid status, to study the effects of the autoimmune status itself (independent of the effects on thyroid parameters).

MATERIALS AND METHODS

Design

This study was embedded in the Generation R Study, a population-based cohort from early fetal life onwards in Rotterdam, the Netherlands, which has been described in detail

previously (20-22). Mothers with a delivery date between April 2002 and January 2006 were enrolled. The Medical Ethics Committee of the Erasmus Medical Center, Rotterdam, approved the study. Written informed consent was obtained from all participants.

Population for analysis

Data on serum TSH and FT4 levels were complete for 5770 women with a live birth pregnancy without congenital anomalies or trisomies. Women with known thyroid disease or thyroid (interfering) medication usage (n = 88) were excluded. Women with known comorbidities (including diabetes, chronic hypertension, hypercholesterolemia, chronic heart disorder, and systemic lupus erythematosus; n = 227), twin pregnancies (n = 62) and pregnancies after fertility treatment (n = 69) were also excluded. From the resulting group of 5324 women, 4464 women had available data on birth weight and were included in one or more analyses. Cord serum TSH and FT4 levels were available in 2724 of their newborns.

Thyroid parameters

Maternal serum samples were obtained in early pregnancy (mean = 13.3 wk; SD = 1.7), and cord serum samples were obtained at birth (mean = 39.9 wk; SD = 1.9) (22). Maximally 3 h after sampling, plain tubes were centrifuged and serum was stored at –80 C (23). TSH and FT4 were determined in maternal and cord serum samples using chemiluminescence assays (Vitros ECI, Ortho Clinical Diagnostics, Rochester, NY). The intra- and interassay coefficients of variation were < 4.1% for TSH and < 5.4% for FT4. Maternal TPOAb was measured using the Phadia 250 immunoassay (Phadia AB, Uppsala, Sweden) and regarded as positive when > 60 IU/mL (22).

Outcome measurements

Information on birth weight was obtained from medical records completed by community midwives and obstetricians. SGA was defined as a gestational age adjusted birth weight below the 2.5th percentile in the study cohort (less than –2.09 standard deviation (SD)). Prematurity was defined as delivery at a gestational age < 37 wks. Low birth weight (LBW) was defined as a birth weight < 2500 grams.

Ultrasound measurements were used to establish gestational age in early pregnancy (planned at gestational age 12 wks), and to estimate fetal weight in mid-pregnancy (planned at gestational age 20 wks) and late-pregnancy (planned at gestational age 30 wks) using the formula of Hadlock et al. (24).

Covariates

Information on maternal age, parity, smoking habits, vomiting during first trimester, socioeconomic status (SES), ethnicity, and comorbidity (including diabetes, chronic

hypertension, hypercholesterolemia, chronic heart disorder, and Systemic Lupus Erythematosus) was obtained by questionnaires during pregnancy. Maternal prenatal smoking was classified as no smoking, smoking until pregnancy, and continued smoking during pregnancy (25). SES was defined by educational level, net household income, and employment status (20). At enrolment maternal height and weight were measured to calculate body mass index (BMI, kg/m^2). Information on fertility treatment and fetal gender was obtained from midwifes and obstetricians.

Statistical analysis

Reference ranges for maternal TSH and FT4 were defined as the range between the 2.5th and 97.5th percentiles, after exclusion of women with positive TPOAbs, known thyroid disease, thyroid (interfering) medication usage, comorbidities, twin pregnancies, and pregnancies after fertility treatment. In mothers with normal range maternal TSH and FT4 levels (2.5th-97.5th percentiles), reference ranges for cord TSH and FT4 were defined as the range between the 2.5th and 97.5th percentiles.

In women with normal range TSH and FT4 levels, the association between FT4 and birth weight was studied using linear regression analyses. FT4 levels were additionally divided in quintiles and studied in relation to birth weight using analysis of covariance (AN(C)OVA). Linear regression was used to study the relation of FT4 with estimated fetal weight in mid-pregnancy and late-pregnancy. We assessed the associations between maternal FT4 quintiles and longitudinally measured SD scores of weight (mid-pregnancy and late-pregnancy estimated fetal weight and birth weight) using unbalanced repeated-measurement analysis, which enables optimal use of available data, taking into account correlations within subjects and assessing both time dependent and independent associations. Repeated measurement analyses were performed with the Proc Mixed module of the Statistical Analysis System (version 9.2; SAS Institute Inc, Cary NC).

Logistic regression and AN(C)OVA were used to assess the associations between FT4 (quintiles) and LBW. The associations between TSH levels, birth weight and estimated fetal weight in mid-pregnancy and late-pregnancy were studied using similar analyses. Maternal TPOAb status (TPOAb-positives vs –negatives) was studied in relation to birth weight, LBW, and estimated fetal weight in mid-pregnancy and late-pregnancy using logistic regression and AN(C)OVA. To additionally test if effects could be due to the autoimmune disease itself (independent of the effects on thyroid parameters), analyses were adjusted for maternal TSH and FT4 levels.

When associations with birth weight were detected, we additionally studied the separate effects on SGA and duration of pregnancy (including prematurity), as low birth weight can either be due to intrauterine growth retardation (SGA) or prematurity.

In newborns with normal range cord FT4 and TSH levels, whose mothers had normal range FT4 and TSH levels, the association between cord FT4 and birth weight was stud-

ied using linear regression. Cord FT4 levels were additionally divided in quintiles and studied in relation to birth weight using AN(C)OVA. The association between cord TSH and birth weight was studied using linear regression. Linear regression was also used to study the relation between cord TSH and FT4. Analyses were additionally corrected for maternal TSH and FT4 levels.

All analyses were repeated using multivariate analyses, correcting for maternal age, ethnicity, SES, parity, smoking during pregnancy, vomiting, newborn gender, as well as for gestational age at weight measurement. Analyses were additionally corrected for maternal BMI.

As outcome measures were correlated (fetal and birth weight endpoints), no multiple testing corrections were performed. Therefore, a p-value threshold of 0.05 was used to declare statistical significance.

RESULTS

Baseline characteristics of the study population are shown in Table 1. The group of newborns in which cord thyroid parameters were available had a higher SES and con-

Table 1. Population characteristics

Characteristic (n = 4464)	Mean (SD)
Maternal age (yr)	29.7 (5.1)
Maternal ethnicity (% western)*	65.4%
Socioeconomic status (low/middle/high)	9.7% / 45.1% / 45.2%
Maternal smoking during pregnancy	17.4%
Maternal BMI (kg/m^2)	24.4 (4.3)
Maternal TSH (mU/L; median (IQR))**	1.34 (0.85;2.02)
Maternal FT4 (pmol/L)**	15.1 (3.5)
Maternal TPOab-positivity**	5.6%
Fetal gender (% male)	50.7%
Estimated fetal weight mid-pregnancy (g)	376.8 (84.1)
Estimated fetal weight late-pregnancy (g)	1612.4 (250.9)
Birth weight (g)	3416.4 (560.2)
LBW (low birth weight)	4.8%
Gestational age at delivery (weeks)	39.9 (1.9)
SGA (small size for gest. age at birth)	2.8%
Cord TSH (mU/L; median (IQR))***	9.42 (6.45;14.30)
Cord FT4 (pmol/L)***	20.9 (3.4)

*53.0% Dutch, 11.5% Surinam/Antillean, 7.8% Turkish, 6.0% Moroccan, 9.2% Other Western, 12.5% Other non-Western.
** Determined at gestational age 13.5 (2.0) wks.
***Based on 2724 newborns.

sisted of more Dutch newborns, compared to the group of newborns in which thyroid parameters were not available (% high SES: 46.7 vs 42.8 %, $P = 0.014$; % Dutch: 56.5 vs 49.8 %, $P = 1.5 \times 10^{-5}$).

Based on the 2.5th and 97.5th percentiles, maternal reference ranges were 0.03 - 4.04 mU/L for TSH, and 10.4 – 22.0 pmol/L for FT4, as reported previously (22). Maternal normal range FT4 quintiles were: 10.38 – 12.80, 12.81 – 14.20, 14.21 – 15.40, 15.41 – 17.00, and 17.01 – 22.00 pmol/L. Cord reference ranges were 3.41 – 33.80 mU/L for TSH, and 15.3 – 28.1 pmol/L for FT4 (22).

Maternal early pregnancy thyroid parameters and birth weight

In mothers with normal range FT4 and TSH levels, maternal FT4 levels were negatively associated with birth weight (β = -15.4 (3.6) g/pmol/L [mean (SE)], $P = 1.6 \times 10^{-5}$). This is illustrated in Figure 1, which shows the birth weight for the maternal normal range FT4 quintiles. Associations remained significant after additional correction for potential confounders, including maternal age, ethnicity, SES, parity, smoking during pregnancy, vomiting, child gender, and gestational age at birth (β = -18.7 (3.5) g/pmol/L, $P = 1.0 \times 10^{-6}$).

Higher maternal normal range FT4 levels were not only associated with a lower birth weight, but also with a lower estimated fetal weight at late-pregnancy (β = -3.3 (1.6) g/pmol/L, $P = 0.048$). There was no association between maternal FT4 levels and estimated fetal weight at mid-pregnancy (data not shown).

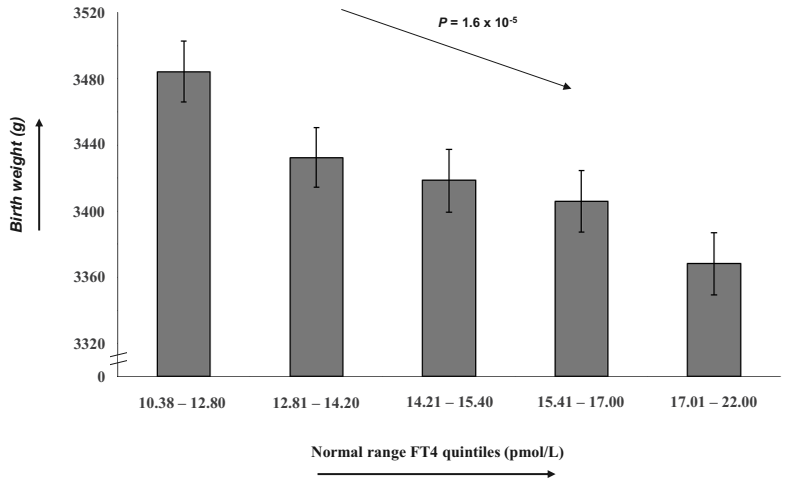

Fig. 1 Maternal early pregnancy normal range free T4 (FT4) quintiles and birth weight in 4,464 mother-child pairs. Analyses were performed in mothers with normal range FT4 and TSH levels, after exclusion of thyroid peroxidase antibody (TPOAb) positives, known thyroid disease or thyroid (interfering) medication usage, comorbidities, twin pregnancies, and pregnancies after fertility treatment. Error bars represent SEs.

Figure 2 presents the estimated differences in SD scores (SDS) for fetal and birth weight for the maternal early pregnancy normal range FT4 quintiles, as compared to the first (lowest) FT4 quintile (10.38-12.80 pmol/L). Higher normal range FT4 levels were associated with a lower weight, except for the second FT4 quintile (12.81-14.20 pmol/L). Compared to the first quintile, differences in weight growth rates were 0.0038 ($P = 0.16$), -0.0011 ($P = 0.69$), and -0.0037 ($P = 0.19$) SDS/week for the second, third and fourth quintiles, respectively. For the fifth quintile this was -0.0058 SDS/week ($P = 0.038$), resulting in a 0.23 SD lower weight at birth, which corresponds to a 116 g lower birth weight.

When maternal FT4 levels were analyzed continuously, higher normal range FT4 levels were associated with an increased risk of SGA newborns (OR (95% CI) = 1.09 (1.01-1.17), $P = 0.03$). No associations with duration of pregnancy (β = -0.002 (0.012) weeks, P=0.86) or prematurity (OR = 1.03 (0.95-1.11), $P = 0.48$) were found.

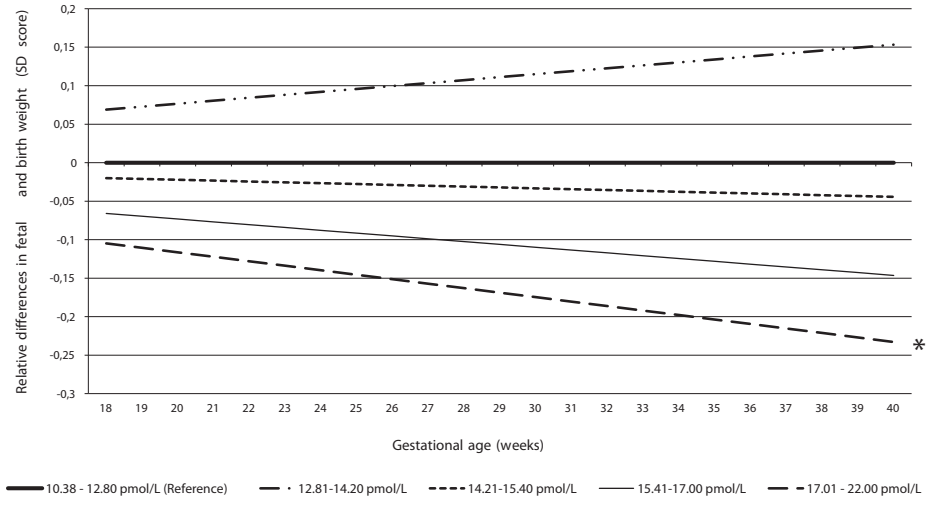

Fig. 2 Standard deviation scores for fetal and birth weight for maternal early pregnancy normal range FT4 quintiles, as compared to the lowest quintile (10.38 – 12.80 pmol/L). Values are based on repeated measurements regression models. * $P < 0.05$

Higher FT4 levels were also associated with an increased risk of LBW newborns (OR = 1.09 (1.03-1.15), $P = 0.005$), which is illustrated for the normal range FT4 quintiles in Figure 3. Mothers in the highest normal range FT4 quintile (17.01-22.00 pmol/L) had a 2.8 times increased risk of a LBW newborn compared to mothers in the lowest quintile (10.38-12.80 pmol/L) (OR = 2.81 (1.65-4.80), $P = 1.5 \times 10^{-4}$). In these mothers, the TSH range was 0.10-2.96 mU/L (median: 1.45 mU/L).

Similar significant associations with SGA, LBW, and late-pregnancy fetal weight were found after additional correction for maternal age, ethnicity, SES, parity, smoking during

pregnancy, vomiting, newborn gender, and gestational age at weight measurement, as well as after additional correction for maternal BMI (data not shown).

Trends towards lower maternal TSH levels and lower birth weight and estimated fetal weights were observed, but did not reach statistical significance (data not shown).

Five point six percent of the women were TPOAb-positive. Maternal TPOAb status was not associated with birth weight, LBW, or fetal weight at mid- and late-pregnancy, nor after correction for maternal TSH and FT4 levels (data not shown).

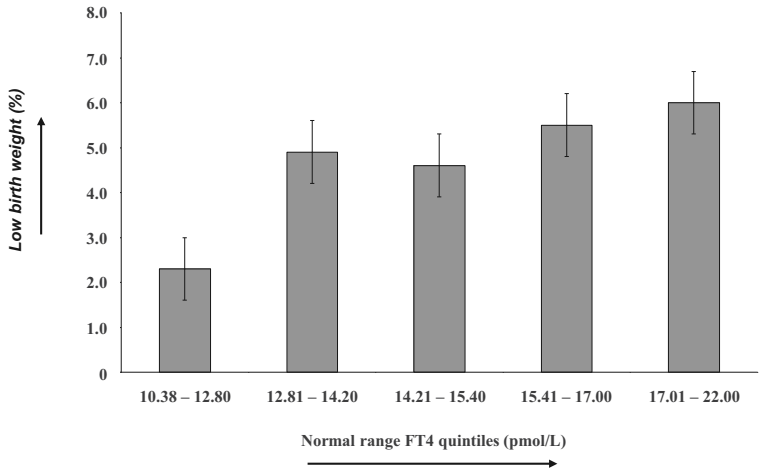

Fig. 3 Maternal early pregnancy normal range FT4 quintiles and low birth weight (< 2500 g) in 4,464 mother-child pairs. Analyses were performed in mothers with normal range FT4 and TSH levels, after exclusion of TPOAb-positives, known thyroid disease or thyroid (interfering) medication usage, comorbidities, twin pregnancies, and pregnancies after fertility treatment. Logistic regression analysis over the entire normal FT4 range (10.38-22.00 pmol/L) resulted in OR = 1.09 (1.03-1.15), $P = 0.005$. Logistic regression analysis over quintile 5 (17.01-22.00 pmol/L) vs quintile 1 (10.38-12.80 pmol/L) resulted in OR = 2.81 (1.65-4.80), $P = 1.5 \times 10^{-4}$. Error bars represent SEs.

Cord thyroid parameters and birth weight

In newborns with normal range cord FT4 and TSH levels, cord FT4 levels were positively associated with birth weight (β = 23.0 (3.2) g/pmol/L, $P = 9.2 \times 10^{-13}$). Figure 4 shows the birth weight for the cord normal range FT4 quintiles. Cord TSH levels were also positively associated with birth weight (β = 4.1 (1.4) g/mU/L, $P = 0.007$). Similar significant associations were found after additional correction for maternal early pregnancy FT4 and TSH levels, maternal age, ethnicity, SES, parity, smoking during pregnancy, vomiting, and newborn gender, as well as after additional correction for maternal BMI (data not shown).

Finally, cord TSH levels were positively associated with cord FT4 levels (β = 0.03 (0.01) pmol/mU, P = 0.001), also after correction for maternal early pregnancy TSH and FT4 levels (β = 0.03 (0.01) pmol/mU, $P = 2.8 \times 10^{-4}$).

Similar significant associations were found after exclusion of SGA newborns (data not shown).

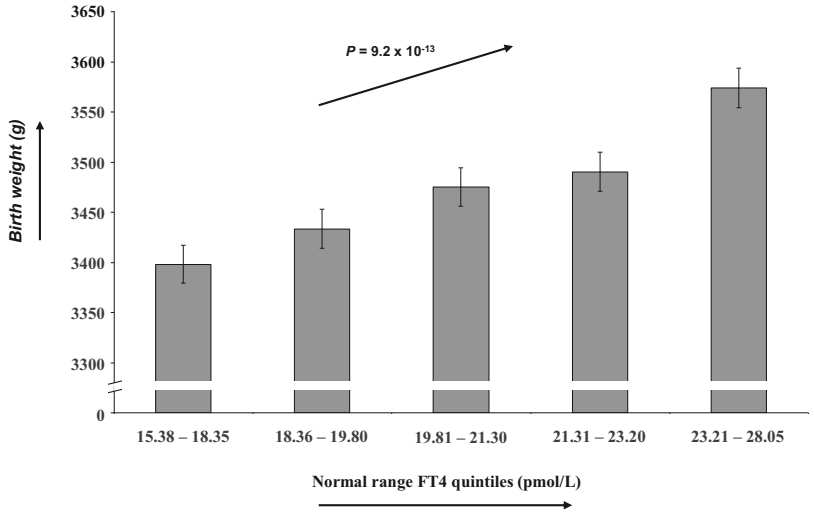

Fig. 4 Cord normal range FT4 quintiles and birth weight in 2,456 newborns. Analyses were performed in newborns with normal range cord FT4 and TSH levels, whose mothers had normal range early pregnancy FT4 and TSH levels, after exclusion of TPOAb-positive mothers, mothers with known thyroid disease or thyroid (interfering) medication usage, comorbidities, twin pregnancies, and pregnancies after fertility treatment. Error bars represent SEs.

DISCUSSION

In the present study, we investigated the effects of early pregnancy maternal thyroid parameters within the normal range and maternal TPOAb status on birth weight, as well as the relations between cord thyroid parameters and birth weight.

Birth weight is often used as a proxy for fetal growth and development, as well as for fetal nutritional status. Low birth weight is associated with neonatal mortality and morbidity, as well as with the occurrence of diseases in later life (4-10). Even mild variations in birth weight within the normal range are known to be associated with later life morbidity (5). A number of studies have investigated the effects of maternal thyroid dysfunction during pregnancy on birth weight (11-15). Most of these studies were performed in mothers with Graves' disease, and showed a substantial increased risk of LBW newborns (11-13). A potential mechanism underlying this observed association is

that hyperthyroid mothers have increased lipid and protein degradation, leading to a state of maternal chronic caloric deficiency, which has been shown to negatively affect birth weight (26, 27). Given the increased risk of LBW newborns in mothers with thyroid dysfunction during pregnancy, it is remarkable to note that limited data are available on the effects of variation in maternal thyroid parameters within the normal range on birth weight. Shields et al. studied the relation between thyroid function during pregnancy and birth weight in 905 mother-child pairs, and found a negative association between maternal FT4 levels at 28 wk gestation and birth weight (28). This is in line with the results from the current study, in which we show a negative association between early pregnancy maternal FT4 levels and birth weight in 4,464 mother-child pairs. We additionally found an increased risk of LBW newborns with higher maternal FT4 levels, as well as a lower estimated fetal weight in late-pregnancy. Similar patterns were observed in our repeated measurements regression analyses.

We did not find significant associations between maternal TSH levels and birth weight. This could be (partially) explained by an interfering role of human chorionic gonadotropin (hCG), which has important placental, uterine and fetal functions, and is an agonist of the TSH receptor leading to increased thyroid hormone production (2, 29).

Low birth weights can be due to intrauterine growth retardation or a shorter duration of pregnancy. We therefore additionally studied the separate effects on SGA and prematurity, and show that higher maternal normal range FT4 levels are associated with an increased risk of SGA newborns, and not with a shorter duration of pregnancy or prematurity.

The effects of maternal early pregnancy FT4 levels on fetal weight, birth weight and the risk of SGA and LBW newborns cannot be explained by a confounding role of maternal BMI since associations remained significant after additional correction for maternal BMI. Potential interfering roles of maternal age, ethnicity, SES, parity, smoking during pregnancy, vomiting, and newborn gender were excluded by correcting the analyses for these factors.

We also took a potentially interfering role of TPOAbs into account. TPOAb-positivity is a common finding in the general population, as well as in pregnant women (30), with a prevalence of 5.6% in the current study. Although various maternal autoimmune diseases have been associated with lower birth weight (16-19), limited data are available on the effects of maternal TPOAb-positivity on birth weight. In the current study, no associations were found between early pregnancy maternal TPOAb-positivity and birth weight, LBW, or with fetal weight at mid- and late-pregnancy. To study possible effects of autoimmunity itself (independent of the effects on thyroid parameters), analyses were additionally adjusted for maternal TSH and FT4 levels, but we did not find any associations. Shields et al. studied the relation between maternal TPOAb-positivity at 28 wk gestation and birth weight in 905 mother-child pairs and did not find any associations either (28). This is also in line with the results of Mannisto et al. who did not

find associations between first trimester maternal TPOAb-positivity and birth weight in 5,763 mother-child-pairs (31). However, an increased risk of LBW newborns was found in this study. The origin of the discrepancy with the current study regarding the LBW risk is currently unknown, and should be clarified in future studies taking the possible role of ethnicity and other concomitant autoimmune diseases into account.

A potential mechanism underlying the observed association between high-normal maternal FT4 levels, fetal weight, birth weight and SGA is the trans-placental delivery of high-normal FT4 levels to the fetus. In a normal functioning fetal hypothalamus-pituitary-thyroid axis, this will be compensated by a decreased production of T4 by the fetal thyroid. However, various factors are known to influence the hypothalamus-pituitary-thyroid axis function, such as common polymorphisms in thyroid hormone pathway genes (32-34).

Limited data are available on the correlations between early pregnancy FT4 levels and FT4 levels later in pregnancy. Lambert-Messerlian et al. found a weak positive correlation ($r = 0.32$) between FT4 levels in the first and second trimesters of pregnancy (35). As there are no other large studies correlating FT4 levels throughout pregnancy, more large studies are needed which also take the third trimester into account. As only early pregnancy (mean = 13.3 wks) FT4 levels were available in the current study, we do not know if all women with high-normal FT4 levels had high-normal FT4 levels during the entire pregnancy. However, in this context it is important to note that Shields et al. (28) also found a lower birth weight in newborns of mothers with a higher FT4 level in mid-pregnancy (mean = 28 wks).

Taken together, we show that maternal high-normal FT4 levels in early pregnancy are associated with lower fetal weight, lower birth weight, and an increased risk of SGA and LBW newborns. These data demonstrate that even mild variation in thyroid function within the normal range can have important consequences for the fetus and newborn, and underline the importance of tight regulation of FT4 levels during pregnancy. The exact mechanism underlying the observed associations should be clarified in future studies, taking the maternal metabolic profile and placental passage of T4 into account.

Our results suggest that it could be beneficial to narrow down the maternal early pregnancy FT4 reference ranges. However, before taking such measures, the effects on the risk of other pregnancy complications need to be considered as well. Low thyroid function has for example been associated with miscarriage, preeclampsia, and delayed child cognitive function (1,3,36). However, little is known about the effects of variation in FT4 levels within the normal range on these endpoints. As we show clear effects of variation in maternal FT4 levels within the normal range on birth weight, our results should prompt others to study the effects of variation in normal range FT4 levels on these other endpoints as well.

Contrary to what might be expected based on the negative association between early pregnancy maternal FT4 levels and birth weight, a positive association between cord FT4 levels and birth weight was found. Recently, Shields et al. studied birth weight in relation to cord FT4 levels in 616 mother-child pairs and found a similar positive associa-

tion (28). Leptin is produced by adipocytes and is known to stimulate the hypothalamus-pituitary-thyroid axis by increasing TRH production (37). In this context, it is interesting to note that in the current study we additionally found a positive association between birth weight and cord TSH levels, as well as a positive association between cord TSH and FT4 levels. We have previously shown a positive association between maternal and cord TSH levels, as well as a positive association between maternal and cord FT4 levels (22). Given these interrelations between maternal and cord thyroid parameters and birth weight, we additionally corrected the cord thyroid parameter and birth weight analyses for maternal thyroid parameters, but no differences in effects were observed. Taken together, these findings suggest that increased leptin production in heavier newborns could play a role in these observed associations, which needs to be clarified in future studies. These studies should also take a possible role for insulin into account, given the complex relations between maternal and newborn thyroid parameters and weight.

In conclusion, we show that maternal high-normal FT4 levels at an early stage of pregnancy are associated with a lower fetal weight and birth weight, as well as with an increased risk of SGA and LBW newborns. We did not find any association between maternal TPOAb status and fetal or birth weight. Finally, positive associations between birth weight and cord TSH and FT4 levels were found, as well as a positive association between cord TSH and FT4 levels.

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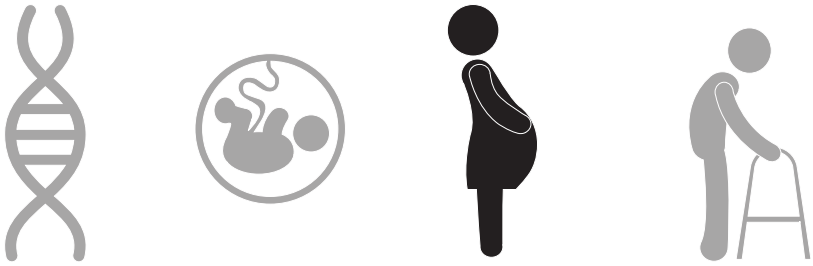
Chapter 6

Maternal early-pregnancy thyroid function is associated with subsequent hypertensive disorders of pregnancy: the Generation R Study.

M. Medici*, T.I.M. Korevaar*, S. Schalekamp-Timmermans, R. Gaillard, Y.B. de Rijke, W.E. Visser, W. Visser, S.M.P.F. de Muinck Keizer-Schrama, A. Hofman, H. Hooijkaas, J.J. Bongers-Schokking, H. Tiemeier, V.W.V. Jaddoe, T.J. Visser, R.P. Peeters, E.A.P. Steegers

Submitted

**Both authors contributed equally to this work.*



ABSTRACT

Context: Hypertensive disorders during pregnancy are associated with a wide range of maternal and fetal complications, and only few risk factors are known for the development of these disorders during pregnancy. Conflicting and limited data are available on the relation between thyroid (dys)function and the risk of hypertensive disorders of pregnancy.

Objective: To study the associations between early-pregnancy thyroid dysfunction, thyroid function within the normal range, and the risk of hypertensive disorders.

Design, Setting and Participants: In early pregnancy, serum TSH, FT4 and TPO-antibody (TPOAb) levels were determined in 5153 pregnant women. The associations of thyroid function with the risk of hypertensive disorders were studied.

Main Outcome Measures: Mean blood pressures and hypertensive disorders, including pregnancy-induced hypertension (PIH; n=209) and preeclampsia (n=136).

Results: Hyperthyroid mothers had a higher risk of hypertensive disorders (OR (95% CI) = 3.40 (1.46-7.91), $P = 0.005$), which was mainly due to an increased risk of PIH (OR = 4.18 (1.57-11.1), $P = 0.004$). Hypothyroidism and hypothyroxinemia were not associated with hypertensive disorders.

Within the normal range, high-normal FT4 levels were associated with an increased risk of hypertensive disorders (OR = 1.62 (1.06-2.47), P=0.03), which was mainly due to an increased risk of preeclampsia (OR = 2.06 (1.04-4.08), P=0.04).
TPOAb status was not associated with hypertensive disorders.

Conclusions: We show that hyperthyroidism and also high-normal FT4 levels during early-pregnancy are associated with an increased risk of hypertensive disorders. These data demonstrate that even mild variation in thyroid function within the normal range can have such effects.

INTRODUCTION

Hypertensive disorders, including pregnancy-induced hypertension (PIH) and (pre) eclampsia, are common during pregnancy with an estimated prevalence of 2-8% (1-3). Various studies have shown that hypertensive disorders are a major cause of both maternal and fetal morbidity and mortality. Amongst others, complications may include renal failure, disseminated intravascular coagulation, cerebrovascular bleeding, intrauterine growth retardation, abruptio placentae, premature delivery and still births (1, 3).

Both hypo- and hyperthyroidism have been shown to have important vascular effects, including endothelial cell dysfunction (4-8). Therefore, a number of studies have investigated the association between thyroid dysfunction and hypertensive disorders during pregnancy (9-19). Most of these studies were of limited sample size, but a number of large studies have also been published on this topic in the last decade (9-12, 15, 16, 19). Some of these studies have found an increased risk of hypertensive disorders in mothers with hypothyroidism (9, 15, 19) or hyperthyroidism (15), while others did not find any associations (10-12, 16). Differences between these studies might have been due to the fact that not all studies controlled for potentially confounding factors, such as thyroid autoimmunity, smoking, body mass index (BMI), ethnicity, socio-economic status, and parity.

More recently, a number of reports have shown that even minor variations in thyroid function can have important effects on pregnancy complications (20-22). In this context it is interesting to note that none of these studies have investigated the effects of variation in thyroid function within the normal range on the risk of hypertensive disorders.

For these reasons, we studied the effects of thyroid function within the normal range, thyroid dysfunction, and thyroid autoimmunity on blood pressure and the risk of hypertensive disorders during pregnancy. This study was carried out in a population-based pregnancy cohort including 5153 women, taking the effects of a wide range of confounding factors into account.

MATERIALS AND METHODS

Design

This study was embedded in the Generation R Study, a population-based cohort from early fetal life onwards in Rotterdam, the Netherlands, which has been described in detail previously (23, 24). Mothers with a delivery date between April 2002 and January 2006 were enrolled. The study was approved by the Medical Ethics Committee of the Erasmus Medical Center, Rotterdam. Written informed consent was obtained from all adult participants.

Population for analysis

8880 women were enrolled in pregnancy in the Generation R Study. TSH and FT4 levels were determined in 5803 pregnant women. Women with twin pregnancies (n = 128), pre-existing thyroid disease (n = 81), thyroid (interfering) medication usage (n = 4) and fertility treatment (n = 68) were excluded. If subsequent pregnancies were recorded in the database only the record of the first pregnancy was used (n = 369 excluded). In total, 5153 women were included in one or more analyses.

Thyroid measurements

Maternal serum samples were obtained in early pregnancy (mean (SD): 13.3 (1.7) wk) (24). Plain tubes were centrifuged and serum was stored at -80 C. TSH and FT4 were determined in maternal serum samples using chemiluminescence assays (Vitros ECI; Ortho Clinical Diagnostics, Rochester, NY). The intra- and interassay coefficients of variation were <4.1% for TSH at a range of 3.97-22.7 mU/L and <5.4% for FT4 at a range of 14.3-25.0 pmol/L. Maternal TPOAbs were measured in 5067 women using the Phadia 250 immunoassay (Phadia AB, Uppsala, Sweden) and regarded as positive when >60 IU/ml (24).

Iodine measurements

Maternal serum and urinary samples were obtained at the same time (mean (SD): 13.3 (1.7) wk). Urinary iodine concentrations were determined in a random subset of 1085 women, which has been described in detail previously (25). Median urinary iodine excretion was used to determine population iodine status as advocated by the WHO (with <150 µg/L as insufficient, 150 – 249 µg/L as adequate, and >500 µg/L as excessive) (26).

Hypertensive disorders

Blood pressure measurements were performed in early, mid and late pregnancy (27). The hypertensive disorders group included women with PIH or preeclampsia. Women who delivered in hospital and who were reported to have experienced PIH or preeclampsia (which included preeclampsia, eclampsia, and/or HELLP syndrome), were selected from hospital registries. Their individual medical records were subsequently studied by qualified medical doctors, who defined PIH or preeclampsia according to the criteria of the International Society for the Study of Hypertension in Pregnancy (ISSHP) (28). Briefly, the following criteria were used to identify woman with PIH: development of systolic blood pressure ≥140 mm Hg and/or diastolic blood pressure ≥90 mmHg after 20 weeks of gestation in a previously normotensive woman. These criteria plus the presence of proteinuria (defined as two or more dipstick readings of 2+ or greater, one catheter sample reading of 1+ or greater, or a 24-hour urine collection containing at least 300 mg of protein) were used to identify women with preeclampsia (29).

Covariates

Ultrasound measurements were used to establish gestational age in early pregnancy (gestational age 12 wks) (23). Information on maternal age, parity, smoking status, socio-economic status (SES) and ethnicity was obtained by questionnaires during pregnancy. Ethnicity was determined by country of origin and was defined according to the classification of Statistics Netherlands. Maternal smoking status was classified as no smoking, smoking until known pregnancy, and continued smoking during pregnancy. SES was defined by educational level, net household income, and employment status (23). At enrolment, maternal height and weight were measured to calculate body mass index (BMI, kg/m^2).

Statistical analysis

Reference ranges were determined by the 2.5th-97.5th percentiles, as described previously (24). Hyperthyroidism was defined as a low (<2.5th percentile) TSH with a high (>97.5th percentile) FT4; subclinical hyperthyroidism as a low TSH with a normal (2.5th – 97.5th percentiles) FT4; hypothyroidism as a high TSH with a low FT4; subclinical hypothyroidism as a high TSH with a normal FT4, and hypothyroxinemia as a low FT4 with a normal TSH. For the normal range TSH and FT4 quintiles, cut-off levels were: TSH: 1st 0.03-0.76 mU/L; 2nd 0.77-1.13 mU/L; 3rd 1.14-1.54 mU/L; 4th 1.55-2.12 mU/L; 5th 2.13-4.03 mU/L; and FT4: 1st 10.4-12.8 pmol/L; 2nd 12.9-14.1 pmol/L; 3rd 14.2-15.4 pmol/L; 4th 15.5-17.0 pmol/L; 5th 17.1-21.9 pmol/L.

Blood pressure levels and their course during pregnancy were analyzed in these groups and compared to the euthyroid group (i.e., women with normal TSH and FT4 levels) using a mixed linear model for repeated measurements which allowed for missing data points (in total 14,125 measurements performed) (30, 31). For the normal range quintile analyses, the 3rd quintile was used as the reference quintile. Analyses were performed using SAS 9.3 (SAS Institute, Cary, NC, USA). Logistic regression analyses were used to calculate the risk of hypertensive disorders in these groups.

Analyses were adjusted for gestational age at venous puncture and blood pressure measurement, maternal age, smoking status, SES, parity, ethnicity, BMI and child gender. Placental weight was not associated with thyroid function. Thyroid function was not associated with maternal comorbidities including diabetes, chronic hypertension, hypercholesterolemia, chronic heart disorder, and systemic lupus erythematosus. Similar results were obtained when women with these comorbidities were excluded.

We used multiple imputation for covariates with missing data. Five imputed data sets were created and pooled for analyses. Smoking, SES, ethnicity (missing due to non-response in 13.0%, 7.1% and 5.7%, respectively), gestational age at blood sampling, and BMI (missing due to not recorded in 2.0% and <1.0%) were added to the model. Furthermore, we added hypertensive disorders, TSH, FT4 and TPOAb levels, maternal age, parity and child gender to the model as prediction variables only. No significant differences in descriptive characteristics were found between the original and imputed datasets. Women

with pre-existing hypertension were excluded from analyses on hypertensive disorders, PIH or blood pressure. Unless stated otherwise, statistical analyses were performed using Statistical Package of Social Sciences version 20.0 for Windows (SPSS Inc. Chicago, IL, USA).

RESULTS

The study population consisted of 5153 women, of which 345 (6.7%) developed a hypertensive disorder. Of these women, 209 (4.1%) developed PIH and 136 (2.6%) developed preeclampsia. Baseline characteristics of the studied population are shown in Table 1. Median urinary iodine excretion was 221 µg/L, indicating an iodine sufficient population (26).

Table 1. Baseline characteristics of 5153 pregnant women from the Generation R Study

Maternal age (yrs (SD))	29.7 (5.1)
Thyroid function parametersa (median)	
TSH (mU/L)	1.34
FT4 (pmol/L)	14.8
TPOAb-positivity (%)	5.5
Urinary Iodine excretiona (median, µg/L)	221
Hypertensive disorderb (N (%))	345 (6.7)
Pregnancy induced hypertension	209 (4.1)
Preeclampsia[c]	136 (2.6)
BMI (kg/m2 (SD))	24.5 (4.4)
Parity (%)	
Nullipara	60.9
Primipara	26.0
Multipara	13.1
Smoking (%)	
Never	72.6
Former	9.4
Active	18.0
Socio-economic status (%)	
Low	10.9
Middle	46.9
High	42.2
Ethnicity (%)	
Dutch	51.1
Moroccan	6.3
Turkish	8.7
Surinamese/Antillean	12.1
Other western	11.9
Other non-western	9.9
Child gender (% boys)	50.8

[a] Blood and urine samples were collected at 13.5 (2.0) (mean (SD)) wks
[b] Includes pregnancy induced hypertension, preeclampsia, eclampsia and HELLP.
[c] Includes preeclampsia, eclampsia and HELLP.

Thyroid dysfunction, normal range thyroid function and mean blood pressure levels

Figure 1 shows the mean systolic and diastolic blood pressures during pregnancy for the thyroid dysfunction and euthyroid groups. The mean blood pressures for the normal-range TSH and FT4 quintiles are shown in Supplemental Figure 1. Minor differences in the course of blood pressures were found among the different groups, but this did not

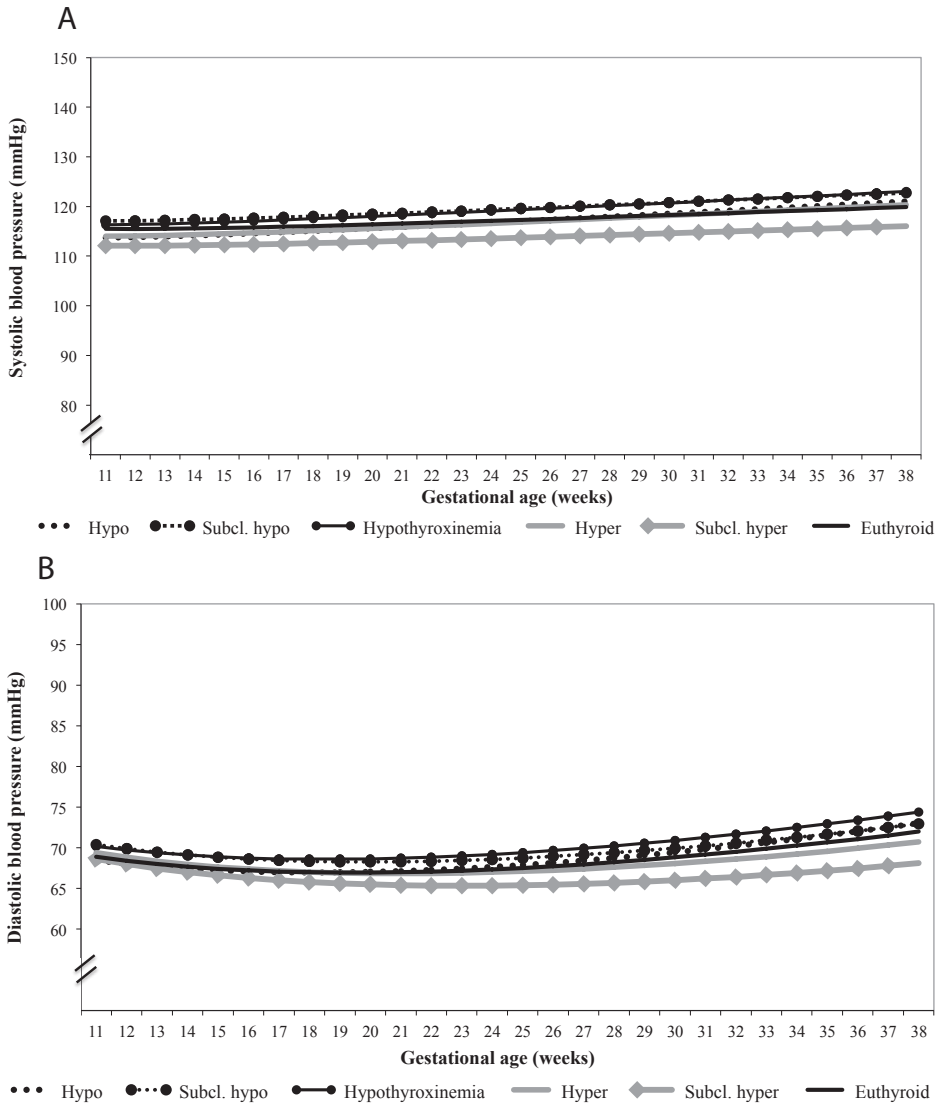


Figure 1. Thyroid dysfunction and mean systolic (A) and diastolic (B) blood pressures during pregnancy. Differences between groups were small and not statistically significant.

result in significant differences in mean blood pressures, except for a small difference in diastolic blood pressures between the fifth and third FT4 quintiles ($P_{adjusted}$ = 0.005).

Thyroid dysfunction, normal range thyroid function and the risk of hypertensive disorders

The relations between thyroid dysfunction, normal range thyroid function and hypertensive disorders are shown in Table 2. Women with hyperthyroidism had a 3.4-fold higher risk of developing a hypertensive disorder. The other studied thyroid dysfunction groups were not associated with hypertensive disorders. Within the normal range, women with high-normal FT4 levels also had an increased risk of hypertensive disorders. No significant associations were found with normal-range TSH levels.

Table 2. Thyroid dysfunction, normal range thyroid function, and the risk of hypertensive disorders during pregnancy.

	Hypertensive disorders		
	% (N)	**OR (95% CI)**	***P***
Overt hypothyroidism	0.0 (0/17)	*NA*	*NA*
Subclinical hypothyroidism	8.5 (14/165)	1.23 (0.69-2.22)	0.47
Hypothyroxinemia	6.4 (9/129)	1.08 (0.53-2.22)	0.83
Overt hyperthyroidism	13.7 (7/51)	3.40 (1.46-7.91)	0.005
Subclinical hyperthyroidism	3.2 (2/62)	0.80 (0.19-3.34)	0.76
Euthyroidism[a] (reference)	6.2 (276/4451)	reference	
Normal range TSH			
1st quintile	6.8 (60/885)	1.36 (0.91-2.03)	0.14
2nd quintile	4.7 (43/907)	0.87 (0.56-1.33)	0.51
3rd quintile (reference)	5.6 (50/894)	reference	
4th quintile	6.3 (56/884)	1.11 (0.74-1.67)	0.61
5th quintile	7.6 (67/881)	1.23 (0.83-1.81)	0.31
Normal range FT4			
1st quintile	6.6 (59/897)	1.28 (0.83-1.96)	0.26
2nd quintile	6.6 (63/956)	1.36 (0.90-2.07)	0.15
3rd quintile (reference)	4.7 (40/848)	reference	
4th quintile	5.8 (51/885)	1.28 (0.83-1.98)	0.27
5th quintile	7.3 (63/865)	1.62 (1.06-2.47)	0.03

All analyses adjusted for gestational age at blood sampling, maternal age, BMI, smoking, SES, parity, ethnicity, and child gender.

[a] Defined as mothers with normal range (2.5th-97.5th percentiles) TSH and FT4 levels.

NA: Not available (no statistics were performed on this group as the number of persons with overt hypothyroidism was low and this group did not include any cases with hypertensive disorders).

Table 3 shows the effects of thyroid dysfunction and normal range thyroid function on PIH and preeclampsia, when analyzed separately. Women with overt hyperthyroidism had a 4.2-fold higher risk of PIH. These women also seemed to have a higher risk of preeclampsia, but this effect was not statistically significant (P = 0.11). Within the normal range, high-normal FT4 levels were associated with a 2.1-fold increased risk of preeclampsia. There were no associations between normal-range TSH levels and PIH or preeclampsia.

Finally, there were no associations between TPOAb-positivity and PIH or preeclampsia (data not shown).

Table 3. Thyroid dysfunction, normal range thyroid function, and the risk of pregnancy induced hypertension or preeclampsia.

	Pregnancy induced hypertension			**Preeclampsia**[a]		
	% (N)	**OR (95% CI)**	***P***	**% (N)**	**OR (95% CI)**	***P***
Overt hypothyroidism	0.0 (0/17)	*NA*	*NA*	5.6 (1/18)	1.84 (0.23-14.7)	0.56
Subclinical hypothyroidism	4.2 (7/165)	0.90 (0.41-2.00)	0.80	4.8 (8/168)	1.80 (0.85-3.82)	0.13
Hypothyroxinemia	4.7 (6/129)	1.23 (0.51-2.93)	0.65	3.8 (5/133)	1.29 (0.50-3.29)	0.60
Overt hyperthyroidism	9.8 (5/51)	4.18 (1.57-11.1)	0.004	5.7 (3/53)	2.68 (0.80-9.00)	0.11
Subclinical hyperthyroidism	1.6 (1/62)	0.72 (0.10-5.31)	0.75	1.6 (1/64)	0.64 (0.09-4.78)	0.66
Euthyroidism[b] (reference)	4.0 (177/4451)	reference		2.5 (113/4527)	reference	
Normal range TSH						
1st quintile	3.8 (34/892)	1.19 (0.71-1.98)	0.51	3.3 (30/905)	1.58 (0.89-2.81)	0.12
2nd quintile	3.7 (33/900)	1.11 (0.66-1.85)	0.70	1.4 (13/916)	0.62 (0.31-1.25)	0.18
3rd quintile (reference)	3.5 (31/894)	reference		2.3 (21/907)	reference	
4th quintile	3.7 (33/884)	1.06 (0.64-1.74)	0.84	2.9 (26/900)	1.22 (0.68-2.20)	0.51
5th quintile	5.2 (46/881)	1.33 (0.83-2.16)	0.24	2.6 (23/899)	1.01 (0.55-1.86)	0.97
Normal range FT4						
1st quintile	4.0 (36/897)	1.03 (0.62-1.72)	0.91	2.8 (26/915)	1.68 (0.85-3.34)	0.14
2nd quintile	4.1 (39/956)	1.11 (0.67-1.83)	0.68	2.7 (26/974)	1.68 (0.85-3.31)	0.14
3rd quintile (reference)	3.5 (30/848)	reference		1.5 (13/862)	reference	
4th quintile	3.7 (33/885)	1.04 (0.62-1.74)	0.89	2.5 (22/897)	1.81 (0.90-3.65)	0.10
5th quintile	4.5 (39/865)	1.25 (0.75-2.06)	0.39	3.0 (26/879)	2.06 (1.04-4.08)	0.04

All analyses adjusted for gestational age at blood sampling, maternal age, BMI, smoking, SES, parity, ethnicity, and child gender.

[a] Includes preeclampsia, eclampsia and HELLP.

[b] Defined as mothers with normal range (2.5th-97.5th percentiles) TSH and FT4 levels.

NA: Not available (no statistics were performed on this group as the number of persons with overt hypothyroidism was low and this group did not include any cases with hypertensive disorders).

DISCUSSION

In the current study, we investigated the effects of normal-range thyroid function and thyroid dysfunction on the risk of hypertensive disorders during pregnancy. We first studied the effects of thyroid (dys)function on mean systolic and diastolic blood pressure levels, but did not find any significant effects. Also within the normal range no effects were observed, except for small differences in diastolic blood pressures between the fifth and third FT4 quintiles, ranging from 0-1 mmHg. Although several studies have investigated the associations between thyroid dysfunction and hypertensive disorders during pregnancy (9-19), no data were so far available on the effects of variation in thyroid function within the normal range on the risk of hypertensive disorders during pregnancy. This study is therefore the first to demonstrate that also within the normal range, women with high-normal FT4 levels have a 2-fold increased risk of preeclampsia.

Most of the studies that investigated the associations between thyroid dysfunction and hypertensive disorders had a limited sample size and showed conflicting results (9-19). The largest study, recently published by Mannisto *et al* (15), investigated the associations between hypo- or hyperthyroidism and hypertensive disorders in a retrospective US cohort of 223,512 pregnancies. An increased risk of preeclampsia was found for both hypo-and hyperthyroidism. Unfortunately, this study lacked information on treatment of thyroid disease during pregnancy and no data on TPOAb status were available. As data were derived from electronic medical records, the authors were not able to study more subtle alterations in thyroid function, including subclinical hypo- and hyperthyroidism and variation in thyroid function within the normal range. In a prospective population-based cohort of 24,883 pregnancies, Wilson *et al.* found positive associations between subclinical hypo- and hyperthyroidism and the risk of PIH, mild preeclampsia and severe preeclampsia (19). However, after adjustment for confounding factors (i.e., maternal age, weight, ethnicity, and parity) the only remaining significant association was between subclinical hypothyroidism and severe preeclampsia. Ashoor *et al.* compared serum thyroid parameters in the first trimester between pregnant women that did or did not develop preeclampsia (9). Although higher TSH and lower FT4 levels were found in 77 pregnant women that would later develop preeclampsia, no data were reported on the prevalence of overt hypothyroidism, subclinical hypothyroidism or hypothyroxinemia in these groups.

In contrast to the studies discussed above, a number of large studies did not find any associations between thyroid dysfunction and hypertensive disorders during pregnancy (10-12, 16). These conflicting results could at least partially be due to the fact that not all studies had data on thyroid medication and various serum thyroid parameter cut-off levels were used to define thyroid disease. In addition, only part of these studies were able to correct for factors which are known to be associated with thyroid parameters

and/or the risk of hypertensive disorders, including maternal BMI, age, parity, smoking, SES and ethnicity. The current population-based study investigates the effects of the entire range of thyroid (dys)function on blood pressure and hypertensive disorders during pregnancy. As thyroid function reference ranges can differ between populations (24), we calculated reference ranges in our own population and in our analyses we took a wide range of potentially interfering factors into account. In this way, we found that pregnant women with hyperthyroidism have a substantially increased risk of hypertensive disorders (13.7 vs 6.2%), mainly due to an increased risk of PIH. Although our study included more than 5,000 pregnant women, there were only few cases with overt hypothyroidism, limiting the statistical power for this group.

Hypertensive disorders during pregnancy are of great importance as they account for 16% of the worldwide maternal deaths (2). Not only have these disorders been associated with an increased risk of maternal and child morbidity and mortality during pregnancy, but also after pregnancy. For example, various studies have shown an increased risk of maternal hypertension, ischemic heart disease, stroke, end-stage renal disease, and mortality in later life (32-34), as well as an increased risk of childhood hypertension, cognitive limitations, and mortality (3, 34-37).

Possible mechanisms by which thyroid hormone may influence the onset of hypertensive disorders during pregnancy come from studies which investigated the cardiovascular effects of thyroid dysfunction. Some of these studies have shown that (subclinical) hypothyroidism is associated with increased vascular resistance, increased blood pressure, ventricular hypertrophy and endothelial cell dysfunction, characterized by decreased nitric oxide production with impaired vasorelaxation (6, 8). Although less is known about the vascular effects of high-normal FT4 levels or hyperthyroidism, a few studies have shown that patients with Graves' hyperthyroidism have a reduction in protective mechanisms against endothelial damage, and show signs of endothelial cell activation and dysfunction (4, 5, 7, 38). These studies suggest that high thyroid hormone levels can lead to endothelial cell dysfunction, which is known to play a pivotal role in the pathophysiology of hypertensive disorders in pregnancy (3). However, the exact mechanisms underlying the associations between high-normal thyroid function, hyperthyroidism and hypertensive disorders during pregnancy need to be clarified in future studies.

Given the wide range of detrimental effects of hypertensive disorders during pregnancy, various studies have tried to identify risk factors in early pregnancy for the development of hypertensive disorders, and it is remarkable to note that only few risk factors have been identified (3). The current study identifies high-normal FT4 levels and hyperthyroidism during early pregnancy as risk factors for hypertensive disorders. To predict which mothers will develop hypertensive disorders during pregnancy, Poon et al. developed a prediction model, including maternal history, uterine artery pulsatility

index, mean arterial pressure, pregnancy-associated plasma protein-A and placental growth factor (39). Future studies should analyze if serum thyroid function tests could increase the sensitivity of this prediction model. Furthermore, given that only few risk factors have been associated with the development of hypertensive disorders during pregnancy, the diagnostic workup after the diagnosis of a hypertensive disorder is limited (3, 40). Our results suggest that it would be useful to add thyroid function testing to this diagnostic workup.

In conclusion, we show that hyperthyroidism and also high-normal FT4 levels during early-pregnancy are risk factors for the development of hypertensive disorders. These data demonstrate that even mild variation in thyroid function within the normal range can have such effects.

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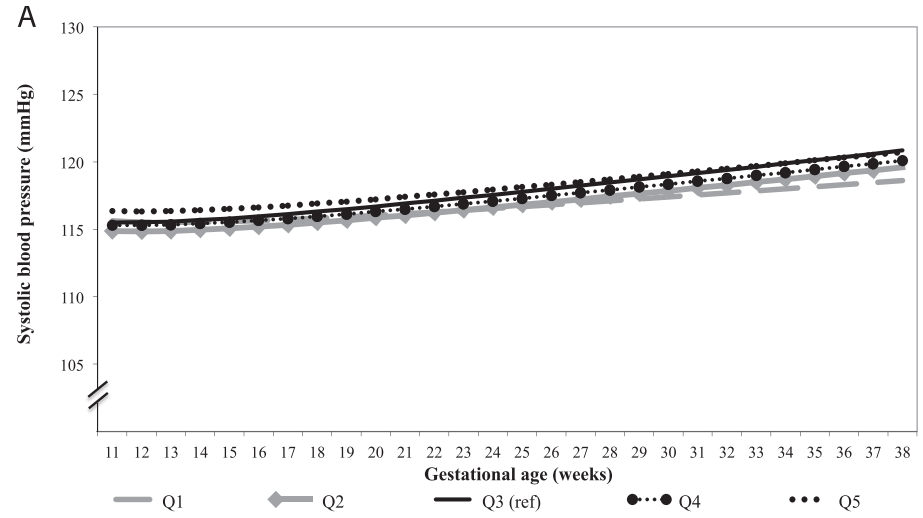
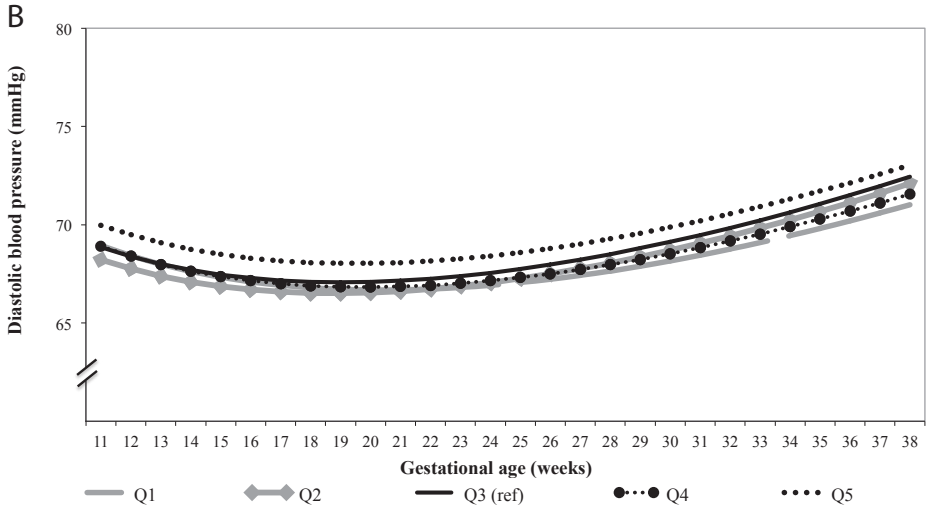
Supplemental Figure 1

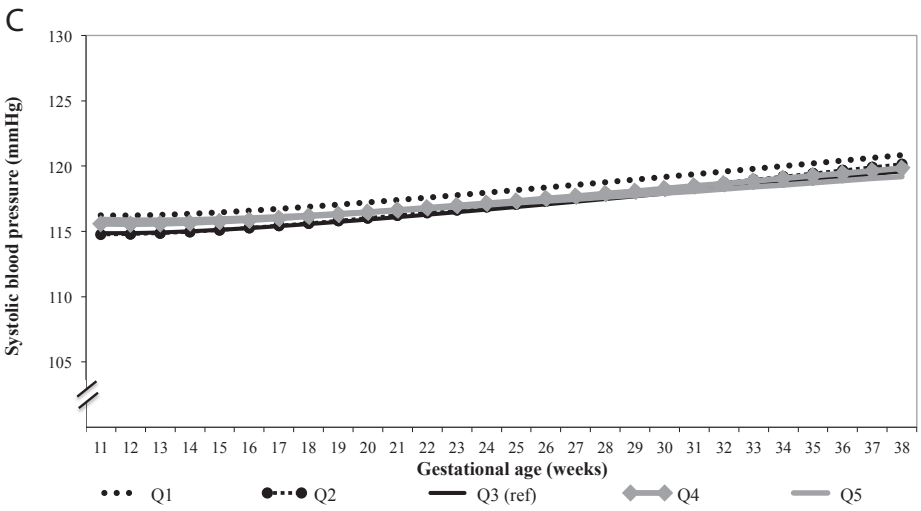


Normal-range TSH quintiles and systolic blood pressure during pregnancy

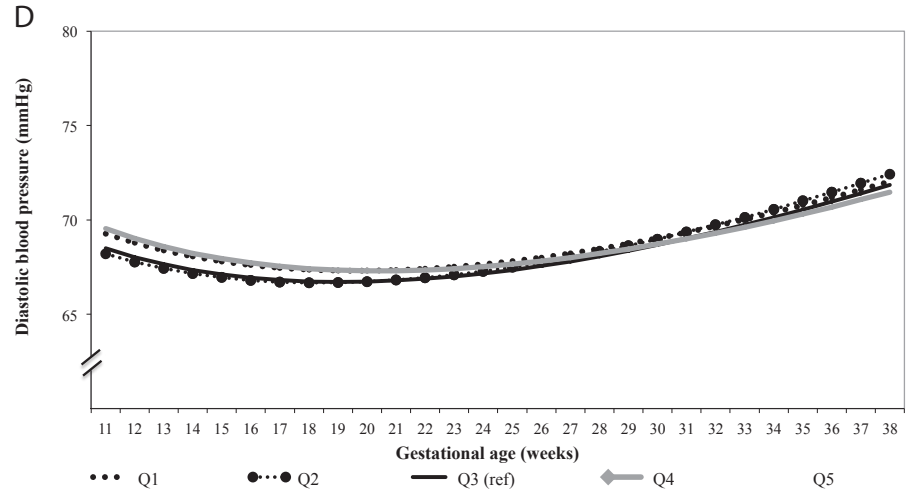


Normal-range TSH quintiles and diastolic blood pressure during pregnancy

Supplemental Figure 1 (continued)



Normal-range FT4 quintiles and systolic blood pressure during pregnancy



Normal-range FT4 quintiles and diastolic blood pressure during pregnancy

Suppl. Fig 1. Normal-range TSH and FT4 quintiles and mean systolic (a+c) and diastolic (b+d) blood pressures during pregnancy. Besides a significant difference in diastolic blood pressures between FT4-Q5 and -Q3 (*P*adjusted = 0.005), differnces were small and not statistically significant.
TSH Quintiles: Q1: 0.03-0.76 mU/L; Q2: 0.77-1.13 mU/L; Q3: 1.14-1.54 mU/L; Q4: 1.55-2.12 mU/L; Q5: 2.13-4.03 mU/L.
FT4 Quintiles: Q1: 10.4-12.8 pmol/L; Q2: 12.9-14.1 pmol/L; Q3: 14.2-15.4 pmol/L; Q4: 15.5-17.0 pmol/L; Q5: 17.1-21.9 pmol/L.

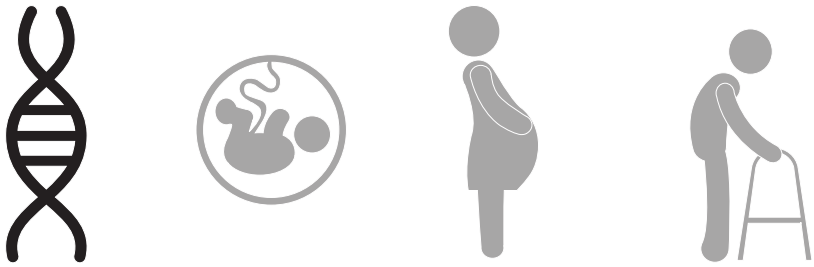



Chapter 7

A large-scale association analysis of 68 thyroid hormone pathway genes with serum TSH and FT4 levels.

M. Medici, W.M. van der Deure, M. Verbiest, S.H. Vermeulen, P.S. Hansen, L.A. Kiemeney, A.R. Hermus, M.M. Breteler, A. Hofman, L. Hegedüs, K.O. Kyvik, M. den Heijer, A.G. Uitterlinden, T.J. Visser, R.P. Peeters

European Journal of Endocrinology 2011; 164:781-8.



ABSTRACT

Objective: Minor variation in thyroid hormone (TH) serum levels can have important effects on various clinical endpoints. Although 45-65% of the inter-individual variation in TH serum levels is due to genetic factors, the causative genes are not well established. We therefore studied the effects of genetic variation in 68 TH pathway genes on serum TSH and FT4 levels.

Design and Methods: 68 genes (1512 polymorphisms) were studied in relation to serum TSH and FT4 levels in 1121 Caucasian subjects. Promising hits ($P < 0.01$) were studied in 3 independent Caucasian populations (2656 subjects) for confirmation. A meta-analysis of all 4 studies was performed.

Results: For TSH, 8 *PDE8B* polymorphisms ($P = 4.10^{-17}$) remained significant in the meta-analysis. For FT4, 2 *DIO1* ($P = 8.10^{-12}$) and 1 *FOXE1* ($P = 0.0003$) polymorphisms remained significant in the meta-analysis. Suggestive associations were detected for 1 *FOXE1* ($P = 0.0028$) and 3 *THRB* ($P = 0.0045$) polymorphisms with TSH, and 1 *SLC16A10* polymorphism ($P = 0.0110$) with FT4, but failed to reach the significant multiple-testing corrected p-value ($P < 0.0022$ and $P < 0.0033$ respectively).

Conclusions: Using a large-scale association analysis, we replicated previously reported associations with genetic variation in *PDE8B*, *THRB* and *DIO1*. We demonstrate effects of genetic variation in *FOXE1* on serum FT4 levels, and borderline significant effects on serum TSH levels. A suggestive association of genetic variation in *SLC16A10* with serum FT4 levels was found. These data provide insight into the molecular basis of inter-individual variation in TH serum levels.

INTRODUCTION

Adequate thyroid hormone (TH) levels are essential for normal growth and differentiation, for the regulation of energy metabolism, and for the physiological function of virtually all human tissues. Epidemiological evidence shows that minor variation in TH serum levels, even within the normal range, can have important effects on clinical endpoints, such as bone mineral density (1), atrial fibrillation (2), metabolic syndrome (3) and cardiovascular mortality (4).

In healthy subjects, serum TSH and TH levels show substantial inter-individual variability leading to wide laboratory reference ranges, whereas the intra-individual variability is within a narrow range, suggesting that every person has its own individual 'set-point' (5). Approximately 45-65% of this inter-individual variation in serum TSH and TH levels is due to genetic factors (6, 7). The causative genes are, however, not well established. Well-known TH pathway genes such as the deiodinases (8-12), TSH receptor (10, 13, 14) and TH transporters (15-17) have been associated with TH serum levels, but their contribution to the overall variation is modest (12, 13). A genome wide linkage scan by Panicker and colleagues identified eight chromosomal loci involved in the control of the pituitary-thyroid axis, but as can be expected from this type of study, the actual genes were not identified (18). Recent genome wide association studies demonstrated associations of polymorphisms located in Phosphodiesterase 8B (19) and the *CAPZB* locus (20) with serum TSH levels.

The identification of new associations in genome wide association studies is hampered by the need for stringent correction for multiple testing, requiring p-values < 5.10^{-7} (21). For this reason, we performed a focused association analysis of 68 candidate genes, known to be involved in TH synthesis, metabolism or transport, in relation to serum TSH and FT4 levels. Promising hits were studied in three independent populations for confirmation.

MATERIALS AND METHODS

The association of serum TSH and FT4 levels with genetic variation in the candidate genes was studied in the Rotterdam Study (22). Promising associations were studied in a Danish twin population (6), the Scan Study (23) and the Nijmegen Biomedical Study (24) for confirmation, after which a meta-analysis of all 4 studies was conducted.

Subjects with serum FT4 levels indicating hypo- or hyperthyroidism were excluded, as common genetic variation is in general thought to play a minor role in the pathogenesis of hypo- and hyperthyroidism. As the role of common genetic variation in subclinical hypo- or hyperthyroidism is less clear, we did not exclude subjects with TSH levels

outside the reference ranges. Positive thyroid peroxidase antibodies (TPOAbs), known thyroid disease and/or thyroid medication usage were excluded from all analyses.

Selection of candidate genes

A selection of candidate genes was made by searching NCBI GenBank for 'thyro' limited by 'human' and 'current' (520 genes). Based on the current literature, an expert in the field (TJV) reviewed these genes, which resulted in a selection of 70 genes with a known role in thyroid hormone synthesis, transport or metabolism. As our cohorts consisted of both men and women, genes on the X-chromosome (i.e., *MCT8* and *TBG*) were excluded, resulting in a final selection of 68 genes (Figure 1).

Study populations

The Rotterdam Study is a prospective population-based cohort study on determinants of chronic diseases in the elderly (22). The study comprised 7983 men and women living in a district of Rotterdam, The Netherlands. Informed consent was obtained from each participant, and the Medical Ethics Committee of the Erasmus Medical Center Rotterdam approved the study. At baseline, all participants were interviewed and underwent extensive physical examination. Serum TSH (TSH Lumitest; Henning, Berlin, Germany), FT4 (chemoluminescence assay; Vitros, ECI Immunodiagnostic System, Ortho-Clinical Diagnostics, Amersham, UK) and TPOAb (ELISA; Milenia, DPC, Los Angeles, USA) levels were determined in 1350 subjects of whom DNA was available. After excluding subjects with serum FT4 levels indicating hypo- or hyperthyroidism, positive TPOAbs, known thyroid disease and/or thyroid medication usage, 1121 subjects were available for analysis.

The Danish twin population is part of a nationwide project (GEMINAKAR) investigating the relative influence of genetic and environmental factors on various traits related to the metabolic syndrome and cardiovascular risk factors. Rationale and design have been described in detail previously (6). In short, a representative sample of self-reported healthy twin pairs was recruited from the population-based Danish Twin Registry (25). In the GEMINAKAR study 1512 men and women (756 twin pairs) were examined. Informed consent was obtained from each participant, and all regional Danish Scientific-Ethical Committees approved the study. At baseline, all participants were interviewed and underwent physical examination. Serum TSH (fluoroimmunometric assay; PerkinElmer/Wallac, Turku, Finland), FT4 (AutoDELFIA; PerkinElmer/Wallac, Turku, Finland) and TPOAb (AutoDELFIA; PerkinElmer/Wallac, Turku, Finland) levels were determined in 905 subjects of whom DNA was available. After applying exclusion criteria, 474 unrelated subjects were included in the present study.

The Scan Study is a prospective population-based cohort study in 1077 men and women, designed to study causes and consequences of age-related brain changes on MRI. Rationale and design have been described in detail previously (23). Informed

consent was obtained from each participant, and the Medical Ethics Committee of the Erasmus Medical Center Rotterdam approved the study. At baseline, all participants were interviewed and underwent physical examination. Serum TSH and FT4 (chemoluminescence assay; Vitros, ECI Immunodiagnostic System, Ortho-Clinical Diagnostics, Rochester, USA) and TPOAb (immunometric assay; DPC, Los Angeles, USA) levels were determined in 854 subjects of whom DNA was available (8). After applying exclusion criteria, 697 subjects were included in the present study.

The Nijmegen Biomedical Study is a population-based survey on lifestyle and medical history in 9350 men and women living in Nijmegen, The Netherlands. Rationale and design have been described previously (24). Informed consent was obtained from each participant, and the Institutional Review Board of the Radboud University Nijmegen Medical Centre approved the study. Serum TSH levels (immunoluminometric assay; Architect, Abbott Diagnostics Division, Hoofddorp, The Netherlands), FT4 levels (chemoluminescence assay; Vitros, ECI Immunodiagnostic System, Ortho-Clinical Diagnostics, Amersham, UK), TPOAb levels (fluoroimmunometric assay; Abbott Diagnostics Division, Hoofddorp, The Netherlands) and Illumina HumanHap370K array (see below) genotype data were available for 1832 subjects (24). After applying exclusion criteria, 1485 subjects were included in the present study.

Genotyping

In all study populations, genomic DNA was extracted from samples of peripheral venous blood according to standard procedures. Subjects in the Rotterdam Study were genotyped using the Illumina HumanHap550K array. All directly genotyped polymorphisms with a minor allele frequency (MAF) ≥ 5% and located within a 20 kb region (10 kb upstream to 10 kb downstream) of each of the 68 candidate genes, were selected. After quality control (QC) and exclusion of polymorphisms with a Hardy-Weinberg equilibrium (HWE) p-value ≤ $1.\ 10^{-6}$ or genotyping call rate < 90%, 1512 polymorphisms were included in the analysis.

Genotypes for replication (46 polymorphisms) were determined using PCR, iPLEX single base primer extension, and matrix assisted laser desorption/ionization – time of flight mass spectrometry in a 384-well format (Sequenom, San Diego, CA, USA; see: http://www.sequenom.com) and ABI Taqman allelic discrimination Assay-on-Demand (Applied Biosystems Inc., Foster City, CA, USA). Polymorphisms with a genotyping call rate < 90% or a deviation from HWE were excluded from the analyses. rs989758, rs13097208, rs13066296 and rs832790 had a genotyping call rate < 90% in both the Danish twin population and the Scan study. For the Nijmegen Biomedical Study, genotype data were available from the Illumina HumanHap370K array (26). Polymorphism imputation after QC was based on Phase II CEU HapMap samples (version 22, build 36) and was done using IMPUTE (27).

Statistical methods

In the Rotterdam Study, associations with serum TSH and FT4 levels were assessed by linear regression using gender- and age-adjusted standardized residuals in PLINKv1.07 (28) and SPSS 15.0 for Windows (SPSS, Inc., Chicago, IL, USA). Due to non-normal distribution, TSH was transformed by the natural logarithm. Polymorphisms that showed significant associations at $P < 0.01$ were genotyped and studied in the Danish twin population, the Scan Study and the Nijmegen Biomedical Study for confirmation, using SPSS 15.0 for Windows and SNPTEST (27). To minimize the influence of inter-assay variation, effect sizes were assessed by linear regression using gender- and age-adjusted standardized residuals. For the polymorphisms that did not reach the p-value threshold of $P < 0.01$ in the Rotterdam Study, there was 80% power to detect differences of 0.23, 0.17 and 0.15 standard deviations in TSH and FT4 levels for MAFs of 10%, 20% and 30%, respectively. This study is therefore powered to detect at least moderate effects.

Meta-analyses based on all 4 populations were conducted using the METAL software package applying inverse-variance weighted fixed-effects methodology (http://www.sph.umich.edu/csg/abecasis/Metal). To control for multiple testing, a p-value threshold for both the TSH and the FT4 meta-analyses was calculated based on the number of

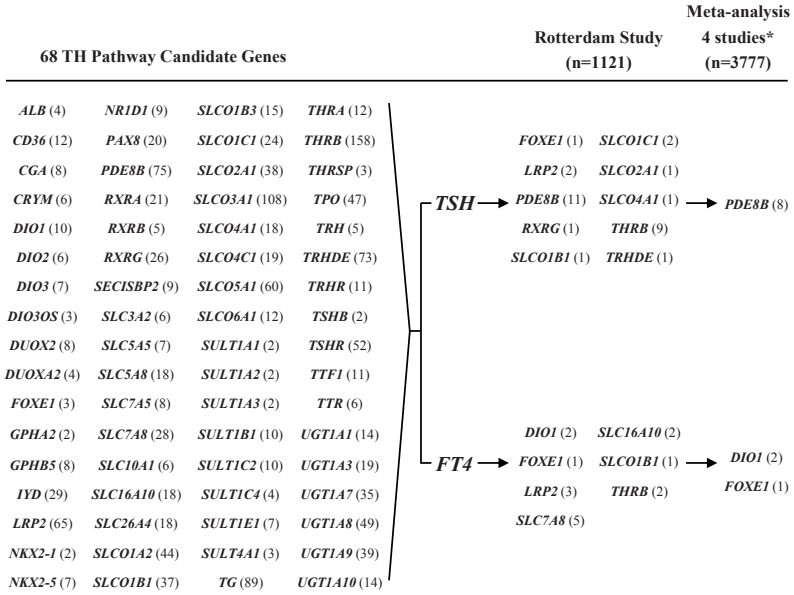


Figure 1. TSH and FT4 flowchart for polymorphisms in 68 thyroid hormone (TH) pathway candidate genes. At each stage, the genes that passed selection are shown, together with the number of polymorphisms (between brackets). Except for 17 imputed polymorphisms in the Nijmegen Biomedical Study, all polymorphisms were directly genotyped. In the Rotterdam Study, associations with $P < 0.01$ were considered significant. P-value thresholds for the TSH and FT4 meta-analyses were respectively $P = 0.0022$ and $P = 0.0033$.

*Rotterdam Study, Danish twin population, Scan Study and Nijmegen Biomedical Study.

independent polymorphisms tested, thereby taking the linkage disequilibrium (LD) structure between these polymorphisms into account. The number of independent polymorphisms was calculated in the Rotterdam Study using PLINKv1.07 (28), for which a LD threshold of $r^2 > 0.2$ was used. To define a p-value threshold to declare statistical significance, we divided $P = 0.05$ by the number of independent tests, which was estimated to be 22.84 for TSH and 15.24 for FT4. Consequently, the p-value thresholds for the TSH and FT4 meta-analyses were respectively $P = 0.0022$ and $P = 0.0033$.

RESULTS

Baseline characteristics of the studied populations are shown in Table 1.

Table 1. Baseline characteristics of the study populations

	Rotterdam Study	Danish Twins	Scan Study	Nijmegen Biomedical Study
Number	1121	474	697	1485
Ethnicity	Caucasian	Caucasian	Caucasian	Caucasian
Women (%)	58.5	46.0	48.8	46.6
Age (years)	69.0 (7.6)	36.0 (10.6)	71.3 (7.1)	61.7 (10.2)
TSH (mU/L)	1.74 (1.20)	1.75 (0.88)	1.32 (0.91)	1.51 (1.13)
FT4 (pmol/L)	16.4 (2.7)	12.9 (1.5)	17.8 (2.8)	13.6 (2.0)

Indicated numbers (mean (SD)) are based on subjects with available genotype data, after applying exclusion criteria.

Figure 1 summarizes the flowchart for the TSH and FT4 analyses, together with the genes that passed selection at each stage of the study. Of the 1512 studied polymorphisms in 68 genes, 30 polymorphisms in 10 genes showed a significant association (i.e., $P < 0.01$) with TSH in the Rotterdam Study. Of these, 8 polymorphisms in *PDE8B* remained significant at $P < 0.0022$ in the meta-analysis of the 4 populations (Table 2). Suggestive associations were detected for 1 polymorphism in *FOXE1* ($P = 0.0028$) and 3 polymorphisms in *THRB* (rs6792725: $P = 0.0087$, rs13097208: $P = 0.0045$, rs13066296: $P = 0.0056$), but failed to reach statistical significance after multiple-testing correction (Table 2).

For FT4, significant associations of 16 polymorphisms in 7 genes were detected in the Rotterdam Study. Of these, 2 polymorphisms in *DIO1* and 1 polymorphism in *FOXE1* remained significant at $P < 0.0033$ in the meta-analysis of the 4 populations (Table 3). In addition, a suggestive association was detected for 1 polymorphism in *SLC16A10*

Table 2. Effects of *FOXE1*, *PDE8B* and *THRB* polymorphisms on serum TSH levels in the meta-analysis of 4 populations

Gene	Polymorphism	Minor allele	Beta (SE)*	*P*
FOXE1	rs1443434	C	0.07 (0.02)	0.0028
PDE8B	rs1382879	G	0.19 (0.02)	5.10^{-17} **
	rs2046045	C	0.20 (0.02)	4.10^{-17} **
	rs9687206	G	0.18 (0.02)	3.10^{-15} **
	rs12515498	G	0.14 (0.03)	9.10^{-8} **
	rs832790	A	0.17 (0.03)	9.10^{-10} **
	rs1351283	G	0.18 (0.02)	2.10^{-14} **
	rs989758	A	0.18 (0.03)	2.10^{-10} **
	rs7714529	A	-0.11 (0.03)	8.10^{-6} **
THRB	rs13097208	A	-0.08 (0.03)	0.0045
	rs13066296	A	-0.11 (0.04)	0.0056
	rs6792725	A	0.06 (0.02)	0.0087

* Effects were calculated using linear regression and expressed in SD of natural logarithm transformed TSH level, corrected for age and gender.
** Reached the significant multiple-testing corrected p-value (i.e., $P < 0.0022$).
Note that the associations of rs832790, rs989758, rs13097208 and rs13066296 are based on data from the Rotterdam Study and the Nijmegen Biomedical Study, as in the Danish twin population and the Scan Study the genotyping call rate was lower than 90%.

($P = 0.0110$), but failed to reach statistical significance after multiple-testing correction (Table 3).

In a separate meta-analysis of only the three replication cohorts (i.e., Danish twin population, Scan Study and Nijmegen Biomedical Study), all polymorphisms in *PDE8B* and *DIO1*, but not *FOXE1*, also showed significant associations with TSH (at $P < 0.0022$) and FT4 (at $P < 0.0033$) (data not shown).

Table 3. Effects of *DIO1*, *FOXE1* and *SLC16A10* polymorphisms on serum FT4 levels in the meta-analysis of 4 populations

Gene	Polymorphism	Minor allele	Beta (SE)*	*P*
DIO1	rs2235544	C	-0.16 (0.02)	8.10^{-12} **
	rs11206244	A	0.16 (0.03)	5.10^{-10} **
FOXE1	rs1443434	C	-0.08 (0.02)	0.0003 **
SLC16A10	rs17606253	G	0.08 (0.03)	0.0110

* Effects were calculated using linear regression and expressed in SD, corrected for age and gender.
** Reached the significant multiple-testing corrected p-value (i.e., $P < 0.0033$).

DISCUSSION

In the present study, we studied the effects of genetic variation in 68 TH pathway genes on serum TSH and FT4 levels in 3777 subjects from 4 independent populations. Previously reported associations with genetic variation in *PDE8B*, *THRB* and *DIO1* were replicated. We demonstrate an effect of genetic variation in *FOXE1* on serum FT4 levels, and a borderline significant effect on serum TSH levels. In addition, a suggestive association of genetic variation in *SLC16A10* with serum FT4 levels was found.

Various genes have been studied in relation to serum TSH and TH levels in recent years, demonstrating that variants in the *PDE8B* (19, 29) and *DIO1* (8, 10-12) genes alter TSH and FT4 levels respectively, and suggesting a similar role for other genes such as *THRB* (19, 30) and *TSHR* (10, 13, 14).

FOXE1, also known as *TTF2* (Thyroid Transcription Factor 2), is a transcription factor in thyroid morphogenesis. Its importance is illustrated in mice with a homozygous inactivation of *FOXE1*, which exhibit a cleft palate and neonatal hypothyroidism due to an ectopic or absent thyroid gland (31). In humans, heterozygous missense mutations lead to neonatal hypothyroidism due to thyroid dysgenesis, cleft palate, choanal atresia and spiky hair, which is referred to as the Bamforth-Lazarus syndrome (32). In our study, genetic variation in *FOXE1* was associated with FT4 levels, and a borderline significant association with TSH levels was found. E.g., in the Rotterdam Study the per-allele effect was -0.30 pmol/L for FT4 and 0.11 mU/L for TSH. The lower FT4 and higher TSH levels in *FOXE1*-rs1443434 risk allele carriers suggest a moderately impaired thyroid development, since more TSH seems to be required to stimulate the thyroid to produce TH. It would therefore be interesting to study thyroid size and morphology in FOXE1-rs1443434 risk allele carriers using ultrasound.

Various studies have identified the *FOXE1* region as a susceptibility locus for thyroid cancer (33-35). A recent genome wide association study identified a polymorphism (i.e., rs965513) which was associated with both thyroid cancer risk and lower serum TSH and T4 levels (33). As this polymorphism is located in a LD region with *FOXE1* as the nearest gene (57 kb distance), the authors concluded that the effects of rs965513 might be mediated through processes involving *FOXE1*. Our data identify a genetic variant (i.e., rs1443434) in the *FOXE1* gene itself that is associated with both serum FT4 and TSH levels, which is in moderate LD with rs965513 ($D' = 0.74$, $r^2 = 0.48$). *FOXE1*-rs1443434 is located in a region of high LD (http://www.hapmap.org), including *FOXE1*-rs1867277, a polymorphism located in the 5'UTR-region of the gene which has been shown to influence transcriptional regulation of *FOXE1* (34). Taken together, the responsible/functional variant is likely to be situated in the *FOXE1* locus, but its exact localization remains to be elucidated in future studies, involving large resequencing of this region.

PDE8B is highly expressed in the thyroid and catalyzes the hydrolysis and inactivation of cAMP (36). A genome wide association study by Arnaud-Lopez et al. reported that genetic variation in *PDE8B* was associated with serum TSH levels (19). Panicker et al. reported similar associations in a recent genome wide association study, which did not reach genome wide significance (20). Besides, genetic variation in *PDE8B* has also been associated with subclinical hypothyroidism in pregnancy (29). In this study, we show in multiple populations that genetic variation in *PDE8B* is associated with TSH levels. As the minor alleles (except for rs7714529) were associated with higher TSH levels, we might speculate that these variants increase PDE8B activity, resulting in lower cAMP levels in response to TSH. Consequently, a higher TSH level will be required to maintain normal levels of TH. This hypothesis is supported by our findings, which show an association of genetic variation in *PDE8B* with TSH but not with FT4 levels. Similar to the results of Arnaud-Lopez et al., most associated polymorphisms in our study are located in intron 1 of the *PDE8B* gene, a region of high LD (see http://www.hapmap.org). PDE8B is also expressed in the adrenal gland and an inactivation mutation in *PDE8B* has been identified in a patient with micronodular adrenocortical hyperplasia, leading to Cushing's syndrome (37). No information was provided about the thyroid state of this patient.

The *DIO1* gene encodes the iodothyronine deiodinase type 1 (D1). D1 is present in liver, kidney and thyroid, and plays a key-role in the production of the active hormone T3 from T4 and in the clearance of the metabolite rT3. *DIO1*-rs2235544 and *DIO1*-rs11206244 are in high LD (see http://www.hapmap.org). Associations of these polymorphisms with serum TH levels have been reported previously (8, 10-12, 20), and are replicated in our study.

The *SLC16A10* gene, encoding for monocarboxylate transporter 10 (MCT10), is a transporter which facilitates both uptake and efflux of T3 and T4 (38). MCT10 has a wide tissue distribution including intestine, kidney, liver, skeletal muscle, heart and placenta (39). In the meta-analysis of the 4 populations, a polymorphism in *MCT10* showed an association with altered serum FT4 levels. This association did not reach statistical significance after multiple-testing correction at $P < 0.0033$. Therefore, despite a low p-value (rs17606253: $P = 0.0110$), replication is needed in future studies. rs17606253 is located in intron 3 of the *SLC16A10* gene, a region of high LD (http://www.hapmap.org). Exons 4 to 6 are also included in this region, coding for part of the transmembrane domain and the C-terminal domain. However, future studies need to clarify the exact functional variant in this region.

The structure of the *SLC16A10* gene is highly homologous to that of the *SLC16A2* (*MCT8*) gene (38). *SLC16A2* is located on the X-chromosome, and was therefore not analysed in this study. Amongst other tissues, MCT8 is highly expressed in brain, and mutations in *MCT8* result in high levels of serum T3 and a syndrome of severe psychomotor retardation, known as the Allan-Herndon-Dudley syndrome (40). So far, no patients with mutations in *MCT10* have been reported. Our results suggest that patients with

mutations in *MCT10* might be biochemically characterized by abnormal FT4 levels, in addition to other features.

We additionally found associations of genetic variation in the *THRB* gene (encoding TH receptor ß) with serum TSH levels. TH action is mediated via the TH receptors alpha and beta (TRα and TRβ). Amongst other tissues, TRβ is expressed in liver and kidney and is the predominant receptor in the negative feedback regulation of the hypothalamus-pituitary-thyroid axis. Mutations in TRβ lead to the TH resistance syndrome, which is biochemically characterized by increased levels of TH and a non-suppressed TSH. Common genetic variation in *THRB* has previously been reported to be associated with serum TSH levels (19, 30). However, results were inconsistent (30) or based on a single population (19). In this study, 3 polymorphisms in low LD and located in intron 1 (rs6792725), intron 5 (rs13097208) and intron 8 (rs13066296) of the *THRB* gene showed associations with altered serum TSH levels. As these associations did not reach statistical significance after multiple-testing correction at $P < 0.0022$ (rs6792725: $P = 0.0087$, rs13097208: $P = 0.0045$, rs13066296: $P = 0.0056$), replication is needed in future studies.

Besides these newly identified and replicated gene variants that are associated with inter-individual variation in serum TH levels, it is interesting to note the absence of associations for all other selected TH pathway gene variants. This could be due to an absence of functional variants in these genes. Alternatively, it could reflect the flexibility of the entire system to correct for functional changes in one of its components. Based on our results, we conclude that high frequency polymorphisms in the majority of these TH pathway genes do not play an important role in inter-individual variation in serum TH levels. However, we cannot exclude potential effects of rare polymorphisms in the genes selected, which could be detected by large resequencing efforts of these regions.

Strengths of our analyses include the large-scale approach with a high number of TH pathway genes studied in relation to both TSH and FT4 levels, the high coverage of genetic variation in the studied genes, and the use of multiple large population-based cohorts.

Our study also has some potential limitations. Promising polymorphisms were selected based on their effects in the Rotterdam Study. Study characteristics might influence these effects and may therefore interfere with the selection of promising hits. However, the Rotterdam Study is not a selection of the general population but a population-based cohort study. To exclude confounding by thyroid disease, we excluded subjects with known thyroid disease or thyroid medication usage. We therefore do not think that these biases have strongly influenced our results.

Finally, we studied polymorphisms with a minor allele frequency higher than 5%. We therefore cannot exclude potential effects of rare polymorphisms in the studied genes on TH serum levels. However, most polymorphisms in the human genome are located within LD blocks, which will be largely covered by our selection of studied polymor-

phisms. We were therefore powered to detect part of the effects of rare polymorphisms in the studied genes on TH serum levels.

In summary, we performed a large-scale candidate gene study of TH pathway genes for serum TSH and FT4 levels, with replication in 3 independent populations. Previously reported associations with *PDE8B*, *THRB* and *DIO1* were replicated. We report a role for *FOXE1* in inter-individual variation in serum FT4 levels, and found a borderline significant association with serum TSH levels. In addition, a suggestive association of genetic variation in *SLC16A10* with serum FT4 levels was found.

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Chapter 8

A meta-analysis of thyroid-related traits reveals novel loci and gender-specific differences in the regulation of thyroid function.

E. Porcu[*], M. Medici*, G. Pistis*, C.B. Volpato, S.G. Wilson, A.R. Cappola, S.D. Bos, J. Deelen, M. den Heijer, R.M. Freathy, J. Lahti, C. Liu, L.M. Lopez, I.M. Nolte, J.R. O'Connell, T. Tanaka, S. Trompet, A. Arnold, S. Bandinelli, M. Beekman, S. Böhringer, S.J. Brown, B.M. Buckley, C. Camaschella, A.J.M. de Craen, G. Davies, M.C.H. de Visser, I. Ford, T. Forsen, T.M. Frayling, L. Fugazzola, M. Gögele, A.T. Hattersley, A.R. Hermus, A. Hofman, J.J. Houwing-Duistermaat, R.A. Jensen, E. Kajantie, M. Kloppenburg, E.M. Lim, C. Masciullo, S. Mariotti, C. Minelli, B.D. Mitchell, R. Nagaraja, R.T. Netea-Maier, A. Palotie, L. Persani, M.G. Piras, B.M. Psaty, K. Räikkönen, J.B. Richards, F. Rivadeneira, C. Sala, M.M. Sabra, N. Sattar, B.M. Shields, N. Soranzo, J.M. Starr, D.J. Stott, F.C.G.J. Sweep, G. Usala, M.M. van der Klauw, D. van Heemst, A. van Mullem, S.H.Vermeulen, J.P. Walsh, R.G.J. Westendorp, E. Widen, G. Zhai, F. Cucca, I.J. Deary, J.G. Eriksson, L. Ferrucci, C.S. Fox, W.J. Jukema, L.A. Kiemeney, P.P. Pramstaller, D. Schlessinger, A.R. Shuldiner, E.P. Slagboom, A.G. Uitterlinden, B. Vaidya, T.J. Visser, B.H.R. Wolffenbuttel, I. Meulenbelt, J.I. Rotter, T.D. Spector, A.A. Hicks, D. Toniolo, S. Sanna[#], R.P. Peeters[#], S. Naitza[#]

[,#] These authors contributed equally to this work.*

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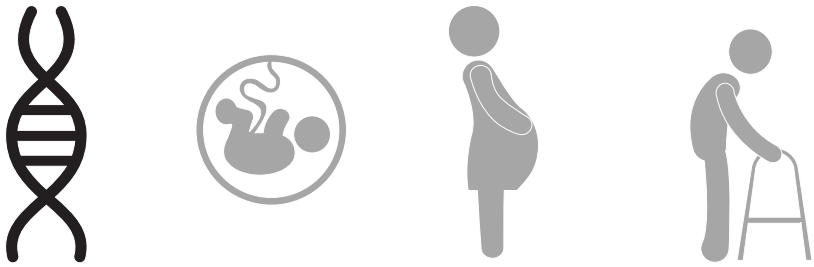

ABSTRACT

Thyroid hormone is essential for normal metabolism and development, and overt abnormalities in thyroid function lead to common endocrine disorders affecting approximately 10% of individuals over the life span. In addition, even mild alterations in thyroid function are associated with weight changes, atrial fibrillation, osteoporosis and psychiatric disorders. To identify novel variants underlying thyroid function we performed a large meta-analysis of genome-wide association studies for serum levels of the highly heritable thyroid function markers TSH and FT4, in up to 26,420 and 17,520 euthyroid subjects, respectively. Here we report 26 independent associations, including several novel loci for TSH (*PDE10A*, *VEGFA*, *IGFBP5*, *NFIA*, *SOX9, PRDM11, FGF7*, *INSR*, *ABO*, *MIR1179*, *NRG1*, *MBIP*, *ITPK1*, *SASH1, GLIS3*) and FT4 (*LHX3*, *FOXE1, AADAT, NETO1/FBXO15, LPCAT2/CAPNS2*). Notably, only limited overlap was detected between TSH and FT4 associated signals, in spite of the feedback regulation of their circulating levels by the hypothalamic-pituitary-thyroid axis. Five of the reported loci (*PDE8B*, *PDE10A, MAF/LOC440389, NETO1/FBXO15* and *LPCAT2/CAPNS2*) show strong gender-specific differences, which offers clues for the known sexual dimorphism in thyroid function and related pathologies. Importantly, the TSH-associated loci contribute not only to variation within the normal range, but also to TSH values outside the reference range, suggesting that they may be involved in thyroid dysfunction. Overall, our findings explain, respectively, 5.64% and 2.30% of total TSH and FT4 trait variance, and improve the current knowledge of the regulation of hypothalamic-pituitary-thyroid axis function and the consequences of genetic variation for hypo- or hyperthyroidism.

INTRODUCTION

Through the production of thyroid hormone (TH), the thyroid is essential for normal development, growth and metabolism of virtually all human tissues. Its critical role in heart, brain, bone, and general metabolism is illustrated by the clinical manifestations of thyroid disease, which affects up to 10% of the population. Low thyroid function (i.e., hypothyroidism) can lead to weight gain, high cholesterol, cognitive dysfunction, depression, and cold intolerance, whereas hyperthyroidism may result in weight loss, tachycardia, atrial fibrillation, and osteoporosis. Mild variation in thyroid function, both subclinical and within the normal range, is associated with these TH-related clinical outcomes as well [1,2,3,4].

The thyroid gland secretes predominantly the pro-hormone thyroxine (T4), which is converted into the active form triiodothyronine (T3) in peripheral tissues. The production of TH by the thyroid gland is regulated by the hypothalamus-pituitary-thyroid (HPT) axis, via a so-called negative feedback loop. Briefly, low levels of serum TH in hypothyroidism result in an increased release of thyroid stimulating hormone (TSH) by the pituitary, under the influence of hypothalamic thyrotropin releasing hormone (TRH) [5]. TSH, a key regulator of thyroid function, stimulates the synthesis and secretion of TH by the thyroid. When circulating TH levels are high, as in hyperthyroidism, TRH and TSH synthesis and secretion are inhibited.

In healthy (euthyroid) individuals, TSH and free T4 (FT4) levels vary over a narrower range than the broad inter-individual variation seen in the general population, suggesting that each person has a unique HPT axis set-point that lies within the population reference range [6]. Besides environmental factors such as diet, smoking and medication, little is known about the factors that influence this inter-individual variation in TSH and FT4 levels [7,8,9]. The heritability of TSH and FT4 has been estimated from twin and family studies at about 65% and 40%, respectively [10,11,12]. However, the underlying genetic variants are not fully established, and the contribution of those discovered so far to the overall variance is modest. Single nucleotide polymorphisms (SNPs) in the *phosphodiesterase type 8B* (*PDE8B*), upstream of the *capping protein (actin filament) muscle Z-line* (*CAPZB*) and, more recently, of the *nuclear receptor subfamily 3, group C, member 2* (*NR3C2*) and of *v-maf musculoaponeurotic fibrosarcoma oncogene homolog* (*MAF/LOC440389*) genes have been implicated in TSH variation by genome-wide association studies (GWAS) [13,14,15], whereas SNPs in the *iodothyronine deiodinase DIO1* have been associated with circulating levels of TH by candidate gene analysis [16,17,18].

To identify additional common variants associated with thyroid function, we performed a meta-analysis of genome-wide association data in 26,420 euthyroid individuals phenotyped for serum TSH and 17,520 for FT4 levels, respectively. In addition, we also assessed gender-specific effects and correlation with subclinical thyroid dysfunction.

METHODS

Ethics statement

All human research was approved by the relevant institutional review boards, and conducted according to the Declaration of Helsinki.

Cohort details

Cohort description, genotyping and statistical methods for individual study cohorts are reported in Text S1 and Table S1 (please see Appendix 1 for supplemental text, tables and figures).

Statistical Analyses

We carried out a meta-analysis including up to 26,523, individuals from 18 cohorts for TSH and up to 17,520 individuals from 15 cohorts for FT4 (see Table 1). FT4 measures were not available for all 21,955 individuals with TSH levels of the 15 participating cohorts. We combined evidence of associations from single GWAS using an inverse variance meta-analysis, where weights are proportional to the squared standard error of the beta estimates, as implemented in METAL [57]. Prior to GWAS, each study excluded individuals with known thyroid pathologies, taking thyroid medication, who underwent thyroid surgery, and with out-of-range TSH values (<0.4 mIU/L and >4 mIU/L), and an inverse normal transformation was applied to each trait (Table S1). Age, age-squared, and gender were fitted as covariates, as well as principal components axes or additional variables, as required (Table S1). Family-based correction was applied if necessary (see Table S1). Uniform quality control filters were applied before meta-analysis, including MAF <0.01, call rate <0.9, HWE $P < 1 \times 10^{-6}$ for genotyped SNPs and low imputation quality (defined as $r^2 < 0.3$ or info < 0.4 if MACH [58] or IMPUTE [59,60] were used, respectively) for imputed SNPs.

Genomic control was applied to individual studies if lambda was > 1.0. The overall meta-analysis showed no significant evidence for inflated statistics (lambda for TSH, FT4 and were 1.05 and1.03 respectively). To evaluate for heterogeneity in effect sizes across populations, we used a chi-square test for heterogeneity, implemented in METAL [57]. The same test was used to evalute heterogeneity related to iodine intake, by comparing effect sizes obtained in a meta-analysis of studies assessing individuals from South Europe (InChianti, MICROS, Val Borbera, SardiNIA, totaling up to 7,488 subjects) with those estimated in a meta-analysis of studies assessing individuals from North America (BLSA, CHS, FHS, OOA, totaling up to 5,407 subjects). Finally, the main meta-analysis was carried out independently by two analysts who obtained identical results.

Conditional analysis

To identify independent signals, each study performed GWA analyses for both TSH and FT4 by adding the lead SNPs found in the primary analysis (19 for TSH, and 4 for FT4, see Table 2) as additional covariates to the basic model, and removing those from the test data set. When lead SNPs were not available, the best proxies (r^2>0.8) were included. We then performed a meta-analysis on the conditional GWAS results, using the same method and filters as described above. We used the standard genome-wide significance cutoff ($P < 5 \times 10^{-8}$) to declare a significant secondary association.

Gender-specific analysis

To identify sex-specific effects, each study performed GWA analyses for each gender separately, using the same covariates and transformation as in the basic model (with the exception of gender covariate). We then performed a meta-analysis on association results using the same method and filters described for the primary analysis. To evaluate sex-specific differences we tested heterogeneity between effect sizes as described above. False-discovery rates (FDRs) on the 26 associated SNPs were calculated with R's p.adjust procedure via the method of Benjamini and Hochberg [24].

Variance explained

The variance explained by the strongest associated SNPs was calculated, for each trait and in each cohort, as the difference of R^2 adjusted observed in the full and the basic models, where the full model contains all the independent SNPs in addition to the covariates. The estimates from each cohort were combined using a weighted average, with weights proportional to the cohort sample size.

Extreme phenotype analysis

To evaluate the impact of the detected variants with clinically relevant TSH levels, we compared the allele frequencies observed in different categories of individuals in a case-control approach. Specifically, we compared individuals in the upper and lower TSH tails (individuals with TSH >4 mIU/L and TSH <0.4 mIU/L, respectively, whom were excluded for the GWAS analyses), as well as individuals in each tail with those in the normal TSH range. In the first case, individuals in the lower tail were considered controls and those in the upper tail cases. In the other two cases, we defined individuals in the normal range as controls and individuals on the two tails cases. To avoid sources of bias, individuals taking thyroid medication and/or with thyroid surgery were excluded. Only unrelated individuals were selected from the family-based cohort SardiNIA, while GEE correction was applied to the TwinsUK dataset. Results from single cohorts were then meta-analyzed. We first assessed the global impact of the 20 TSH- and 6 FT4-associated variants by defining a genotype-risk score (GRS) for each individual as the weighted sum

of TSH- and FT4-elevating alleles, with weights proportional to the effect estimated in the meta-analysis. For each comparison, we then calculated quartiles from the global distribution (cases + controls) of the genotype score and used quartile 1 as the baseline reference to compare the number of cases and controls in the other quartiles. In addition, for TSH-associated variants we conducted single SNP comparisons. GRS quartile and single SNP analyses were performed by each study separately. Cohort specific results were then meta-analyzed for both the GRS score and single SNP results only if they had at least 50 cases and 50 controls. Specifically, cohorts included were: CHS, Lifelines, PROSPER, RS, SardiNIA and TwinsUK.

Bivariate analysis

Bivariate analysis was carried out with the software poly [26] in the SardiNIA cohort using the same individuals included in the GWAS and considering the same covariates and transformation for TSH and FT4 levels.

Table 1. Descriptive statistics of all cohorts.

Cohort	Subjects (N)	Age (mean (SD))	Age (range)	Men (%)	TSH (mean (SD))	FT4 (mean (SD))
BLSA	593	69.9 (15.4)	22-98	54.8	2.1 (0.9)	1.1 (0.2)
CHS	1,655	74.6 (4.9)	67-94	41.8	2.1 (0.9)	1.2 (0.2)
FHS	2,140	47.4 (10.0)	21-77	49.9	1.6 (0.8)	NA
GARP	290	60.3 (7.5)	42-79	20.3	1.9 (0.8)	1.2 (0.2)
HBCS	454	60.9 (2.8)	56-68	51.3	1.8 (0.8)	1.1 (0.1)
InChianti	951	68.4 (15.4)	21-102	45.3	1.5 (0.8)	1.4 (0.3)
LBC1921	401	79.0 (0.6)	77-80	44.0	1.7 (0.8)	1.1 (0.2)
LBC1936	834	69.5 (0.8)	67-71	54.0	1.7 (0.8)	1.2 (0.2)
LifeLines	1,306	45.0 (10.0)	20-79	44.6	2.7 (4.1)	1.3 (0.2)
LLS	736	59.1 (6.8)	30-75	45.9	1.7 (0.8)	1.2 (0.2)
MICROS	1,047	44.6 (16.5)	8-94	45.7	1.9 (0.9)	1.0 (0.2)
NBS	1,617	61.5 (10.3)	27-78	50.7	1.6 (1.1)	1.1 (0.2)
OOA	1,025	49.9 (16.7)	20-97	57.8	2.2 (0.5)	NA
PROSPER	4,402	75.3 (3.4)	69-83	49.1	1.9 (0.8)	1.3 (0.2)
RS	1,346	68.7 (7.4)	55-93	40.7	1.6 (0.8)	1.3 (0.2)
SardiNIA	4,087	42.5 (17.7)	14-101	46.9	1.7 (0.8)	1.3 (0.2)
TwinsUK	2,133	46.6 (12.5)	18-82	0	1.4 (0.7)	1.1 (0.1)
ValBorbera	1,403	53.6 (18.3)	18-102	46.9	1.5 (0.8)	NA

The table shows descriptive statistics of all cohorts included in the meta-analysis. TSH is reported in mIU/L and FT4 in ng/dl. SD, standard deviation. NA, not available

RESULTS

To identify common genetic variants associated with serum TSH and FT4 levels, we carried out a meta-analysis of genome-wide association results from 18 studies for TSH and 15 studies for FT4 levels, which assessed the additive effect of ~ 2.5 million genotyped and HapMap-imputed SNPs in relation to those traits in individuals of European ancestry. For cohort description see Table 1 and Table S1 (supplemental text, tables and figures are included in Appendix 1). In order to avoid bias due to the presence of thyroid pathologies, prior to analysis we excluded all individuals with TSH values outside the normal range (TSH<0.4 mIU/L and TSH>4.0 mIU/L) and those taking thyroid medication for known thyroid pathologies whenever the relevant information was available. Our meta-analysis was thereby carried out in up to 26,420 and 17,520 euthyroid subjects, respectively for TSH and FT4. Additional exclusion criteria used by individual cohorts are detailed in Table S1.

Using the standard genome-wide threshold of 5 X 10^{-8}, we observed significant associations for SNPs at 23 loci, of which 19 were associated with TSH, and 4 with FT4 (Figure S1). The results are presented in Table 2 and Figure 1-5. In Table S2 single cohort results for each GW significant SNP are reported.

For TSH, 4 signals confirmed previously described loci with proxy SNPs at *PDE8B* (*P* = 1.95 X 10^{-56}, r^2 = 0.94 with the reported rs4704397), *CAPZB* (*P* = 3.60 X 10^{-21}, r^2 = 1 with the reported rs10917469) and *NR3C2* (*P* = 9.28 X 10^{-16}, r^2 = 0.90 with the reported rs10028213), whereas the signal was coincident at *MAF/LOC440389* (*P* = 8.45 X 10^{-18}) [13,14,15]. The remaining signals were in or near 15 novel loci: *PDE10A (phosphodiesterase type 10A, P* = 1.21 X 10^{-24}), *VEGFA* (*Vascular endothelial growth factor*, *P* = 6.72 X 10^{-16}), *IGFBP5* (*insulin-like growth factor binding protein 5, P* = 3.24 X 10^{-15}), *SOX9 (sex determining region Y-box 9*, *P* = 7.53 X 10^{-13}), *NFIA* (*nuclear factor I/A*, *P* = 5.40 X 10^{-12}), *FGF7* (*fibroblast growth factor 7*, *P* = 1.02 X 10^{-11}), *PRDM11* (*PR domain containing 11*, *P* = 8.83 X 10^{-11}), *MIR1179* (*microRNA 1179*, *P* = 2.89 X 10^{-10}), *INSR* (*insulin receptor*, *P* = 3.16 X 10^{-10}), *ABO (ABO glycosyltransferase*, *P* = 4.11 X 10^{-10}), *ITPK1* (*inositol-tetrakisphosphate 1-kinase*, *P* = 1.79 X 10^{-9}), *NRG1* (*neuregulin 1*, *P* = 2.94 X 10^{-9}), *MBIP* (*MAP3K12 binding inhibitory protein 1*, *P* = 1.17 X 10^{-8}), *SASH1* (*SAM and SH3 domain containing 1*, *P* = 2.25 X 10^{-8}), *GLIS3* (*GLIS family zinc finger 3*, *P* = 2.55 X 10^{-8}) (Figure 1-4).

For FT4, we confirmed the *DIO1* locus (*P* = 7.87 X 10^{-32}), with the same marker previously reported in candidate gene studies [17,18], and identified 3 additional novel loci, *LHX3* (*LIM homeobox 3*, *P* = 2.30 X 10^{-14}), *FOXE1* (*forkhead box E1*, *P* = 1.50 X 10^{-11}) and *AADAT* (*aminoadipate aminotransferase*, *P* = 5.20 X 10^{-9}) (Figure 5). The most associated SNP at the *FOXE1* locus, rs7045138, is a surrogate for rs1443434(r^2 = 0.97), previously only suggestively associated with FT4 levels [18], and is also correlated with SNPs re-

Table 2. Independent SNPs associated with TSH and FT4 serum levels.

Gene	SNP	Chr	Position	A1/A2	Freq A1	Effect	StdErr	*P*	N	Het *P*
TSH levels										
PDE8B	rs6885099	5	76566105	A/G	0.594	-0.141	0.009	1.95×10^{-56}	26042	0.520
PDE10A	rs753760	6	165966473	C/G	0.691	0.100	0.010	1.21×10^{-24}	25988	0.363
CAPZB	rs10799824	1	19713761	A/G	0.161	-0.113	0.012	3.60×10^{-21}	26031	0.042
MAF/LOC440389	rs3813582	16	78306854	T/C	0.674	0.082	0.010	8.45×10^{-18}	25948	0.292
VEGFA	rs9472138	6	43919740	T/C	0.285	-0.079	0.010	6.72×10^{-16}	25767	0.017
VEGFA	rs11755845	6	44012758	T/C	0.266	-0.065	0.010	1.68×10^{-10}	25710	0.417
NR3C2	rs10032216	4	149888956	T/C	0.781	0.087	0.011	9.28×10^{-16}	26053	0.504
IGFBP5	rs13015993	2	217333768	A/G	0.736	0.078	0.010	3.24×10^{-15}	26016	0.605
SOX9	rs9915657	17	67639131	T/C	0.541	-0.064	0.009	7.53×10^{-13}	25692	0.349
NFIA	rs334699	1	61393084	A/G	0.052	-0.141	0.021	5.40×10^{-12}	25757	4.05×10^{-3}
FGF7	rs10519227	15	47533656	A/T	0.245	-0.072	0.011	1.02×10^{-11}	25988	0.098
PRDM11	rs17723470	11	45184143	T/C	0.279	-0.065	0.010	8.83×10^{-11}	26054	0.833
MIR1179	rs17776563	15	86920108	A/G	0.322	-0.060	0.010	2.89×10^{-10}	25758	0.452
INSR	rs4804416	19	7174848	T/G	0.569	-0.057	0.009	3.16×10^{-10}	25632	0.438
ABO	rs657152	9	135129086	A/C	0.343	0.058	0.009	4.11×10^{-10}	25765	1.22×10^{-4}
ITPK1	rs11624776	14	92665344	A/C	0.660	-0.064	0.011	1.79×10^{-9}	23482	0.845
NRG1	rs7825175	8	32535816	A/G	0.210	-0.066	0.011	2.94×10^{-9}	25996	0.711
MBIP	rs1537424	14	35643769	T/C	0.608	-0.052	0.009	1.17×10^{-8}	25478	0.333
SASH1	rs9497965	6	148562985	T/C	0.415	0.051	0.009	2.25×10^{-8}	25980	0.444
GLIS3	rs1571583	9	4257209	A/G	0.249	0.057	0.010	2.55×10^{-8}	25766	0.118
FT4 levels										
DIO1	rs2235544	1	54148158	A/C	0.510	0.138	0.012	7.87×10^{-32}	17226	0.193
LHX3	rs7860634	9	138229500	A/G	0.530	0.102	0.013	2.30×10^{-14}	14529	0.067
FOXE1	rs7045138	9	99631284	T/C	0.553	0.098	0.015	1.50×10^{-11}	10997	0.457
AADAT	rs11726248	4	171290094	A/G	0.106	0.111	0.019	5.20×10^{-9}	17515	0.972
LPCAT2/CAPNS2	rs6499766	16	54161629	A/T	0.478	0.056	0.012	1.18×10^{-6}	17489	0.269
NETO1/FBXO15	rs7240777	18	69318732	A/G	0.5632	-0.049	0.012	3.13×10^{-5}	17146	7.84×10^{-3}

The table shows the association results for SNPs that reached genome-wide level ($p < 5 \times 10^{-08}$) in the main meta-analysis. SNPs at *LPCAT2/CAPNS2* and *NETO1/FBXO15* reached the GW threshold in the gender-specific meta-analysis (see Table 3), and here the p-value in the main meta-analysis is reported. For each SNP, the best candidate gene is showed, as well as its genomic position in build 36, the effect allele (A1) and the other allele (A2), its combined frequency across studies and its standard error, the effect size and its standard error, the p-value for association, the number of samples analyzed, and the p-values for heterogeneity of effects across the cohorts meta-analyzed. Effect sizes are standardized, so they represent the estimated phenotypic change, per each copy of the effect allele, in standard deviation units.

cently reported to be associated with both low serum TSH and FT4 levels ($r^2 = 0.59$ with rs965513) [19], as well as with hypothyroidism ($r^2 = 0.59$ with rs7850258) [20].

At each locus, a single variant was sufficient to explain entirely the observed association, except for the *VEGFA* locus, which contained an independent signal located 150 kb downstream of the gene, detected by conditional analyses (Figure 1F and Table 2).

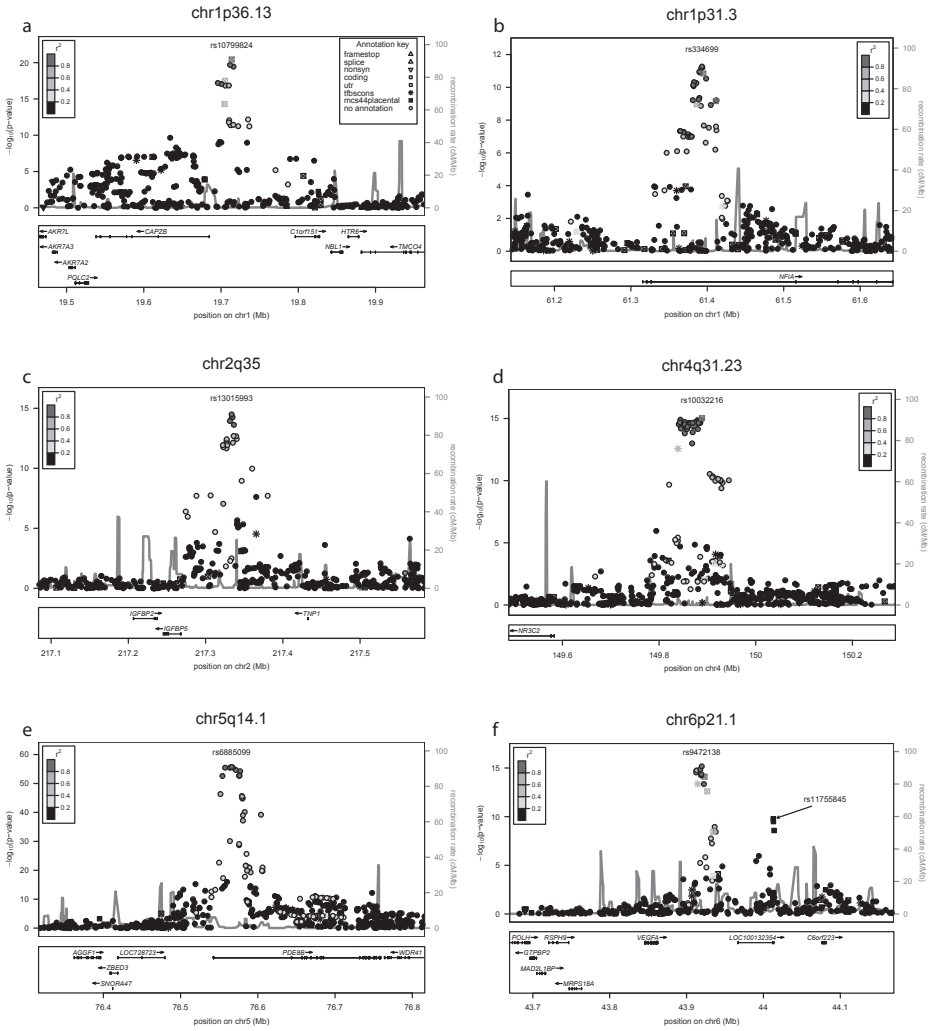

Figure 1. Regional association plots showing genome-wide significant loci for serum TSH. In each panel (A-F), the most significant SNP is indicated (purple circle). In panel F, an independent signal at the associated locus is indicated with an arrow. The SNPs surrounding the most significant SNP are color-coded to reflect their LD with this SNP as in the inset (taken from pairwise r^2 values from the HapMap CEU database build 36/hg18). Symbols reflect genomic functional annotation, as indicated in the legend [61]. Genes and the position of exons, as well as the direction of transcription, are noted in lower boxes. In each panel the scale bar on the Y-axis changes according to the strength of the association. A color figure is available at: http://www.plosgenetics.org/article/info%3Adoi%2F10.1371%2Fjournal.pgen.1003266

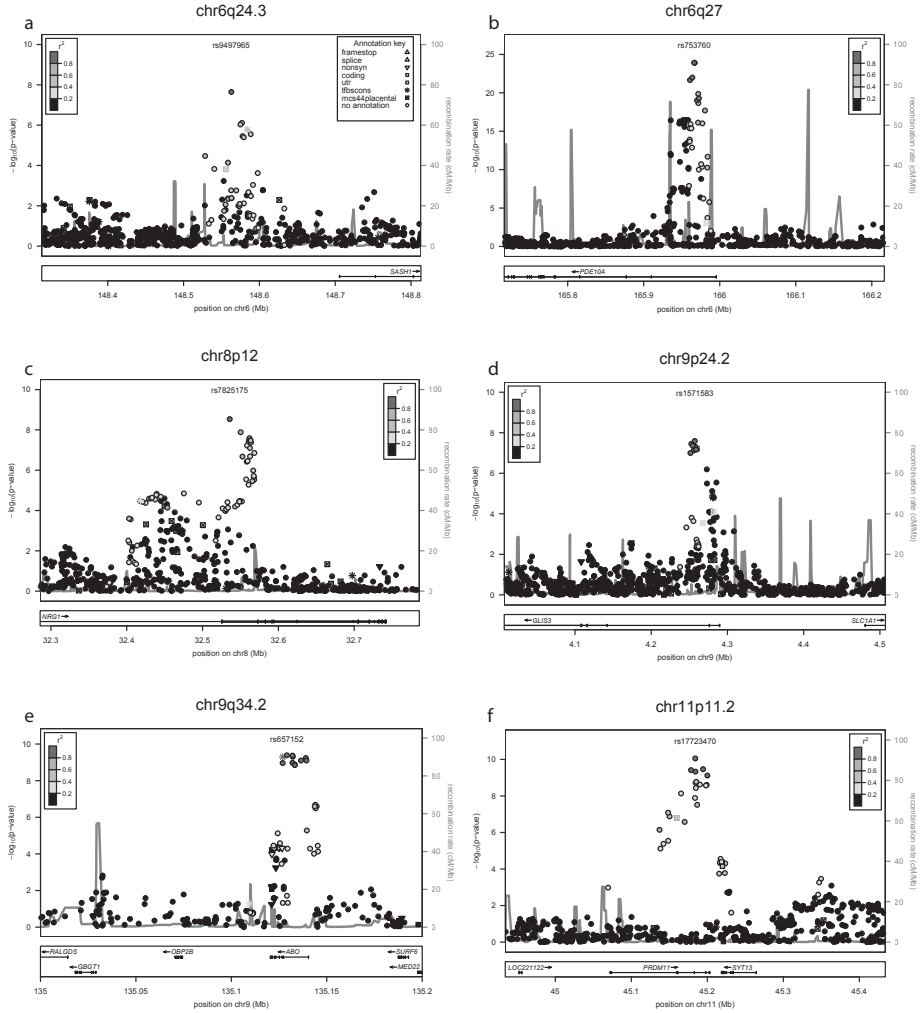

Figure 2. Regional association plots showing genome-wide significant loci for serum TSH. In each panel (A-F), the most significant SNP is indicated (purple circle). The SNPs surrounding the most significant SNP are color-coded to reflect their LD with this SNP as in the inset (taken from pairwise r^2 values from the HapMap CEU database build 36/hg18). Symbols reflect genomic functional annotation, as indicated in the legend [61]. Genes and the position of exons, as well as the direction of transcription, are noted in lower boxes. In each panel the scale bar on the Y-axis changes according to the strength of the association. A color figure is available at: http://www.plosgenetics.org/article/info%3Adoi%2F10.1371%2Fjournal.pgen.1003266

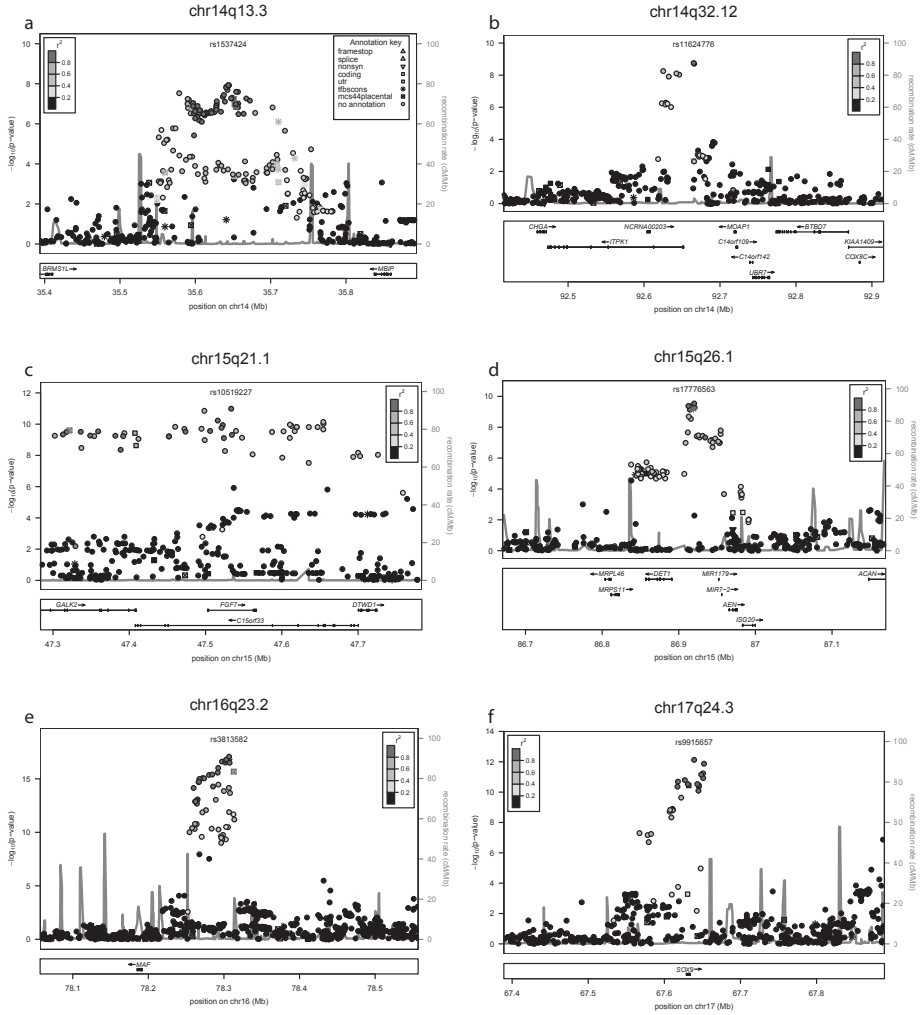

Figure 3. Regional association plots showing genome-wide significant loci for serum TSH. In each panel (A-F), the most significant SNP is indicated (purple circle). The SNPs surrounding the most significant SNP are color-coded to reflect their LD with this SNP as in the inset (taken from pairwise r^2 values from the HapMap CEU database build 36/hg18). Symbols reflect genomic functional annotation, as indicated in the legend [61]. Genes and the position of exons, as well as the direction of transcription, are noted in lower boxes. In each panel the scale bar on the Y-axis changes according to the strength of the association.
A color figure is available at: http://www.plosgenetics.org/article/info%3Adoi%2F10.1371%2Fjournal.pgen.1003266

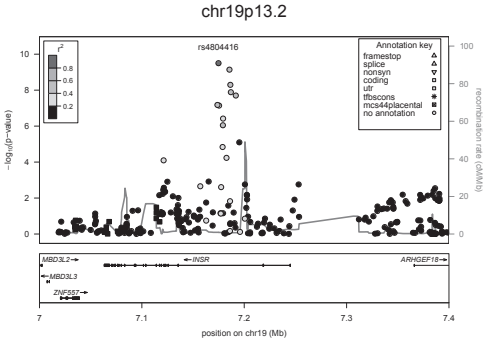

Figure 4. Regional association plot showing the genome-wide significant *INSR* locus for serum TSH. In panel A, the most significant SNP is indicated (purple circle). The SNPs surrounding the most significant SNP are color-coded to reflect their LD with this SNP as in the inset (taken from pairwise r^2 values from the HapMap CEU database build 36/hg18). Symbols reflect genomic functional annotation, as indicated in the legend [61]. Genes and the position of exons, as well as the direction of transcription, are noted in lower boxes. In each panel the scale bar on the Y-axis changes according to the strength of the association. A color figure is available at: http://www.plosgenetics.org/article/info%3Adoi%2F10.1371%2Fjournal.pgen.1003266

Of all 24 independent markers, significant evidence for heterogeneity ($P<0.002$, corresponding to a Bonferroni threshold of 0.5/24) was only observed at *ABO* ($P = 1.22 \times 10^{-4}$). Iodine nutrition, which may profoundly affect thyroid function, is quite different in some of the cohorts under study (i.e., Europe vs North America). To test whether the observed heterogeneity could be attributable to different iodine intake, we combined cohorts from South Europe (an iodine-deficient region) and compared effect sizes with those observed in a meta-analysis of North American samples (an iodine-replete region). Interestingly, the effect size of the top marker at *ABO* was three times larger in Europeans vs North American, and this difference remained significant after Bonferroni correction ($P = 7.0.9 \times 10^{-4}$) (Table S3). However, the relation of the *ABO* SNP, a tag for the blood group O, to iodine intake remains to be determined.

Gender-specific analyses

Given the reported clinical differences in thyroid function in males and females [21,22,23], we searched for gender-specific loci by whole-genome sex-specific meta-analysis, analyzing males and females separately in each cohort. Some of the loci detected in the main meta-analysis were seen at genome-wide significance level only in females (*NR3C2, VEGFA, NRG1* and *SASH1*) or in males (*MAF/LOC440389, FGF7*, *SOX9*, *IGFBP5*) with either the same top SNP or one surrogate, but effect sizes at their variants were significantly gender-specific only at *PDE8B*, *PDE10A* and *MAF/LOC440389,* considering a false discovery rate of 5% [24]. In addition, effects at *MAF/LOC440389* were significantly different also at the more stringent Bonferroni threshold of 1.9×10^{-3} (= 0.05/26), and close to significance at *PDE8B* and *PDE10A* (Table 3). At these latter loci, the TSH-elevating alleles

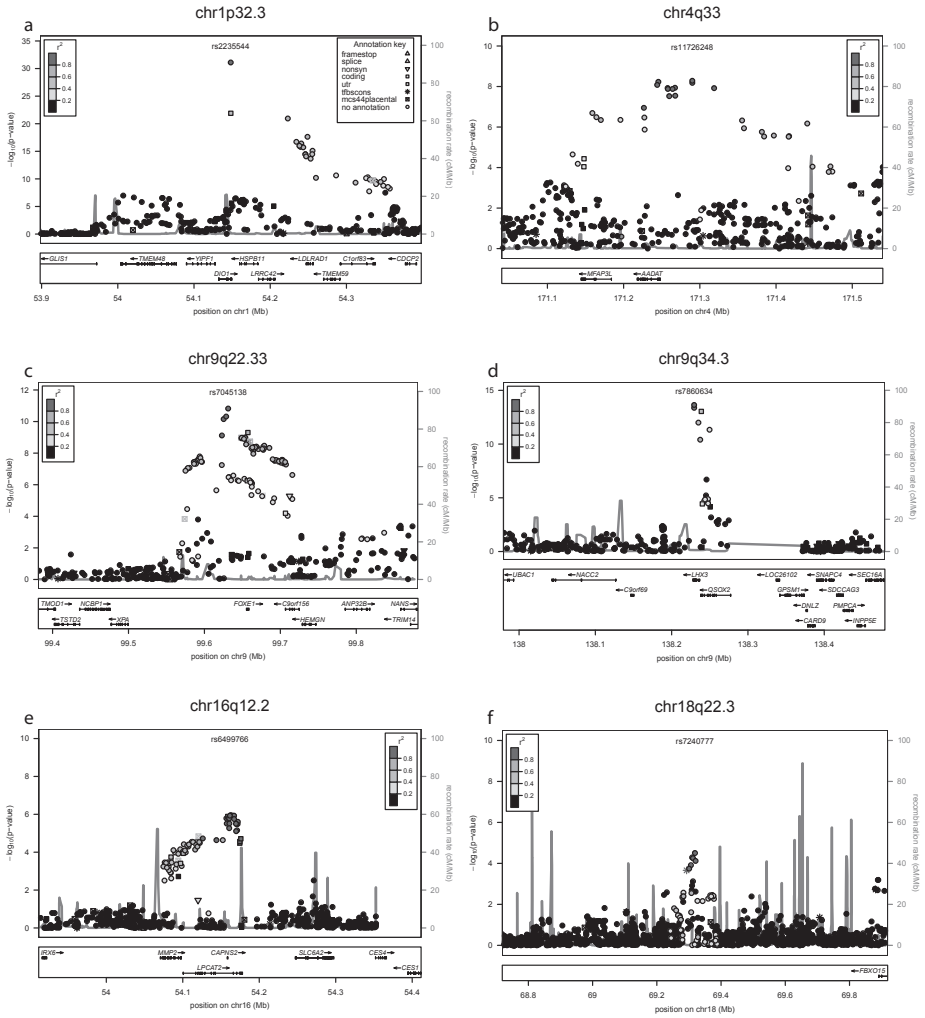

Figure 5. Regional association plots showing genome-wide significant loci for serum FT4. In each panel (A-F), the most significant SNP is indicated (purple circle). The SNPs surrounding the most significant SNP are color-coded to reflect their LD with this SNP as in the inset (taken from pairwise r^2 values from the HapMap CEU database build 36/hg18). Symbols reflect genomic functional annotation, as indicated in the legend [61]. Genes and the position of exons, as well as the direction of transcription, are noted in lower boxes. The scale bar on the Y-axis changes according to the strength of the association. A color figure is available at: http://www.plosgenetics.org/article/info%3Adoi%2F10.1371%2Fjournal.pgen.1003266

showed a stronger impact on trait variability in males compared to females (Figure 6). In addition, the gender specific meta-analysis for FT4, revealed a novel female-specific locus on chromosome 18q22, and a novel male-specific locus on chromosome 16q12.2, that had not been detected in the main meta-analysis (Table 3, Figure 6 and Figure S2). The female-specific signal (rs7240777, P = 3.49 X 10^{-8}) maps in a "gene desert" region,

with the nearest genes *NETO1* (*neuropilin (NRP) and tolloid (TLL)-like 1*), located, about 550 kb upstream and *FBXO15* (*F-box only protein 15*) 500 kb downstream (Figure 5D). The male-specific association is located in intron 11 of the *LPCAT2* (*lysophosphatidylcholine acyltransferase 2*) gene, and near *CAPNS2* (*calpain, small subunit 2*) (rs6499766, $P = 4.63 \times 10^{-8}$), a gene which may play a role in spermatogenesis [25]. The FT4-elevating alleles in the *NETO1/FBXO15* and *LPCAT2/CAPNS2* were fully gender-specific, i.e. there was no effect in males and in females, respectively ($P > 0.01$).

Overall, the 20 TSH and the 6 FT4 associations account, respectively, for 5.64% and 2.30% of total trait variance.

Common loci regulating TSH and FT4 levels

To explore overlap between TSH- and FT4-associated loci and their involvement in the HPT-negative feedback loop, we assessed the associations of the top TSH-associated SNPs on FT4 levels, and *vice versa*. For the SNPs in or near *PDE8B*, *MAF/LOC440389*, *VEGFA*, *IGFBP5*, *NFIA*, *MIR1179*, *MBIP* and *GLIS3* the TSH-elevating allele appeared to be associated with decreasing FT4 levels ($P < 0.05$, Table S4). However, after application of Bonferroni correction (threshold for FT4 association of TSH SNPs, $P = 2.5 \times 10^{-3}$), none of these reciprocal associations remained significant.

By contrast, a positive relationship was seen for one of the FT4 associated loci, since the variant at the *LHX3* locus was significantly associated with higher levels of both FT4 and TSH ($P = 5.25 \times 10^{-3}$, with Bonferroni threshold 0.05/6=0.008).

As the presence of reciprocal associations between TSH and FT4 regulating SNPs would be expected from physiology, we tested the power of our study to detect such a relationship. Power calculation for the top SNP at *PDE8B*, which has the largest effect on TSH levels, revealed that our meta-analysis only has 9% power to detect an association of FT4 at a Bonferroni $P = 2.5 \times 10^{-3}$. We also carried out a bivariate analysis in the SardiNIA study using poly software to estimate specific contributions [26]. This analysis showed that most of the observed negative feedback correlation is due to environmental factors (environmental correlation = -0.130, genetic correlation = -0.065).

Association of loci with hypothyroidism and hyperthyroidism

To assess possible clinical implications, we investigated whether the variants identified in individuals without overt thyroid pathologies (i.e., with TSH levels within the normal range and not taking thyroid medication) were also associated in individuals with abnormal TSH values (i.e., outside the reference range), who were not included in the initial meta-analysis as potentially affected by thyroid pathology. Towards this, we first assessed the global impact of TSH- and FT4-associated SNPs on the risk of increased or decreased TSH levels by comparing weighted genotype risk score (GRS) quartiles in the individuals with abnormal TSH values that were discarded for the GWAS analyses. For

Table 3. Top associated SNPs and their effect on males and females separately.

			Females				Males					
Gene	**SNP**	**A1/A2**	**Effect**	**StdErr**	***P***	**N**	**Effect**	**StdErr**	***P***	**N**	***Het P***	**FDRs**
TSH levels												
PDE8B	**rs6885099**	A/G	-0,120	0.012	6.09×10^{-24}	14509	-0.168	0.013	2.70×10^{-38}	11533	**7.12 X 10-3**	**0.037**
PDE10A	**rs753760**	C/G	0.076	0.013	4.64×10^{-9}	14473	0.131	0.014	5.85×10^{-20}	11515	**5.40 X 10-3**	**0.035**
CAPZB	rs10799824	A/G	-0.123	0.016	2.69×10^{-14}	14504	-0.099	0.017	1.17×10^{-8}	11527	0.309	0.618
MAF/LOC440389	**rs3813582**	T/C	0.055	0.013	1.75×10^{-5}	14449	0.115	0.014	6.17×10^{-17}	11499	**1.50 X 10-3**	**0.019**
VEGFA	rs9472138	T/C	-0.090	0.013	6.30×10^{-12}	14291	-0.065	0.014	4.89×10^{-6}	11476	0.208	0.450
VEGFA	rs11755845	T/C	-0.058	0.014	1.98×10^{-5}	14250	-0.076	0.015	2.98×10^{-7}	11460	0.368	0.683
NR3C2	rs10032216	T/C	0.106	0.014	1.72×10^{-13}	14519	0.069	0.016	1.12×10^{-5}	11534	0.092	0.294
IGFBP5	rs13015993	A/G	0.069	0.013	1.71×10^{-7}	14491	0.095	0.015	7.60×10^{-11}	11525	0.193	0.450
SOX9	rs9915657	T/C	-0.063	0.012	1.46×10^{-7}	14241	-0.068	0.013	2.39×10^{-7}	11451	0.793	0.896
NFIA	rs334699	A/G	-0.143	0.027	1.80×10^{-7}	14253	-0.149	0.030	5.93×10^{-7}	11504	0.874	0.909
FGF7	rs10519227	A/T	-0.051	0.014	3.80×10^{-4}	14462	-0.095	0.015	6.09×10^{-10}	11526	0.036	0.156
PRDM11	rs17723470	T/C	-0.069	0.013	2.92×10^{-7}	14519	-0.056	0.015	1.45×10^{-4}	11535	0.519	0.710
MIR1179	rs17776563	A/G	-0.053	0.013	3.70×10^{-5}	14305	-0.069	0.014	6.16×10^{-7}	11453	0.395	0.684
INSR	rs4804416	T/G	-0.058	0.012	1.76×10^{-6}	14205	-0.058	0.013	1.12×10^{-5}	11427	0.969	0.969
ABO	rs657152	A/C	0.054	0.013	1.31×10^{-5}	14290	0.067	0.014	1.01×10^{-6}	11475	0.498	0.710
ITPK1	rs11624776	A/C	-0.053	0.015	3.29×10^{-4}	12255	-0.069	0.015	2.61×10^{-6}	11227	0.453	0.693
NRG1	rs7825175	A/G	-0.084	0.015	1.64×10^{-8}	14475	-0.049	0.016	2.36×10^{-3}	11521	0.113	0.294
MBIP	rs1537424	T/C	-0.054	0.012	1.26×10^{-5}	14091	-0.050	0.013	1.59×10^{-4}	11387	0.848	0.909
SASH1	rs9497965	T/C	0.067	0.012	3.36×10^{-8}	14462	0,031	0.013	0.023	11518	0.046	0.171
GLIS3	rs1571583	A/G	0.041	0.014	2.86×10^{-3}	14290	0.074	0.015	9.82×10^{-7}	11476	0.104	0.294
FT4 levels												
DIO1	rs2235544	A/C	0.130	0.015	2.62×10^{-18}	10019	0.143	0.018	4.59×10^{-15}	7201	0.605	0.786
LHX3	rs7860634	A/G	0.098	0.018	5.01×10^{-8}	7665	0.108	0.019	1.72×10^{-8}	6864	0.715	0.845
FOXE1	rs7045138	T/C	0.093	0.020	3.10×10^{-6}	5801	0.105	0.021	4.96×10^{-7}	5196	0.679	0.840
AADAT	rs11726248	A/G	0.123	0.024	4.03×10^{-7}	10252	0.093	0.029	1.47×10^{-3}	7263	0.440	0.693
LPCT2/CAPNS2	**rs6499766**	A/T	0.030	0.015	0.040	10231	0.099	0.018	4.63×10^{-8}	7258	**3.42 X 10-3**	**0.029**
NETO1/FBXO15	**rs7240777**	A/G	-0.083	0.015	3.49×10^{-8}	9963	-0.001	0.018	0.950	7183	**5.64 X 10-4**	**0.014**

The table shows the association results in males and females separately for all independent SNPs associated with TSH and FT4 in the main meta-analysis (Table 2), as well as for the marker found to be associated only in females in the gender-specific meta-analysis. The last two columns report the p-value (*Het P*) and the false discovery rates (FDRs) for differences of effect sizes. SNPs with significantly different effect sizes at 5% FDRs and/or Bonferroni threshold ($p=1.9 \times 10^{-3}$) are highlighted in bold. SdtErr, standard error; A1, effect allele; A2 other allele.

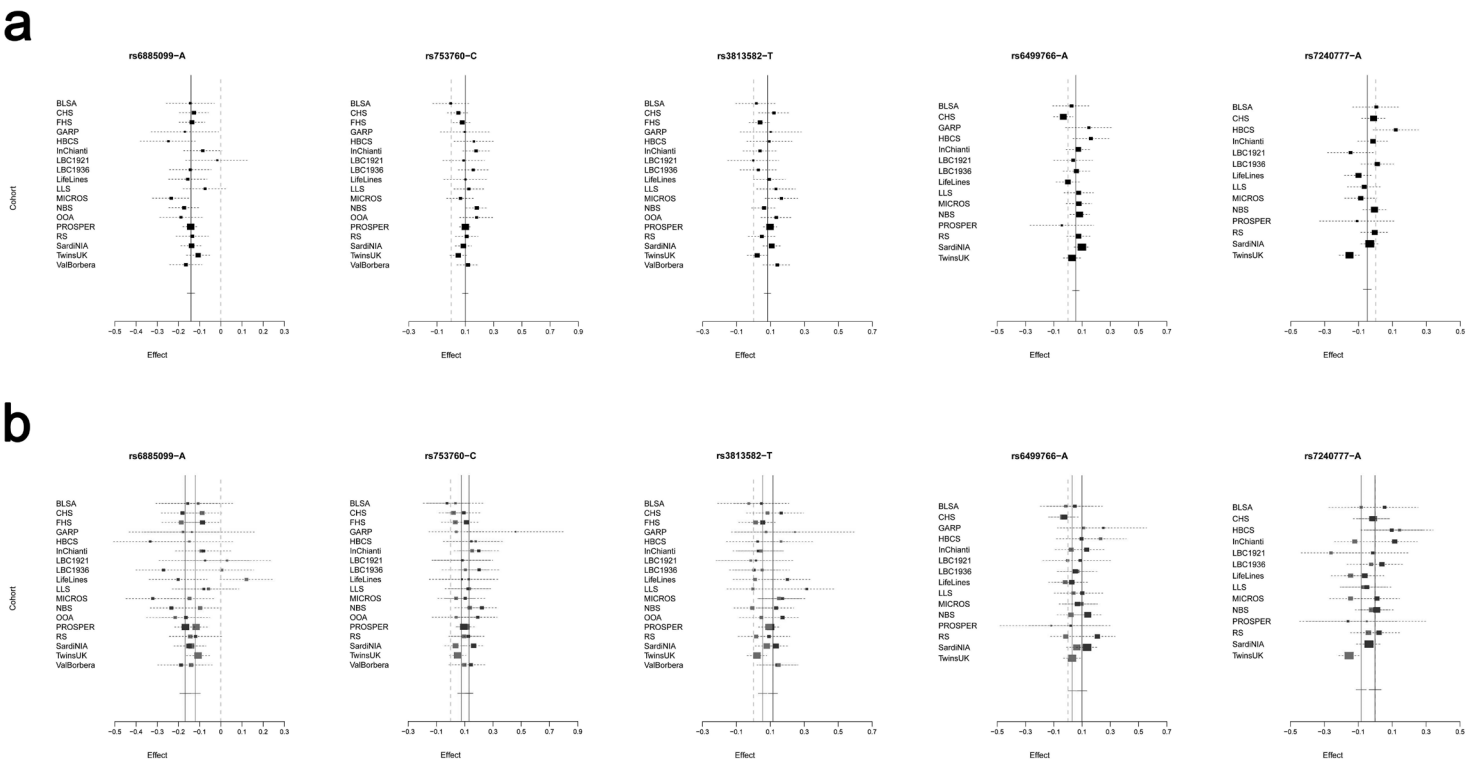

Figure 6. Forest plot of SNPs with gender-specific effects. Squares represent the estimated per-allele beta-estimate for individual studies (a) and in males and females separately (b). The area of the square is inversely proportional to the variance of the estimate. Diamonds represent the summary beta estimates for the subgroups indicated. Horizontal lines represent 95% confidence intervals. In b, red and blue dotted lines represent, respectively, females and males. A color figure is available at: http://www.plosgenetics.org/article/info%3Adoi%2F10.1371%2Fjournal.pgen.1003266

the TSH-associated SNPs, the odds of increased TSH levels were 6.65 times greater in individuals with a GRS in the top quartile compared to individuals in the bottom quartile ($P = 3.43 \times 10^{-20}$) (Table 4, top panel, lower vs upper tail). When we compared subjects with high TSH values with subjects within the normal TSH reference range, subjects with a GRS in the top quartile had odds of an elevated TSH 2.37 times greater than for subjects in the bottom quartile ($P = 1.06 \times 10^{-17}$) (Table 4). With regard to low TSH values versus the normal range, the odds ratio was 0.26 ($P = 5.43 \times 10^{-13}$) (Table 4, top panel, lower vs normal tail). By contrast, with the FT4-associated SNPs we found no significant associations for any of the tested comparisons (data not shown).

We also assessed the 20 independent TSH SNPs individually in relation to the risk of abnormal TSH levels by case-control meta-analysis in subjects with high (cases) versus low (controls) TSH values. This analysis showed that variants at *PDE8B*, *CAPZB*, *FGF7*, *PDE10A, NFIA* and *ITPK1* loci are significantly associated (Bonferroni threshold $P = 2.5 \times 10^{-3}$) with abnormal TSH levels (Table 4, bottom panel). *PDE8B*, *CAPZB* and *FGF7* were also strongly associated with the risk of decreased TSH levels in an analysis of individuals with low (cases) versus normal range TSH (controls). In addition, variants at *VEGFA* were also significantly associated in this comparison. Finally, when individuals with high TSH values were analyzed versus controls, the *NR3C2* locus appeared significantly associated in addition to *PDE8B* and *CAPZB*.

Association of TSH lead SNPs in pregnant women

Normal thyroid function is particularly important during pregnancy and elevated TSH levels are implicated in a number of adverse outcomes for both mother and offspring. We therefore assessed whether the TSH lead SNPs were also associated with elevated TSH during pregnancy, when increased TH production is necessary. We tested 9 of the 20 lead TSH variants (or their proxies, see Text S1) in a cohort of 974 healthy pregnant women at 28 weeks gestation [27] and found, as expected, that mean TSH levels were correlated with the number of TSH-elevating alleles ($P = 3.0 \times 10^{-12}$, Table S5). Effect size estimates in pregnant women were not significantly different when compared to those of women in the main gender-specific meta-analysis (heterogeneity P value > 0.05), suggesting that the effects of the TSH-elevating alleles are no greater during pregnancy (data not shown). However, there was evidence of association between the number of TSH-raising alleles and subclinical hypothyroidism in pregnancy, both in the whole sample (OR per weighted allele: 1.18 [95%CI: 1.01, 1.37], $P = 0.04$) and in TPO antibody-negative women (1.29 [95%CI: 1.08, 1.55], $P = 0.006$) (Table S6).

Table 4. TSH associated SNPs in extreme phenotype categories.

Quartile – based analysis			UPPER vs LOWER				UPPER vs NORMAL				LOWER vs NORMAL			
			OR	StdErr	*P* value	N (cases/controls)	OR	StdErr	*P* value	N (cases/controls)	OR	StdErr	*P* value	N (cases/controls)
Quartile1			—	—	—	141/169	—	—	—	215/2699	—	—	—	143/1842
Quartile2			2.16	0.17	7.09×10^{-6}	194/108	1.31	0.10	0.010	280/2635	0.52	0.15	9.74×10^{-6}	77/1913
Quartile3			3.20	0.18	1.51×10^{-10}	219/86	1.43	0.11	7.00×10^{-4}	311/2595	0.48	0.15	8.31×10^{-7}	71/1913
Quartile4			6.65	0.21	3.43×10^{-20}	256/49	2.37	0.10	1.06×10^{-17}	447/2467	0.26	0.19	5.43×10^{-13}	38/1947
Single marker analysis														
Gene	SNP	A1/ A2	OR	StdErr	*P* value	N (cases/controls)	OR	StdErr	*P* value	N (cases/controls)	OR	StdErr	*P* value	N (cases/controls)
PDE8B	**rs6885099**	G/A	1.84	0.09	**7.33 X 10-11**	920/440	1.20	0.04	**3.61 X 10-5**	1363/11207	0.62	0.08	**2.85 X 10-8**	362/8426
PDE10A	**rs753760**	C/G	1.35	0.09	**1.74 X 10-3**	913/440	1.13	0.05	0.012	1356/11201	0.84	0.08	0.027	362/8420
CAPZB	**rs10799824**	G/A	1.78	0.11	**1.34 X 10-7**	915/440	1.27	0.06	**2.40 X 10-4**	1358/11185	0.68	0.09	**3.23 X 10-5**	362/8404
MAF/LOC440389	rs3813582	T/C	1.16	0.09	0.101	918/441	1.08	0.05	0.131	1361/11228	0.93	0.08	0.348	363/8447
VEGFA	**rs9472138**	C/T	1.29	0.09	5.21×10^{-3}	919/441	1.11	0.05	0.042	1362/11228	0.78	0.08	**2.02 X 10-3**	363/8447
VEGFA	rs11755845	C/T	1.15	0.10	0.147	919/441	1.10	0.05	0.064	1362/11223	0.90	0.09	0.236	363/8442
NR3C2	**rs10032216**	T/C	1.27	0.10	0.022	920/441	1.26	0.06	**5.12 X 10-5**	1363/11229	0.91	0.09	0.290	363/8448
IGFBP5	rs13015993	A/G	1.19	0.10	0.086	920/440	1.12	0.05	0.020	1363/11224	0.92	0.09	0.317	362/8443
SOX9	rs9915657	C/T	1.15	0.09	0.105	911/440	1.04	0.04	0.326	1354/11211	0.91	0.08	0.193	362/8430
NFIA	**rs334699**	G/A	1.85	0.20	**2.10 X 10-3**	912/437	1.38	0.11	4.56×10^{-3}	1355/11181	0.83	0.16	0.265	359/8400
FGF7	**rs10519227**	T/A	1.51	0.10	**3.09 X 10-5**	902/429	1.15	0.06	0.015	1345/11029	0.70	0.09	**4.84 X 10-5**	351/8248
PRDM11	rs17723470	C/T	1.21	0.10	0.056	920/441	0.97	0.05	0.558	1363/11228	0.78	0.09	3.33×10^{-3}	363/8447
MIR1179	rs17776563	G/A	1.24	0.09	0.021	899/431	1.05	0.05	0.258	1342/11016	0.91	0.08	0.255	353/8235
INSR	rs4804416	G/T	1.14	0.09	0.123	920/441	1.10	0.04	0.034	1363/11229	0.97	0.08	0.677	363/8448
ABO	rs657152	A/C	1.12	0.10	0.247	919/441	1.04	0.04	0.358	1362/11219	0.90	0.09	0.228	363/8438
ITPK1	**rs11624776**	C/A	1.36	0.10	**2.44 X 10-3**	907/439	1.13	0.05	0.015	1350/11150	0.82	0.09	0.028	361/8369
NRG1	rs7825175	G/A	1.24	0.11	0.051	904/440	1.20	0.06	3.85×10^{-3}	1347/11151	0.89	0.10	0.223	362/8370
MBIP	rs1537424	C/T	1.26	0.09	0.012	920/441	1.12	0.04	0.012	1363/11229	0.87	0.08	0.082	363/8448
SASH1	rs9497965	T/C	1.08	0.09	0.401	907/441	1.02	0.04	0.623	1350/8129	0.98	0.08	0.823	363/5348
GLIS3	rs1571583	A/G	0.97	0.10	0.769	916/438	1.06	0.05	0.273	1359/11206	1.01	0.09	0.891	360/8425

The table shows results for the quartile-based GRS scores (top panel) and single marker (bottom panel) analyses in extreme phenotype categories, defined as TSH > 4 mIU/L (UPPER) or TSH < 0.4 mIU/L (LOWER). NORMAL, individuals with TSH within the normal range. OR, odds ratio; StdErr, standard error. A1, effect allele; A2 other allele. SNPs reaching the Bonferroni significance threshold are highlighted in bold.

DISCUSSION

We report 26 independent SNPs associated with thyroid function tests in euthyroid subjects, 21 of which represent novel signals (16 for TSH and 5 for FT4). Overall they explain 5.64% and 2.30% of the variation in TSH and FT4 levels, respectively.

We observed that carriers of multiple TSH-elevating alleles have increased risk of abnormal TSH levels, and also found association between the number of TSH-elevating alleles and subclinical hypothyroidism in pregnancy. These results are potentially clinically relevant, because abnormal TSH values are the most sensitive diagnostic markers for both overt and subclinical thyroid disease [4]. The variants identified in the current study, or those in LD with them, may thus contribute to the pathogenesis of thyroid disease. Of note, we found eight loci significantly associated with abnormal TSH levels (*PDE8B*, *PDE10, CAPZB, VEGFA*, *NR3C2, FGF7*, *NFIA* and *ITPK1*), of which two were specifically associated with either abnormally low (*VEGFA*) or elevated (*NR3C2*) TSH values, suggesting differential mechanisms for the contribution of these variants to hyper- and hypothyroidism, respectively. Interestingly, the mineralocorticoid receptor *NR3C2* gene has recently been found to be up-regulated in adult-onset hypothyroidism [28], and *PDE8B* and *CAPZB* have been suggestively associated with hypothyroidism by GWAS [29]. Alternatively, it may be that carriers of these alleles are healthy individuals who may be misdiagnosed as having thyroid disease because their genetically determined TSH concentrations fall outside the population-based reference range. More research is required to determine which of these interpretations is correct, and the relevance of these variants as markers for thyroid dysfunction or thyroid-related clinical endpoints.

The evidence for gender-specific differences at several TSH and FT4 regulatory loci is intriguing. They included variants at *PDE8B*, *PDE10A*, and *MAF/LOC440389*, which showed significantly stronger genetic effects with pituitary-thyroid function in males, and variants at *NETO1/FBX015* and *LPCAT2/CAPNS2* which seems to have an effect only in females and males, respectively. Sex differences in the regulation of thyroid function have generally been linked to the influence of sex hormones and autoimmune thyroid disease, resulting in a higher prevalence of thyroid dysfunction in women, without clear understanding of underlying molecular mechanisms [21,22,23]. Our study suggests that differential genes and mechanisms are potentially implicated in the regulation of thyroid function in men and women. Given the impact of thyroid function on several disease outcomes as well as male and female fertility and reproduction, clarifying the underlying associations may provide additional insight for future interventions.

Although it is well known that TSH and FT4 levels are tightly regulated through a negative feedback loop involving the HPT axis, we detected significant overlap between TSH and FT4 signals only at the *LHX3* locus, which was primarily associated in our study with FT4. The *LHX3* allele is associated with an increase of both TSH and FT4, which is

consistent with the essential role of this transcription factor in pituitary development. Inactivating mutations in *LHX3* cause the combined pituitary hormone deficiency-3 syndrome [CPHD3 (MIM#221750)] [30,31], characterized by low TSH and FT4 levels. The positive association of the *LHX3* variant with both TSH and FT4 suggests an effect of this allele at the level of the HPT-axis, resulting in an increased exposure to thyroid hormone throughout life. In contrast, although several of the TSH-elevating alleles appeared to be associated with decreasing FT4 levels, none of these reciprocal associations remained significant after Bonferroni correction. Lack of loci associated in a reciprocal manner with both TSH and FT4 is somewhat puzzling, as their presence would be expected from physiology. However, these findings are consistent with initial reports by Shields et al. [27] and more recent findings by Gudmundsson et al. [32]. A power analysis showed that our study – in spite of being one of the larger conducted so far on these traits – is underpowered to detect an inverse relationship between TSH and FT4 variants, considering a Spearman rank correlation of -0.130 between these traits [12]. As a consequence, contrasting studies on smaller sample sizes may also lack power and cannot be considered robust when testing this relationship [33]. In addition, we estimated that most of the observed negative feedback correlation is due to environmental factors; so it is unlikely that negative feedback is controlled by a genetic locus with large effect. This observation can rationalize the lack of reciprocal, significant associations detected for both TSH and FT4 in this and other studies, and further supports the crucial role of the HPT-axis in maintaining normal levels of thyroid hormone.

At present the relationship between the associated variants and specific mechanisms involved in regulating TSH and FT4 levels has not been established, but we have identified strong candidates at the majority of the loci by literature-mining approaches, as detailed below and in Table 5.

Most of the 16 novel loci implicated in the regulation of TSH are highly represented in the thyroid with the exception of *PRDM11*, expressed in brain, *ABO*, in blood, and *MIR1179*. *PDE10A* encodes a cAMP-stimulated phosphodiesterase, which was previously only suggestively associated with TSH levels and hypothyroidism [13,34], although the tested variants were weakly correlated with our top signal ($r^2 = 0.55$ with rs2983521 and $r^2 = 0.15$ with rs9347083). The presence of linkage at this gene in families reaching accepted clinical criteria of thyroid dysfunction reinforces the observation that variants in this gene may contribute to clinical thyroid disorders [34]. *PDE10A*, together with *PDE8B* and *CAPZB*, emerged in our study as the strongest currently known genetic determinants of this trait. Both *PDE8B* and *PDE10A* are implicated in cAMP degradation in response to TSH stimulation of thyrocytes. In addition, the activity of both *PDE10A* and *CAPZB* appear modulated by cAMP [35,36]. These three genes most likely act in a pathway that leads to cAMP-dependent thyroid hormone synthesis and release, thus highlighting a critical role of cAMP levels in thyroid function. For the other TSH-associated loci (*VEGFA*,

IGFBP5, *SOX9, NFIA, FGF7, PRDM11, MIR1179, INSR*, *ABO*, *ITPK1*, *NRG1*, *MBIP*, *SASH1* and *GLIS3*), hypotheses can be formulated based on the published literature (see Table 5), but further studies will be necessary to clarify the exact biological mechanisms and the specific genes involved at each locus. The association of TSH levels with *IGFBP5*, *INSR* and *NR3C2* is, however, an indication of a specific role of the growth hormone/insulin-like growth factor (GH/IGF) pathway in thyroid function. Remarkably, expression of *IGFBP5* is tightly regulated by cAMP, again underlying the pivotal role of this second messenger in determining net TSH levels [37].

For FT4, the *DIO1*, *FOXE1* and *LHX3* identified loci have strong biological support as potential effectors. While both *DIO1* and *FOXE1* were previously associated with FT4 levels and hypothyroidism by candidate gene analysis and functional studies [17,18,19,38,39,40,41], association at *LHX3* is novel and is consistent with the essential role of this transcription factor in pituitary development (see above) [30,31,42,43]. Consistent with the role of pituitary in growth, this locus has also recently been associated with height in Japanese [44]. The associations of *AADAT*, *NETO1*/*FXBO15* and *LPCT2/CAPNS2* with FT4 levels are currently less clear. It may be relevant that AADAT catalyzes the synthesis of kynurenic acid (KYNA) from kynurenine (KYN), a pathway that has been associated with the induction in brain of proinflammatory cytokines that are known to activate the hypothalamo-pituitary-adrenal (HPA) axis, in turn affecting the HPT axis and thyroid function, including FT4 levels [45,46,47,48,49].

Additional pathway analyses by MAGENTA[50], GRAIL[51], and IPA (Ingenuity® Systems, www.ingenuity.com) to look for functional enrichment of the genes mapping to the regions associated with TSH, FT4 or both, yielded no novel interactions. However, IPA highlighted an over-representation of genes implicated in developmental processes (11/26, $P = 6.27 \times 10^{-6}$ - 8.85×10^{-3}) and cancer (16/26 loci, $P = 2.44 \times 10^{-6}$ - 9.30×10^{-3}). This is consistent with the notion that a normally developed thyroid gland is essential for both proper function and thyroid hormone synthesis, and that defects in any of the essential steps in thyroid development or thyroid hormone synthesis may result in morphologic abnormalities, impaired hormonogenesis and growth dysregulation. It is also interesting to note that 11 of the 20 TSH signals and 3 of the 6 FT4 signals are connected in a single protein network, underlying the biological interrelationship between genes regulating these traits (Figure S3).

While our manuscript was in preparation, a GWAS of comparable sample size was published on levels of TSH in the general Icelandic population, which confirmed 15 of our reported loci (E. Porcu et al., 2011, ESHG, abstract), and inferred a role for three TSH-lowering variants in thyroid cancer [32]. Four additional TSH loci identified by Gudmundsson and colleagues were also associated in our sample-set of euthyroid individuals with $p < 0.05$ and consistent direction of effects (*VAV3*, *NKX2-3*, *TPO* and *FOXA2*). Finally, 2 loci (*SIVA1*, *ELK3)* could not be tested because the corresponding SNPs or any

Table 5. Candidate genes at newly discovered loci for TSH and FT4 levels.

SNP	Region	Gene	Position	Trait	Function
rs753760	6q26	*PDE10A*	intron 1	TSH	Encodes a dual specificity phosphodiesterase abundant in the thyroid, which can hydrolyze both cAMP and cGMP to the corresponding nucleoside 5′ monophosphate, but has higher affinity for cAMP, and is more efficient with cAMP as substrate. This gene was previously suggestively associated with TSH levels and hypothyroidism and linkage has been observed over this gene in families with individuals reaching the clinical criteria for sub-clinical and clinical thyroid disorders [13,34]. The top SNP is only weakly correlated with previously reported variants (r2=0.449 with rs2983521 and r2=0.184 with rs9347083), but is a perfect proxy of a SNP recently reported in association with TSH [32].
rs9472138; rs11755845	6p12	*VEGFA*	intergenic	TSH/FT4	Encodes a growth factor implicated in angiogenesis, which acts as an important regulator of both benign and malignant processes in the thyroid [62]. Angiogenesis is particularly critical for thyroid function, as the local microvasculature exerts an essential role in the continuous supply of iodine, the key element of thyroid hormone synthesis. In response to a reduction in intracellular iodine concentration, thyrocytes rapidly release angiogenic signals, including an increase in *VEGFA* expression and secretion [63,64]. Notably, thyroid hormone stimulation in rat brain has been shown to induce *VEGFA* upregulation [65], which is consistent with the nominal association of the *VEGFA* locus with FT4 levels observed in this study. The two independent signals, rs9472138 and rs11755845, associated with TSH levels map 40 kb downstream of *VEGFA*, which is the nearest gene in the region. SNPs in this region were recently reported in association with TSH levels (r2=0.874 and r2=0.947) [32].
rs13015993	2q33-36	*IGFBP5*	intergenic	TSH	It belongs to a protein family that interacts with insulin-like growth factors (IGFs) and plays a major role in regulating cell proliferation, differentiation, apoptosis and transformation. *IGFBP5* is significantly over expressed in thyroid papillary carcinoma [54,66]. Studies *in vitro* showed that TSH, through cAMP, inhibits *IGFBP5* transcription, whereas enhanced production of IGFBP5 is correlated with inhibition of thyroid function[67];[68]. In addition, *IFGBP5* has been found up-regulated in response to thyroid hormone in bone, where it interacts with the growth hormone/insulin-like growth factor (GH/IGF) system to contribute to bone formation [69], suggesting that thyroid hormone may potentate the effect of IGF-1 at the receptor level. The top SNP maps about 60 kb upstream of *IGFBP5*. A proxy of this SNP has been recently found associated with TSH and FT4 levels (rs737308, r2=0.927) [32].
rs9915657	17q23	*SOX9*	3′UTR	TSH	Encodes a transcription factor involved in chondrocyte differentiation and male sex determination, although other specific functions are known. The TA domain of SOX9, which is expressed both in the pituitary and in the thyroid, has been reported to interact with a component of the thyroid hormone receptor complex (TRAP230) [70]. How this interaction could affect TSH levels is at present unclear. The top SNP maps 5 kb downstream of *SOX9*, which is the nearest gene in the region.

rs334699	1p31.3-p31.2	*NFIA*	intron 3	TSH	Encodes a member of the NF1 (nuclear factor 1) family of transcription factors. NFI proteins have been implicated in regulating developmental processes by their specific expression pattern during embryonic development and by analysis of NFI-deficient mice [71]. In addition, they play crucial roles in the transcription of many cellular genes. Members of this family, including NFIA, have been shown to interact with thyroid transcription factor 1 (TTF1) [72], a transcription factor essential for thyroid-specific gene expression [73]. The top SNP maps in intron 3 of the gene. A proxy of this SNP has been recently found associated with TSH levels (rs334725, r2=1) [32].
rs10519227	15q21.2	*FGF7*	intron 2	TSH	Encodes a member of the fibroblast growth factor (FGF) family. FGF family members are involved in a variety of biological processes, including embryonic development, cell growth, morphogenesis, tissue repair, tumor growth and invasion. FGF signals play a role in the development of the thyroid gland and mice deficient for corresponding receptors show thyroid agenesis [74]. The top SNP maps in intron 2 of *FGF7*. A proxy of this SNP has been recently associated by GWAS with thyroid volume and goiter (rs4338740, r2=0.874) [36].
rs17723470	11p11	*PRDM11*	intron 2	TSH	Encodes a member of the family of PR-domain genes involved in human cancers [75]. The function of *PRDM11* and its correlation with TSH levels is unclear; however the association with low TSH values in the extreme phenotype analysis supports a role of this gene in this trait. The top SNP maps in intron 2 of the gene and is moderately correlated with a SNP associated with TSH levels (rs7128207, r2=0.55) [32].
rs17776563	15q25.3	*MIR1179*	intergenic	TSH	Encodes a microRNAs (miRNAs), which are short non-coding RNAs involved in post-transcriptional regulation of gene expression in multicellular organisms by affecting both the stability and translation of mRNAs. The associated SNP maps about 30 kb upstream of the gene, which is the nearest gene in the region.
rs4804416	19p13.3-13.2	*INSR*	intron 2	TSH	Encodes the insulin receptor precursor, which is post-translationally cleaved after removal of the precursor signal peptide into two chains (alpha and beta) that are covalently linked. Binding of insulin to the insulin receptor (INSR) stimulates glucose uptake. Two transcript variants encoding different isoforms have been found for this gene. INSR isoforms appear overexpressed in thyroid tumors, where they interact with insulin homolog IGFs (I and II), which act as potent mitogenic and antiapoptotic factors in a variety of human malignancies, and supporting a specific role of the GH/IGF pathway in thyroid function. The top SNP maps in intron 2 of the INSR, and is moderately correlated (rs10420008, r2=0.435) with a variant recently associated with TSH levels [32].
rs657152	9q34.2	*ABO*	intron 1	TSH	Encodes proteins related to the blood group system, ABO, which determines the individual blood group. The associated SNP is in intron 1 of the gene and is a tag of the O blood group allele, caused by a deletion of guanine-258 near the N-terminus of the protein which results in a frameshift and translation of an almost entirely different protein. The correlation of TSH with ABO blood groups is currently unclear. *ABO* has been found associated with several phenotypes, including serum levels of different molecules [76]. It was reported that serum levels of TSH vary in individuals with ABO blood types, and that blood group O may be associated with hyperthyroidism [77].

rs11624776	14q31	*ITPK1*	intergenic	TSH	Encodes the enzyme inositol 1,3,4-trisphosphate 5/6-kinase, which catalyzes the rate-limiting step in the formation of higher phosphorylated forms of inositol in mammalian cells. ITPK1 plays a pivotal role in inositol metabolism and mice producing reduced levels of ITPK1 develop neural tube defects [78]. Its role in the regulation of TSH levels is at present unclear. Of note, inositol phosphates/Ca2+ cascades mediates TSH action on thyroid hormone synthesis [79] The associated SNP is located about 15 kb upstream of the gene, which is the nearest gene in the region.
rs7825175	8p12	*NRG1*	intron 2	TSH	Encodes neoregulin 1, a glycoprotein that interacts with the NEU/ERBB2 receptor tyrosine kinase to increase its phosphorylation on tyrosine residues. *NRG1* is a signaling protein that mediates cell-cell interactions and plays critical roles in the growth and development of multiple organ systems. Its gene dysregulation has been linked to cancer, schizophrenia and bipolar disorder. The associated SNP maps in intron 2 of the gene and is only weakly correlated with a variant recently associated with TSH levels and thyroid cancer risk (rs2439302, r2=0.257) [32].
rs1537424	14q13.3	*MBIP*	intergenic	TSH	Encodes MAP3K12/MUK-binding inhibitory protein, a MAP3K regulator during osmolarity sensing and stress signaling that localizes in either the cytoplasm or nucleus [80]. SNPs in this locus have been recently reported as associated with TSH levels and thyroid cancer risk [32]. The top associated SNP maps at about 190 kb downstream of *MBIP*, which is the nearest gene in the region. Recently, a long, intergenic, noncoding RNA gene (lincRNA) named *Papillary Thyroid Carcinoma Susceptibility Candidate 3* (*PTCSC3*) has been mapped 3.2 kb downstream of rs944289 (r2= 0.708 with our top SNP), whose expression is strictly thyroid specific and acts as a PTC tumor suppressor gene [81].
rs9497965	6q24.3	*SASH1*	intergenic	TSH	Encodes a member of the SLY-family of signal adapter proteins and is a candidate tumor suppressor in breast and colon cancer. However, the biological function of SASH1 and its involvement in malignant transformation remain largely unknown. Of note, SASH1 has been identified as a downstream target of the insulin/IGF1/PI 3-kinase signaling pathway[82], which appears implicated in TSH levels in the current study. The associated SNP is located about 130 kb upstream of *SASH1*, which is the nearest gene in the region.
rs1571583	9p24.2	*GLIS3*	intron 2	TSH	Encodes a nuclear protein with five C2H2-type zinc finger domains, which is a member of the GLI-similar zinc finger protein family. GLIS3 functions as both a repressor and activator of transcription and is specifically involved in the development of pancreatic beta cells, the thyroid, eye, liver and kidney. Mutations in this gene have been associated with neonatal diabetes and congenital hypothyroidism (NDH) [52]. The top SNP at this locus maps in intron 2 of the gene.

rs7860634	9q34.3	*LHX3*	intron 6	FT4/TSH	Encodes a transcription factor with an essential role in pituitary development [30]. Mutations in *LHX3* cause the combined pituitary hormone deficiency-3 (CPHD3) syndrome (OMIM #221750), characterized by a complete deficit in growth hormone, prolactin, gonadotropin, and TSH, a rigid cervical spine leading to limited head rotation, as well as an extended spectrum with variable sensorineural hearing loss and ACTH deficiency [31,42,43], which is consistent with its association also with TSH levels observed in this study. This locus has been also recently associated with height in Japanese[44] The top SNP maps in intron 6 of the gene.
rs7045138	9q22	*FOXE1*	intergenic	FT4	Encodes a transcription factor crucial for thyroid morphogenesis. Mutations in *FOXE1* cause the Bamforth-Lazarus syndrome (MIM #241850), characterized by thyroid dysgenesis with neonatal hypothyroidism, cleft palate, choanal atresia and spiky hair [40,41]. In addition, *FOXE1* is a susceptibility locus for thyroid cancer [19,83,84,85]. The top SNP maps 25 kb upstream of *FOXE1* and is also associated in our study with FT4 levels. SNPs in the *FOXE1* locus were previously associated with FT4 levels in a recent candidate gene analysis (rs1443434, r2=0.776) [18], as well as by GWAS with both low serum TSH and T4 levels (rs965513, r2=1) [19], and hypothyroidism (rs7850258, r2=0.625) [20].
rs716822	4q33	*AADAT*	intron 4	FT4	AADAT catalyzes the synthesis from kynurenine (KYN) of kynurenic acid (KYNA), which is implicated in the pathophysiology of several diseases of the central nervous system involving inflammation-induced brain injury [46,47,48,49]. The KYN pathway has been associated with the induction of proinflammatory cytokines in the brain, which are known to activate the hypothalamo-pituitary-adrenal (HPA)-axis, involved in stress response and affecting the HPT-axis and thyroid function, including FT4 levels [45]. The top SNP maps in intron 4 of the gene.
rs7240777	18q22	*NETO1/ FBXO15*	intergenic	FT4	The top SNP maps in a gene desert region, with *NETO1* located about 550 kb upstream and *FBXO15* about 500 kb downstream. None of these genes has a clear role in thyroid function. *NETO1* is expressed in brain and encodes a predicted transmembrane protein containing two extracellular CUB domains followed by a low-density lipoprotein class A (LDLa) domain. A similar gene in mice plays a critical role in spatial learning and memory by regulating the function of synaptic N-methyl-D-aspartic acid receptor complexes in the hippocampus. *FBOX15* encodes a member of the F-box protein family characterized by an approximately 40-amino acid F-box motif. SCF complexes, formed by SKP1 (MIM#601434), cullin (see CUL1; MIM#603134), and F-box proteins, act as protein-ubiquitin ligases. F-box proteins interact with SKP1 through the F box, and they interact with ubiquitination targets through other protein interaction domains.
rs6499766	16q12.2	*LPCAT2/ CAPNS2*	intron 11	FT4	*LPCAT2* encodes a member of the lysophospholipid acyltransferase family. The enzyme may function to catalyze both the biosynthesis of platelet-activating factor and of glycerophospholipid precursors from arachidonyl-CoA and lysophosphatidylcholine. The encoded protein may function in membrane biogenesis and production of platelet-activating factor in inflammatory cells. The associated SNP maps in intron 11 of the gene, and is also near *CAPNS2*, which is contained in the *LPCAT2* gene. None of these genes has a clear role in thyroid function. Of note, calpain subunit genes (*CAPNS1* and *CAPNS2*) play important functions in mammalian reproduction [25].

The table lists genes of interest in the novel associated regions. For each associated region, the reported gene either contains the lead SNP or is in closest physical proximity with the lead SNP.

surrogate (r2 > 0.5) were not available in our data set (Table S7). Our study shows that most of the loci described in Icelanders are reproducible in other populations of European origin; differences in sample size, phenotype definition (i.e., selection of euthyroid subjects vs general population) and in the genetic map used to detect associations most likely explain non-overlapping genome-wide significant signals. Among them, the reported signals at *SOX9*, *ABO*, *SASH1*, *GLIS3* and *MIR1179* will need to be confirmed in other studies; but one of them - *GLIS3*- is a prime candidate, because it is involved in congenital hypothyroidism [52]. Interestingly, despite the use of variants detected through whole-genome sequencing in Icelanders, the top signals at seven overlapping loci (*PDE8B*, *PDE10A*, *CAPZB*, *MAF*/*LOC440389*, *VEGFA*, *NR3C2*, *IGFBP5*) were either coincident or in high LD (r2>0.9) with those detected in our HapMap-based meta-analysis. Thus, such variants are likely to be the causative ones.

In conclusion, our study reports the first GWAS meta-analysis ever carried out on FT4 levels, adds to the existing knowledge novel TSH- and FT4-associated loci and reveals genetic factors that differentially affect thyroid function in males and females. Several detected loci have potential clinical relevance and have been previously implicated both in Mendelian endocrine disorders (*LHX3* [MIMM#221750], *FOXE1*[MIMM#241850], *PDE8B* [MIMM#614190], *NR3C2* [MIMM#177735], *INSR* [MIMM#609968], *GLIS3* [MIMM#610199]) and thyroid cancer (*FOXE1* [19], *VEGFA* [53], *IGFBP5* [54], *INSR* [55], *NGR1* [32], *MBIP* [32], *FGF7* [56]). Furthermore, the TSH-associated variants were found to contribute to TSH levels outside the reference range. Overall, our findings add to the developing landscape of the regulation of hypothalamic-pituitary-thyroid axis function and the consequences of genetic variation for hypo- or hyperthyroidism.

WEB RESOURCES

The URLs for data presented herein are as follows:
METAL, http://www.sph.umich.edu/cgs/abecasis/metal
MACH, http://www.sph.umich.edu/cgs/abecasis/MaCH/
IMPUTE, https://mathgen.stats.ox.ac.uk/impute/impute.html
LocusZoom, http://www.sph.umich.edu/cgs/abecasis/locuszoom
HapMap, http://www.hapmap.org
Online Mendelian Inheritance in Man (OMIM), http://www.omim.org/

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Chapter 9

Identification of Novel Genetic Loci Associated with Thyroid Peroxidase Antibodies and Clinical Thyroid Disease

M. Medici*, E. Porcu*, G. Pistis*, A. Teumer, S.J. Brown, R.A. Jensen, R. Rawal, G.L. Roef, T.S. Plantinga, S.H. Vermeulen, J. Lahti, M.J. Simmonds, L.L.N. Husemoen, R.M. Freathy, B.M. Shields, D. Pietzner, R. Nagy, L. Broer, L. Chaker, T.I.M. Korevaar, M.G. Plia, C. Sala, U. Völker, J.B. Richards, F.C. Sweep, C. Gieger, T. Corre, E. Kajantie, B. Thuesen, Y.E. Taes, W.E. Visser, A.T. Hattersley, J. Kratzsch, A. Hamilton, W. Li, G. Homuth, M. Lobina, S. Mariotti, N. Soranzo, M. Cocca, M. Nauck, C. Spielhagen, A. Ross, A. Arnold, M. van de Bunt, S. Liyanarachchi, M. Heier, H.J. Grabe, C. Masciullo, T.E. Galesloot, E.M. Lim, E. Reischl, P.J. Leedman, S. Lai, A. Delitala, A.P. Bremner, D.I.W. Philips, J.P. Beilby, A. Mulas, M. Vocale, G. Abecasis, T. Forsen, A. James, E. Widen, J. Hui, H. Prokisch, E.E. Rietzschel, A. Palotie, P. Feddema, S.J. Fletcher, K. Schramm, J.I. Rotter, A. Kluttig, D. Radke, M. Traglia, G.L. Surdulescu, H. He, J.A. Franklyn, D. Tiller, B. Vaidya, T. de Meyer, T. Jørgensen, J.G. Eriksson, P.C. O'Leary, E. Wichmann, A.R. Hermus, B.M. Psaty, T. Ittermann, A. Hofman, E. Bosi, D. Schlessinger, H. Wallaschofski, N. Pirastu, Y.S. Aulchenko, A. de la Chapelle, R.T. Netea-Maier, S.C.L. Gough, H. Meyer zu Schwabedissen, T.M. Frayling, J. Kaufman, A. Linneberg, K. Räikkönen, J.W.A. Smit, L.A. Kiemeney, F. Rivadeneira, A.G. Uitterlinden, J.P. Walsh, C. Meisinger, M. den Heijer, T.J. Visser, T.D. Spector, S.G. Wilson, H. Völzke, A.R. Cappola, D. Toniolo, S. Sanna[#], S. Naitza[#], R.P. Peeters[#]

[,#] Authors contributed equally to this work*

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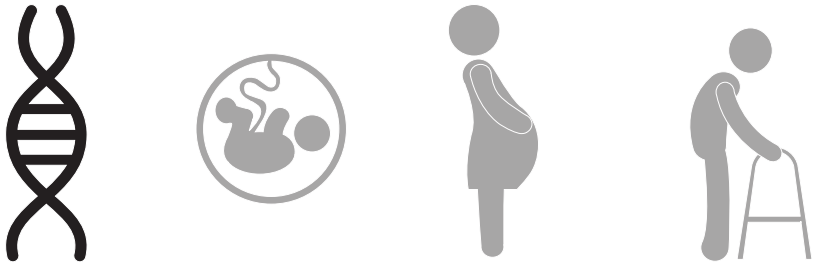

ABSTRACT

Autoimmune thyroid diseases (AITD) are common, affecting 2-5% of the general population. Individuals with positive thyroid peroxidase antibodies (TPOAbs) have an increased risk of autoimmune hypothyroidism (Hashimoto's thyroiditis), as well as autoimmune hyperthyroidism (Graves' disease). As the possible causative genes of TPOAbs and AITD remain largely unknown, we performed GWAS meta-analyses in 18,297 individuals for TPOAb-positivity (1769 TPOAb-positives and 16,528 TPOAb-negatives) and in 12,353 individuals for TPOAb serum levels, with replication in 8,990 individuals.

Significant associations ($P<5x10^{-8}$) were detected at *TPO*-rs11675434, *ATXN2*-rs653178, and *BACH2*-rs10944479 for TPOAb-positivity, and at *TPO*-rs11675434, *MAGI3*-rs1230666, and *KALRN*-rs2010099 for TPOAb levels. Individual and combined effects (genetic risk scores) of these variants on (subclinical) hypo- and hyperthyroidism, goiter and thyroid cancer were studied. Individuals with a high genetic risk score had, besides an increased risk of TPOAb-positivity (OR: 2.18, 95% CI 1.68-2.81, $P=8.1x10^{-8}$), a higher risk of increased thyroid-stimulating hormone levels (OR: 1.51, 95% CI 1.26-1.82, $P=2.9x10^{-6}$), as well as a decreased risk of goiter (OR: 0.77, 95% CI 0.66-0.89, $P=6.5x10^{-4}$). The *MAGI3* and *BACH2* variants were associated with an increased risk of hyperthyroidism, which was replicated in an independent cohort of patients with Graves' disease (OR: 1.37, 95% CI 1.22–1.54, $P=1.2x10^{-7}$ and OR: 1.25, 95% CI 1.12-1.39, $P=6.2x10^{-5}$). The *MAGI3* variant was also associated with an increased risk of hypothyroidism (OR: 1.57, 95% CI 1.18-2.10, $P=1.9x10^{-3}$).

This first GWAS meta-analysis for TPOAbs identified five newly associated loci, three of which were also associated with clinical thyroid disease. With these markers we identified a large subgroup in the general population with a substantially increased risk of TPOAbs. The results provide insight into why individuals with thyroid autoimmunity do or do not eventually develop thyroid disease, and these markers may therefore predict which TPOAb-positives are particularly at risk of developing clinical thyroid dysfunction.

INTRODUCTION

Autoimmune thyroid disease (AITD), including Hashimoto's thyroiditis and Graves' disease, is one of the most common autoimmune diseases, affecting 2-5% of the general population [1,2,3]. Thyroid dysfunction has been associated with osteoporosis, depression, atrial fibrillation, heart failure, metabolic syndrome, and mortality [4,5,6,7,8,9,10,11]. High serum antibodies against the enzyme thyroid peroxidase (TPO), which is located in the thyroid and plays a key role in thyroid hormone synthesis, are present in 90% of patients with Hashimoto's thyroiditis [12,13], the most frequent cause of hypothyroidism and goiter. Although TPO antibodies (TPOAbs) are a useful clinical marker for the detection of early AITD, it remains controversial if these antibodies play a causative role in the pathogenesis of Hashimoto's thyroiditis [14,15,16].

Interestingly, TPOAb-positive persons also have an increased risk of developing autoimmune hyperthyroidism (Graves' disease) [17,18], which is caused by stimulating antibodies against the thyroid stimulating hormone (TSH) receptor [19]. Numerous studies have shown that Graves' hyperthyroidism and Hashimoto's thyroiditis show co-inheritance [17,20,21]. Finally, thyroid autoimmunity is the most common autoimmune disorder in women of childbearing age, and TPOAb-positive women have an increased risk of developing pregnancy complications such as miscarriage and pre-term delivery [17,18,22,23,24,25,26].

The prevalence of TPOAb-positivity in the general population ranges from 5-24%, but it is currently unknown why these people develop TPOAbs, nor is it known why not all individuals with thyroid autoimmunity develop clinical thyroid disease [27,28]. It is estimated that around 70% of the susceptibility to develop thyroid autoantibodies is due to genetic factors [29]. In this context it is remarkable to note that little is known about the genetic factors that determine TPOAb-positivity and the risk of AITD.

We therefore performed a genome wide association study (GWAS) meta-analysis for TPOAbs in the general population in 18,297 individuals from 11 populations. Newly identified genetic variants were studied in relation to subclinical and overt hypo- and hyperthyroidism, goiter, thyroid autoimmunity during pregnancy and thyroid cancer risk.

MATERIALS AND METHODS

Study cohorts

For the TPOAb GWAS stage 1 and 2 analyses, and the hypothyroidism, hyperthyroidism and goiter analyses, individuals were recruited from 16 independent community-based and family studies. For the Graves' disease analyses, cases were recruited from the United Kingdom Graves' disease cohort and controls from the British 1958 Birth Cohort. Thyroid

cancer cases and controls were recruited from the Nijmegen and Ohio thyroid cancer cohorts. A detailed description of the original cohorts contributing samples is provided in Table 1 and in the Supplementary text. The supplementary text, tables and figures are available in Appendix 2. All participants provided written informed consent and protocols were approved by the institutional review boards or research ethics committees at the respective institutions, and conducted according to the Declaration of Helsinki.

Phenotype definitions

Serum TPOAb levels were determined with a range of assays. TPOAb-positives were defined as subjects with TPOAb levels above the assay-specific TPOAb-positivity cut-off, as defined by the manufacturer (Table 1). Serum TSH and free thyroxine (FT4) levels were determined using a range of assays (Table 1). Assay-specific TSH and FT4 reference ranges were used, as provided by the manufacturer (Table 1). Overt hypothyroidism was defined as a high TSH (i.e., a TSH level above the TSH reference range) and a low FT4. Increased TSH was defined as a high TSH, including persons with overt hypothyroidism or subclinical hypothyroidism (i.e., high TSH with a normal FT4). Overt hyperthyroidism was defined as a low TSH and a high FT4. Decreased TSH was defined as a low TSH, including persons with subclinical or overt hyperthyroidism.

The diagnosis of goiter is described in the Supplementary Material, and the diagnosis of Graves' disease and thyroid cancer in the respective cohorts have been described previously [41].

Genotyping

Samples were genotyped with a range of GWAS genotyping arrays (Supplementary Table S1). Sample and SNP quality control procedures were undertaken within each study. For each GWAS, over 2.5 million SNPs were imputed using CEU samples from Phase 2 of the International HapMap project (www.hapmap.org). Genotyping procedures in the stage 2, Graves' disease and thyroid cancer populations are described in the Supplementary Material.

Association analyses

The heritabilities of TPOAb-positivity and serum TPOAb levels were estimated, as described in the Supplementary Material.

In stage 1, we performed a GWAS on TPOAb-positivity as well as a GWAS on continuous TPOAb levels. Persons taking thyroid medication were excluded. Each SNP was tested for association with TPOAb-positivity using logistic regression analyses, adjusting for age and sex. For cohorts with family structure, we approximated the probability of being affected with a linear mixed model adjusting for age and sex. The produced model was used to predict the expected proportion of "risk" (effective) alleles in cases and controls, hence giving the means to estimate odds ratios. Only unrelated individuals were

considered for the SardiNIA cohort. For the GWAS of continuous TPOAb levels, samples with a TPOAb level lower than the minimum TPOAb assay detection limit (Table 1) were excluded. TPOAb levels were natural log-transformed, and sex-specific, age adjusted standardized residuals were calculated. Each SNP was tested for association with these TPOAb level residuals using linear regression analyses (additive model), correcting for relatedness in studies with family structure. See Supplementary Table S1 for the software used for these analyses.

Before meta-analysis, SNPs with a minor allele frequency (MAF) < 1% or a low imputation quality were excluded (Supplementary Material), after which the results of each GWAS were combined in a population size weighted z-score based meta-analysis using METAL [71]. Genomic control was applied to individual studies if $\lambda > 1.0$.

In stage 2, we followed-up stage 1 GWAS significant SNPs, as well as promising SNPs not reaching GWAS significance, in an attempt to reach GWAS significant associations by increasing sample size (Supplementary Material). Results from stage 1 and 2 were combined in a population size weighted z-score based meta-analysis using METAL [71]. A z-score based meta-analysis was used to reduce bias that might be induced by different assays. As this method does not provide betas, and we wanted to provide a rough estimate of the actual effect sizes for convenience, we calculated betas using the fixed effects (inverse variance based) meta-analysis method. Heterogeneity was tested, applying bonferroni based *P*-value thresholds of $P = 0.004$ for the TPOAb-positivity analyses and $P = 0.005$ for the TPOAb level analyses.

All studies assessed and, if present, corrected for population stratification using principal-component analysis (PCA) and/or multidimensional-scaling (MDS), with the exception of SardiNIA and ValBorbera where the high isolation substantiates a lack of stratification (Table S1) [72,73]. Lambda values were all ~1, indicating that population stratification was overall properly accounted for (Table S1). To fully remove residual effects, we applied genomic correction to studies were lambda was > 1. The final meta-analyses reported a lambda of 1.01 for both the TPOAb-positivity and the TPOAb level GWAS, thus no genomic correction was applied.

The variances explained by the GWAS significant SNPs were calculated. We subsequently studied the individual as well as the combined effects of the GWAS significant SNPs on the risk of clinical thyroid disease, as specified in the Supplementary Material. In short, to study combined effects, a genetic risk score was calculated for every person as the weighted sum of TPOAb risk alleles. The associations between the individual SNPs, genetic risk scores and the risk of abnormal thyroid function tests were studied using logistic regression analyses. Logistic regression analyses were used to study the associations with goiter, Graves' disease and thyroid cancer (Supplementary Material). The results of each study were combined in a population size weighted z-score based meta-analysis using METAL [71].

Table 1. Population characteristics and serum TPOAb, TSH, and FT4 level measurements specifications.

		Sample characteristics						TPOAb specifications			TSH specifications		FT4 specifications	
Study	Ethnic group (origin)	N with TPOAb and GWAS data	N using thyroid medication	N case-control approach (cases/ controls)	N continuous approach	Men (%)	Age (yrs) Mean (SD)	TPOAb-positivity (%)	TPOAb-positivity cut-off	Assay (Detection range)	TSH Median (IQR)	Assay (normal range)	FT4 Mean (SD)	Assay (normal range)
Stage 1														
BHS	Caucasian (Australia)	1363	47	1316 (197 / 1119)	1316	43%	53.0 (17.2)	15.0%	35	Immulite 2000 chemiluminescent immunoassay (5-5000)	1.3 (0.9;1.9) mU/L	Immulite 2000 chemiluminescent immunoassay (0.4 - 4.0 mU/L)	16.9 (2.5) pmol/L	Immulite 2000 chemiluminescent immunoassay (9 – 23 pmol/L)
CHS	Caucasian (USA)	2024	0	2024 (281 / 1743)	1817	41%	74.8 (5.1)	13.9%	34	Chemiluminescent immunoassay (5-600)	2.3 (1.5;3.5) mU/L	Chemiluminescent immunoassay (0.27 - 4.2 mU/L)	1.2 (0.2) ng/dL	Chemiluminescent immunoassay (0.93 - 1.7 ng/dL)
HBCS	Caucasian (Finland)	526	29	497 (75/422)	497	50%	61.0 (2.8)	15.1%	12	Chemiluminescent immunoassay (0-1000)	2.0 (1.2;2.4) mU/L	Chemiluminescent immunoassay (0.49 – 4.67 mU/L)	14.1 (1.6) ng/dL	Chemiluminescent immunoassay (0.71 – 1.85 ng/dL)
KORA	Caucasian (Germany)	1765	49	1475 (74/ 1401)	1475	45%	60.5 (8.9)	5.0%	200	Chemiluminescent immunoassay (1-3000)	1.5 (0.6;2.5) mU/L	Chemiluminescent immunoassay (0.4 – 4.3 mU/L)	18.9 (2.6) pmol/L	Chemiluminescent immunoassay (11 – 25 pmol/L)
NBS	Caucasian (Netherlands)	1829	26	1829 (287/1542)	1829	50%	61.5 (10.3)	15.7%	12	Fluoro-immunometric assay (2.6-1000)	1.3 (0.9;2.0) mU/L	Immuno-luminometric assay (0.4 - 4.0 mU/L)	13.5 (2.4) pmol/L	Chemiluminescent immunoassay (8.0 - 22.0 pmol/L)
RS	Caucasian (Netherlands)	1627	50	1577 (137 / 1440)	210	40%	70.2 (5.6)	8.7%	35	Chemiluminescent immunoassay (5-5000)	1.2 (0.6:2.5) mU/L	Chemiluminescent immunoassay (0.4 – 4.3 mU/L)	18.4 (2.4) pmol/L	Chemiluminescent immunoassay (11 –25 pmol/L)
SardiNIA	Caucasian (Italy)	4686	154	972 (108/864)	1257	49%	56.9 (12.5)	11.1%	35	Chemiluminescent immunoassay (5-1000)	1.3 (0.8:2.0) mU/L	Chemiluminescent immunoassay (0.4 - 4.0 mU/L)	1.3 (0.2) ng/dL	Chemiluminescent immunoassay (0.3 –2.4 ng/dl)
SHIP	Caucasian (Germany)	4096	293	3803 (265 / 3538)	1818	52%	49.3 (16.3)	7.0%	60	Chemiluminescent immunoassay (1-3000)	0.7 (0.4;1.0) mU/L	Chemiluminescent immunoassay (0.3 – 3.0 mU/L)	12.8 (3.8) pmol/L	Chemiluminescent immunoassay (7.7 – 23.2 pmol/L)

SHIP-Trend	Caucasian (Germany)	986	99	887 (36 / 851)	887	46%	49.5 (13.7)	4.1%	200	Chemiluminescent immunoassay (1-3000)	1.2 (0.8;1.6) mU/L	Chemiluminescent immunoassay (0.36 – 3.74 mU/L)	-	-
TwinsUK	Caucasian (UK)	2455	86	2369 (461 / 1893)	774	0%	46.9 (12.5)	19.5%	6	Chemiluminescent immunoassay (0.5-1000)	1.3 (0.9;1.8) mU/L	Chemiluminescent immunoassay (0.4 – 4.0 mU/L)	13.6 (1.9) pmol/L	Chemiluminescent immunoassay (9 – 19 pmol/L)
ValBorbera	Caucasian (Italy)	1661	90	1571 (161 / 1410)	452	46%	54.3 (18.4)	10.2%	60 and 50	Two chemiluminescent immunoassays (5.5-3000 ; 6-7500)	1.4 (0.9;2.0) mU/L	Chemiluminescent immunoassay (0.34 - 5.60 mU/L)	-	-
Stage 2														
Asklepios	Caucasian (Belgium)	2418	109	2309 (245/2064)	2185	50%	45.9 (5.9)	10.6%	35	Chemiluminescent immunoassay (5-600)	1.5 (1.1;2.1) mU/L	Chemiluminescent immunoassay (0.3 – 4.2 mU/L)	1.31 (0.2) ng/dL	Chemiluminescent immunoassay (0.9 –1.7 ng/dl)
CARLA	Caucasian (Germany)	1753	270	1483 (186/1297)	1190	60%	64.2 (10.2)	12.5%	28	Chemiluminescent immunoassay (5-600)	0.9 (0.6;1.2) mU/L	Chemiluminescent immunoassay (0.4 – 3.8 mU/L)	16.3 (2.5) pmol/L	Chemiluminescent immunoassay (12.8-20.4 pmol/L)
EFSOCH	Caucasian (UK)	1289	-	1289 (97 / 1192)	1233	64%	34.2 (5.9)	7.5%	34	Chemiluminescent immunoassay (5-600)	1.9 (1.3;2.6) mU/L	Chemiluminescent immunoassay (0.4–4.5 mU/L)	16.0 (2.4) pmol/L	Chemiluminescent immunoassay (11–24 pmol/L)
Health2006 Study	Caucasian (Danish)	3287	-	3287 (204/3083)	3285	45%	49.3 (13.0)	6.2%	100	Chemiluminescent immunoassay (1-3000)	1.7 (1.0;2.0) mU/L	Chemiluminescent immunoassay (0.4–3.7 mIU/L)	12.4 (1.8) pmol/L	Chemiluminescent immunoassay (9.8–18.8 pmol/L)
SardiNIA2	Caucasian (Italy)	1387	30	765 (104/661)	375	41%	46.6 (17.4)	13.6%	35	Chemiluminescent immunoassay (5-1000)	1.6 (1.0;2.2) mIU/ml	Chemiluminescent immunoassay (0.4 - 4.0 mU/L)	1.3 (0.2) ng/dl	Chemiluminescent immunoassay (0.3 –2.4 ng/dl)

Various bioinformatic tools were searched for evidence for functional relevance of the GWAS significant SNPs and pathway analyses were performed on the Stage 1 lead SNPs (see Supplementary Material).

RESULTS

Characteristics of the studied populations are shown in Table 1 and the Supplementary Material S1. Heritability estimates in the family-based cohorts SardiNIA, TwinsUK and Val Borbera were, respectively, 0.65, 0.66, and 0.54 for TPOAb-positivity, and 0.43, 0.66, and 0.30 for TPOAb levels.

Loci associated with TPOAb-positivity and TPOAb levels

See Table 1 and Supplementary Figure S1 for TPOAb measurements and Supplementary Table S1 for genotyping procedures. The supplementary text, tables and figures are available in Appendix 2. In most autoimmune diseases, both the presence and the level of autoantibodies are relevant for the disease onset [18,30,31]. Furthermore, different pathophysiological processes may be involved in the initiation and severity of the autoimmune response. We therefore performed a GWAS on TPOAb-positivity (including 1769 TPOAb-positives and 16,528 TPOAb–negatives), as well as a GWAS on continuous TPOAb levels (including 12,353 individuals) in stage 1. See Supplementary Figures S2 and S3 for QQ (quantile-quantile) and Manhattan plots.

In stage 2, we followed-up 20 stage 1 SNPs (P <5x10^{-6}; 13 TPOAb-positivity and 10 TPOAb level SNPs, with 3 SNPs overlapping) in 5 populations, including up to 8,990 individuals for TPOAb-positivity (922 TPOAb-positives and 8068 TPOAb–negatives) and 8,159 individuals for TPOAb level analyses (see Supplementary Material S1). Results of the combined stage 1 and 2 meta-analyses, including heterogeneity analyses, are shown in Supplementary Tables S2 and S3. Regional association plots are shown in Supplementary Figures S4 and S5. In the combined stage 1 and 2 meta-analyses GWAS significant associations (P <5x10^{-8}) were observed near *TPO* (Chr 2p25; rs11675434), at *ATXN2* (Chr 12q24.1; rs653178), and *BACH2* (Chr 6q15; rs10944479) for TPOAb-positivity, and near *TPO* (rs11675434), at *MAGI3* (Chr 6q15; rs1230666), and *KALRN* (Chr 3q21; rs2010099) for TPOAb levels (Table 2 and Figure 1). The TPOAb level meta-analysis P-values for the 3 GWAS significant TPOAb-positivity loci were: *TPO*-rs11675434: P = 7.4 x 10^{-13}, *ATXN2*-rs653178: P = 1.3 x 10^{-7}, and *BACH2*-rs10944479: P = 2.0 x 10^{-4}.

As the 3 GWAS significant loci for TPOAb levels also showed associations with TPOAb-positivity (*TPO*-rs11675434*:* OR, 1.21 [95% CI, 1.15-1.28)], P = 1.5x10^{-16}; *MAGI3*-rs1230666*:* OR, 1.23 [95% CI, 1.14-1.33], P = 1.5x10^{-6}; *KALRN*-rs2010099*:* OR, 1.24 [95% CI, 1.12-1.37], P = 7.4x10^{-5}), we subsequently studied the (combined) effects of these 5 SNPs on clinical thyroid

Table 2. Newly identified loci associated with TPOAb-positivity and/or serum TPOAb levels reaching genome wide significance

				Alleles				Stage 1 + 2 meta-analysis: up to 2691 cases and 24,596 controls	
TPOAb-positivity	**SNP**	**Chr.**	**Position (Build 36)**	**Risk**	**Other**	**RAFa**	**Nearby gene**	**OR (95% CI)b**	***P* value**
	rs11675434	2	1386822	T	C	0.39	*TPO*	1.21 (1.15-1.28)	1.5×10^{-16}
	rs653178	12	110492139	C	T	0.40	*ATXN2*	1.14 (1.08-1.19)	9.9×10^{-10}
	rs10944479	6	90937114	A	G	0.16	*BACH2*	1.25 (1.14-1.37)	4.0×10^{-8}
				Alleles				**Stage 1 + 2 meta-analysis: up to 20,512 subjects**	
TPOAb levels	**SNP**	**Chr.**	**Position (Build 36)**	**Risk**	**Other**	**RAFa**	**Nearby gene**	**β (SE)c**	***P* value**
	rs11675434	2	1386822	T	C	0.39	*TPO*	0.0202 (0.0046)	7.4×10^{-13}
	rs1230666	1	113974933	A	G	0.16	*MAGI3*	0.0269 (0.0064)	1.8×10^{-8}
	rs2010099	3	125782947	C	T	0.91	*KALRN*	0.0240 (0.0076)	3.1×10^{-8}

Chr., chromosome

[a] Risk allele frequency: Weighted mean frequency of the risk allele across all included cohorts.

[b] Adjusted for age and gender

[c] Expressed in sd of natural logarithm transformed serum TPOAb level, adjusted for age and gender.

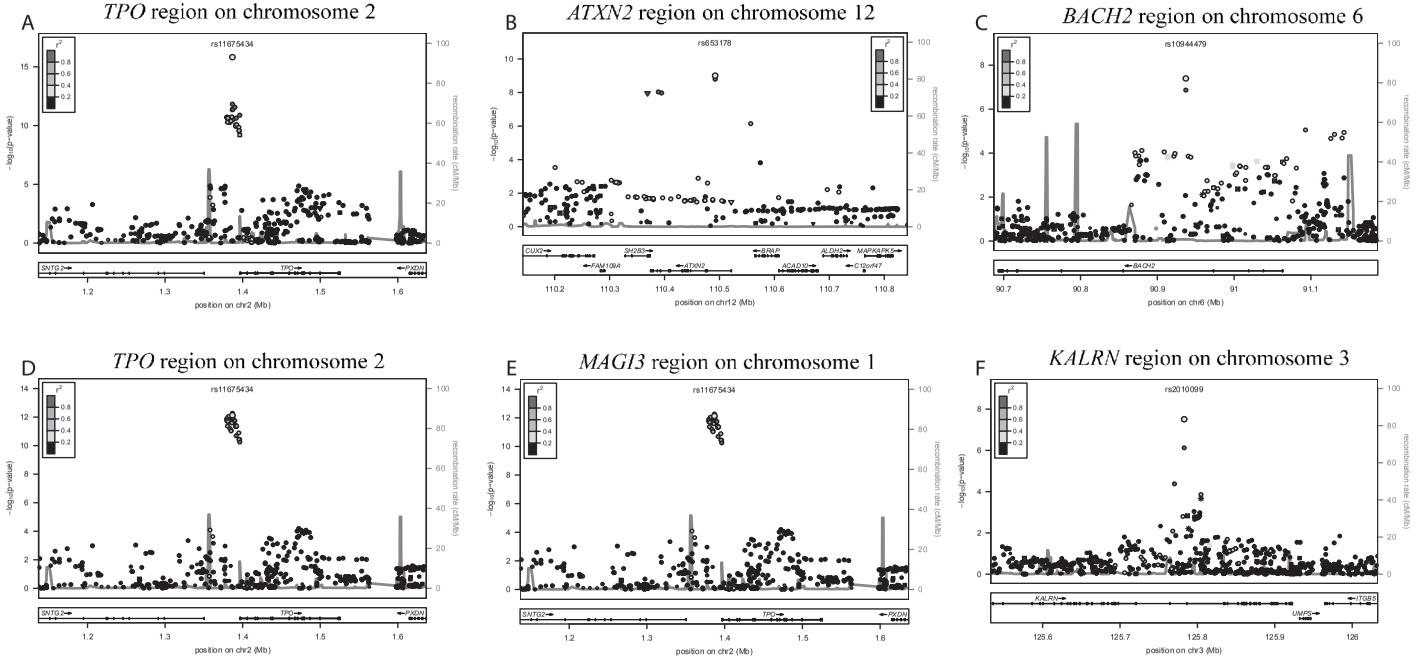

Figure 1. Genome wide association studies meta-analyses: Loci associated with TPOAb-positivity (a-c) and TPOAb levels (d-f) on a genome-wide level of significance. Regional association plots of the genome-wide significant loci associated with TPOAb-positivity (a-c) and TPOAb levels (d-f). The y-axis on the left indicates the $-\log_{10}$ *P* value for the association with TPOAb –positivity (a-c) or TPOAb levels (d-f). Single nucleotide polymorphisms (SNPs) are plotted on the x-axis according to their chromosomal position against the association with the phenotype on the y-axis. The most significant stage 1 SNP is indicated in purple. The combined stage 1 and 2 result of this SNP is indicated in yellow. The SNPs surrounding the most significant SNP are color-coded to reflect their LD with this SNP. Symbols reflect functional genomic annotation, as indicated in the legend. The blue y-axes on the right of each plot indicate the estimated recombination rates (based on HapMap Phase II); the bottom of each panel shows the respective annotated genes at the locus and their transcriptional direction. Mb, megabases
A color figure is available at: http://www.plosgenetics.org/article/info%3Adoi%2F10.1371%2Fjournal.pgen.1004123

Table 3. Genetic risk score and the risk of TPOAb-positivity

GRS Quartile	% TPOAb-positivity (N cases/total)	OR (95% CI)a	*P* value
1 (reference)	5.4 % (89 / 1637)	-	-
2	7.0 % (114 / 1637)	1.29 (0.98-1.69)	0.07
3	9.0 % (152 / 1695)	1.64 (1.26-2.13)	1.3×10^{-4}
4	10.4 % (158 / 1523)	2.18 (1.68-2.81)	8.1×10^{-8}

GRS, genetic risk score (based on rs11675434, rs653178, rs10944479, rs1230666, rs2010099).
[a] Adjusted for age and gender

disease. Genetic risk scores were calculated as described in the Supplementary Material. The variance explained by these 5 SNPs was 3.1 % for TPOAb-positivity and 3.2 % for TPOAb levels. Subjects with a high genetic risk score had a 2.2 times increased risk of TPOAb-positivity compared to subjects with a low genetic risk score ($P = 8.1 \times 10^{-8}$) (Table 3).

Table S4 shows the stage 1 TPOAb-positivity and TPOAb level meta-analyses results for GWAS significant SNPs reported in previous GWAS on thyroid related phenotypes.

Associations with hypo- and hyperthyroidism

The associations between the 5 GWAS significant SNPs and the risk of abnormal thyroid function tests are shown in Table 4. *MAGI3*- rs1230666 was associated with an increased risk of overt hypothyroidism and increased TSH levels below the Bonferroni threshold (i.e., $P = 0.05/5 = 0.01$). Borderline significant signals were observed at *BACH2*- rs10944479 with a higher risk of increased TSH levels as well as overt hyperthyroidism ($P = 0.011$ and $P = 0.012$), and at the *KALRN*-rs2010099 SNP with a lower risk of decreased TSH levels ($P = 0.010$).

Furthermore, a higher genetic risk score was associated with a higher risk of increased TSH levels (Supplementary Table S5). No effects of the genetic risk score on the risk of overt hypothyroidism, hyperthyroidism or decreased TSH levels were observed.

Associations with goiter

Individuals with a high genetic risk score had a 30.4% risk of sonographically-proven goiter, compared to 35.2% in subjects with a low score ($P = 6.5 \times 10^{-4}$) (Table 5). None of the individual SNPs was significantly associated with goiter risk.

Thyroid autoimmunity during pregnancy

As autoimmunity significantly changes during pregnancy [25], we additionally studied these effects in an independent pregnant population. Pregnant women with a high genetic risk score had a 2.4 times increased risk of TPOAb-positivity compared to women with a low score (10.3% vs 4.8%, $P = 0.03$). These women did not have a higher risk of increased TSH levels. However, a borderline significant signal with a lower risk of increased TSH levels was observed at *ATXN2*- rs653178 (OR, 0.54 [95% CI, 0.34-0.87], $P = 0.012$).

Table 4. Newly identified TPOAb associated loci and the risk of thyroid disease in stage 1 and 2 populations

		Alleles		Increased TSH (1110 cases / 19,189 controls)		Hypothyroidism (173 cases / 15,940 controls)		Decreased TSH (967 cases / 19,297 controls)		Hyperthyroidism (78 cases / 14,901 controls)	
Nearby gene	**SNP**	**Risk**	**Other**	**OR (95% CI)**	***P* value**	**OR (95% CI)a**	***P* value**	**OR (95% CI)a**	***P* value**	**OR (95% CI)a**	***P* value**
TPO	rs11675434	T	C	1.08 (0.99-1.18)	0.08	1.14 (0.91-1.42)	0.26	1.02 (0.93-1.11)	0.68	1.10 (0.81-1.49)	0.54
ATXN2	rs653178	C	T	1.01 (0.98-1.04)	0.68	1.25 (1.01-1.54)	0.04	1.01 (0.97-1.04)	0.70	1.00 (0.74-1.33)	0.99
BACH2	rs10944479	A	G	1.17 (1.04-1.32)	0.011	1.37 (1.00-1.88)	0.05	0.91 (0.80-1.03)	0.15	1.80 (1.14-2.85)	0.012
MAGI3	rs1230666	A	G	1.23 (1.09-1.39)	9.0×10^{-4}	1.57 (1.18-2.10)	1.9×10^{-3}	1.08 (0.96-1.22)	0.22	1.61 (0.99-2.60)	0.05
KALRN	rs2010099	C	T	1.05 (0.90-1.23)	0.52	0.80 (0.54-1.20)	0.28	0.82(0.71-0.95)	0.010	0.69 (0.39-1.24)	0.21

All analyses adjusted for age and gender.
ATXN2-rs653178 is in high LD with *SH2B3*- rs3184504
MAGI3-rs1230666 is in high LD with *PTPN22*-rs2476601

Table 5. Newly identified TPOAb associated loci, genetic risk scores and the risk of goiter

Individual SNPs (2205 goiter cases / 4532 controls)						Genetic risk scores			
Nearby gene	**SNP**	**Risk allele**	**Other allele**	**OR (95% CI)a**	***P* value**	**GRS Quartile**	**% Goiter (N cases/total)**	**OR (95% CI)a**	***P* value**
TPO	rs11675434	T	C	0.95 (0.88-1.02)	0.17	1 (reference)	35.2 % (588 / 1669)	-	-
ATXN2	rs653178	C	T	0.95 (0.88-1.03)	0.22	2	33.7 % (570 / 1691)	0.92 (0.79-1.06)	0.21
BACH2	rs10944479	A	G	0.94 (0.85-1.05)	0.28	3	31.6 % (530 / 1675)	0.84 (0.72-0.98)	0.03
MAGI3	rs1230666	A	G	0.90 (0.81-1.00)	0.05	4	30.4 % (517 / 1702)	0.77 (0.66-0.89)	6.5×10^{-4}
KALRN	rs2010099	C	T	0.93 (0.81-1.05)	0.23				

GRS, genetic risk score (based on rs11675434, rs653178, rs10944479, rs1230666, rs2010099).
[a] Adjusted for age, gender, and body surface area
ATXN2-rs653178 is in high LD with *SH2B3*- rs3184504
MAGI3-rs1230666 is in high LD with *PTPN22*-rs2476601

Associations with thyroid disease in independent populations

Graves' disease

As *MAGI3*- rs1230666 and *BACH2*- rs10944479 showed promising associations (i.e., $P \leq 0.05$) with hyperthyroidism in our meta-analyses, we tested these SNPs in an independent population of 2478 patients with Graves' disease and 2682 controls (see Supplementary Material for further details). Both were associated with an increased risk of Graves' disease (*MAGI3*- rs1230666: OR, 1.37 [95% CI, 1.22–1.54]; $P = 1.2 \times 10^{-7}$; *BACH2*- rs10944479: OR, 1.25 [1.12-1.39]; $P = 6.2 \times 10^{-5}$).

Thyroid cancer

Supplementary Table S6 shows the associations of the 5 GWAS significant SNPs with thyroid cancer. No statistically significant associations were detected, but a borderline significant signal with an increased risk of thyroid cancer was observed at *ATXN2*- rs653178 (OR, 1.32 [95% CI, 1.02-1.70], $P = 0.03$).

Pathway analyses

Ingenuity Pathway Analyses (IPA; Ingenuity Systems, Ca, USA) and GRAIL analyses [32] were performed to identify potential pathways involved in AITD, the results of which are shown in Supplementary Tables S7 and S8, and Figure S6. The identified top pathways involved cell death, survival, movement, and OX40 signalling.

DISCUSSION

This is the first GWAS meta-analysis investigating the genetics of TPOAbs in the normal population in up to 18,297 individuals from 11 populations with replication in up to 8,990 individuals from 5 populations. We identified 5 GWAS significant loci associated with TPOAb-positivity and/or levels.

The most significant hit for both TPOAb-positivity and TPOAb levels was located near the *TPO* gene itself. TPO is a membrane-bound protein located on the apical membranes of the thyroid follicular cell, catalyzing key reactions in thyroid hormone synthesis [33]. Mutations in *TPO* have been found in patients with congenital hypothyroidism [34,35]. Although TPOAbs are valid clinical biomarkers of AITD, they are generally considered to be secondary to the thyroid damage inflicted by T-cells.

The *FOXE1* gene has been previously associated with hypothyroidism [36,37] and is known to regulate transcription of *TPO* [38]. In this context it is interesting to note that we did not find any associations of the variant near *TPO* with hypothyroidism. Most genes that have been associated with AITD (predominantly Graves' disease) by candidate gene and GWAS

studies so far are located in the HLA class I and II regions, or in genes involved in T-cell (i.e., *CTLA-4*, *PTPN22*) or other autoimmune responses [28,39]. Until now, the *TPO* gene itself had not been associated with AITD, except in one recent candidate gene analysis in a small cohort (n=188) without replication [40]. A variant near *TPO* (rs11694732), which is in LD with rs11675434 (r2=0.97 in HapMap2), has previously been associated with TSH levels by Gudmundsson et al [41]. However, various other GWAS on serum TSH and FT4 levels have not found any significant associations in or near this locus, including a recent similar sized GWAS by Porcu et al [42].

Three of the other four loci identified here are located in or are in linkage disequilibrium (LD) with genes previously associated with other autoimmune diseases. Rs1230666 is located in intron 9 of *MAGI3*, encoding a protein that modulates activity of AKT/PKB. AKT/PKB is expressed in the thyroid and regulates apoptosis [43], which seems to play an important role in the development of AITD [44,45]. In addition, rs1230666 is in LD with rs2476601 (r2=0.70 in HapMap2), a variant causing a R620W substitution in *PTPN22*. PTPN22 is a lymphoid-specific intracellular phosphatase involved in the T-cell receptor signaling pathway. Variations in *PTPN22*, and specifically R620W, are associated with various autoimmune disorders including type 1 diabetes, rheumatoid arthritis, systemic lupus erythematosus and Graves' disease [46,47,48,49]. The associations of the *MAGI3* locus with TPOAb-positivity and Graves' disease may therefore also be explained by linkage with disease-associated variants in *PTPN22* [50]. Of note, the association signal at rs2476601 is one order weaker than that of the top variant rs1230666.

The *BACH2* locus has been implicated in the susceptibility to several autoimmune diseases, including celiac disease, type 1 diabetes, vitiligo, Crohn's disease, and multiple sclerosis [46,51,52,53,54]. A recent candidate gene analysis associated the *BACH2* locus with an increased risk of AITD, including Hashimoto's thyroiditis and Graves' disease [55]. However, the associations were not significant when Hashimoto's thyroiditis and Graves' disease were studied separately. *BACH2* is specifically expressed in early stages of B-cell differentiation and represses different immunoglobulin genes [56]. Interestingly, BACH2 can bind to the co-repressor SMRT (silencing mediator of retinoid and thyroid receptor), which may suggest a more direct effect on thyroid hormone secretion and action as well.

Polymorphisms in *ATXN2* have been associated with multiple neurodegenerative diseases, including spinocerebellar ataxia and Parkinson's disease [57,58,59]. Different epidemiological studies have associated thyroid dysfunction with cerebellar ataxia [60,61]. Furthermore, the identified SNP in *ATXN2* has been previously associated with renal function, serum urate levels and blood pressure [62,63,64]. However, this SNP is in high LD with rs3184504 (r2=0.873), a variant causing a Trp262Arg substitution of *SH2B adaptor protein 3* (*SH2B3)*. *SH2B3* encodes the adaptor protein LNK, a key negative regulator of cytokine signaling playing a critical role in hematopoiesis. This variant is associated with susceptibility to several autoimmune diseases, including celiac disease, type 1 diabetes, vitiligo, and rheumatoid arthritis [46,51,53,65], suggesting more relevance for TPOAb levels than ATXN2. This is sup-

ported by a recent study which showed that variants in LD with *SH2B3*, *BACH2*, and *PTPN22* are associated with TPOAb levels in patients with type 1 diabetes [66].

Whereas the above four loci are located in genes involved in the immune response or the autoantigen, the *KALRN* (*Kalirin*) gene encodes a multi-domain guanine nucleotide exchange factor for GTP-binding proteins of the Rho family. The relation of *KALRN* with levels of TPOAbs is unclear. This gene has recently been found to be associated with megakaryopoiesis and platelet formation [67], which may suggest a function in the immune system [68]. We furthermore performed pathway analyses on the stage 1 TPOAb-positivity and TPOAb level lead SNPs, and identified the cell death, survival and movement pathway as an important pathway for TPOAbs. This finding is supported by previous studies, which show an important role for apoptosis in the development of AITD [44,45]. Another top pathway involved was the OX40 signalling pathway, and it is of interest to note that OX40 is a T-cell activator promoting the survival of CD4+ T-cells at sites of inflammation [69].

Our results have potential clinical relevance for several reasons. Genetic risk scores based on these novel common (risk allele frequencies: 9-40%) TPOAb-associated SNPs enabled us to identify a large subgroup in the general population with a two-fold increased risk of TPOAb-positivity (10.4% vs 5.4%). These individuals also have a higher risk of increased TSH levels and a lower risk of goiter, suggesting an advanced stage of destruction of the thyroid due to autoimmune processes. Furthermore, pregnant women with high genetic risk scores had a 2.4 times increased risk of TPOAb-positivity during pregnancy. In this context it is interesting to note that TPOAb-positive pregnant women have an increased risk of miscarriages and preterm births independent of thyroid function [70].

Associations with thyroid disease were also found on an individual SNP level. The *MAGI3* SNP was associated with a substantially increased risk of hypothyroidism, and the *BACH2* SNP showed a borderline significant association ($P = 0.011$) with a higher risk of increased TSH levels, which includes subjects with subclinical and overt hypothyroidism. Furthermore, both loci were significantly associated with an increased risk of Graves' hyperthyroidism in an independent population. To predict which patients with first or second degree relatives with documented Hashimoto's or Graves' disease will develop clinical thyroid disease, a clinical algorithm has been developed (i.e., the THEA score) [18]. Future studies should analyze if these genetic markers increase the sensitivity of the THEA score. Graves' hyperthyroidism and Hashimoto's thyroiditis co-segregate in families and subjects with TPOAbs have an increased risk of both diseases [17,18,20,21,22,26]. The current study provides insight into this phenomenon by showing that specific loci associated with TPOAbs and (subclinical) hypothyroidism, *i.e. MAGI3* and *BACH2*, are also associated with Graves' hyperthyroidism in an independent case-control study.

The prevalence of TPOAb-positivity in the general population is high (5-24%), but it is currently unknown why part of the individuals with thyroid autoimmunity develop clinical thyroid disease whereas others do not [27,28]. In this context it is interesting to note that the

TPOAb-associated SNPs located in *TPO* and *ATXN2* were not associated with clinical thyroid disease. This suggests that the TPOAbs in these individuals may be of less clinical relevance, providing insight into why TPOAb-positive individuals do or do not eventually develop clinical thyroid disease.

Our study has some limitations. The validity of the results is restricted to individuals from populations of European ancestry. Future GWASs in populations from non-European descent will be required to determine to which extent our results can be generalized to other ethnic groups. Secondly, we did not perform conditional analyses to further identify secondary association signals within the identified loci, nor did we perform functional studies for the identified variants. Further research is therefore needed to unravel the exact biological mechanism behind the observed associations. The fact that various TPOAb assays were used across the participating cohorts could lead to bias. We therefore used TPOAb-positivity cut-off values as provided by the respective assay manufacturer, instead of using one fixed cut-off value. This is also of clinical importance as in clinical practice most institutions rely on the TPOAb-positivity cut-off as provided by the assay manufacturer. Furthermore, we did not detect heterogeneity in our results, supporting the fact that results obtained with different assays can be combined across cohorts using the z-score based meta-analysis. Finally, as AITD coincides with other autoimmune diseases, our results could be driven by indirect associations with other autoimmune diseases. However, AITD is the most common autoimmune disease in the general population. We furthermore show that carriage of multiple risk alleles is associated with an increased risk of thyroid dysfunction, which underlines the clinical importance of our findings.

In conclusion, this first GWAS for TPOAbs identified five newly associated loci, three of which were also associated with clinical thyroid disease. Furthermore, we show that carriage of multiple risk variants is not only associated with a substantial increased risk of TPOAb-positivity, but also with a higher risk of increased TSH levels (including subclinical and overt hypothyroidism) and a lower risk of goiter. These genetic markers not only help to identify large groups in the general population with an increased risk of TPOAb-positivity, but may also predict which TPOAb-positive persons are particularly at risk of developing clinical thyroid disease.

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Chapter 10

A large-scale population-based analysis of common genetic variation in the thyroid hormone receptor α locus and bone.

M. Medici, F. Rivadeneira, W.M. van der Deure, A. Hofman, J.B. van Meurs, U. Styrkársdottir, C.M. van Duijn, T. Spector, D.P. Kiel; GEFOS Consortium, A.G. Uitterlinden, T.J. Visser, R.P. Peeters

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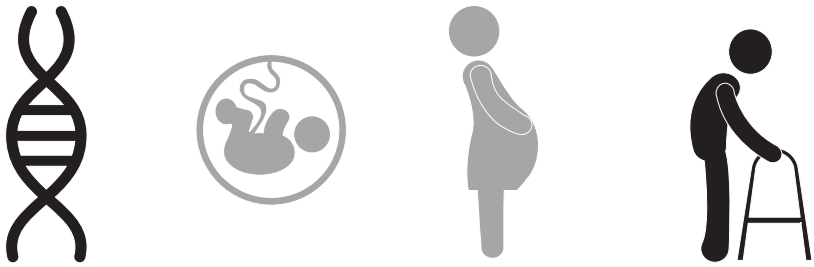

ABSTRACT

Background: Thyroid hormone receptor alpha (TRα) is the predominant TR in bone, and mice with a mutant *TRα* have a delayed bone development and osteosclerosis in adulthood. *REV-ERBα* is a circadian clock gene located on the opposite chromosomal strand of *TRα*, which overlaps and interferes with *TRα* expression. Although these data suggest an important role for the *TRα/REV-ERBα* locus in bone health, limited data are available on the effects of genetic variation in this locus on human bone. We therefore studied the effects of genetic variation in the *TRα/REV-ERBα* locus on human bone.

Methods: 14 Polymorphisms, covering the *TRα/REV-ERBα* locus, were genotyped and studied in multiple Caucasian populations. Associations with bone mineral density (BMD) were studied in GEFOS (n = 19,195). Associations with incident osteoporotic fracture risk were studied in RS1 and RS2 (n = 8,131). Associations with vertebral fracture risk, BMD change and bone geometry were studied in RS1 (n = 5,974). Analyses were corrected for multiple testing.

Results: None of the studied polymorphisms were associated with BMD, fracture risk, BMD change or geometric outcomes. The study was powered to detect small differences in BMD (0.04-0.05 SD), and at least moderate differences in vertebral (RR = 1.42) and incident osteoporotic (RR = 1.23) fracture risk, BMD change (0.13 SD) and geometric outcomes (0.10 SD).

Conclusions: Genetic variation in the *TRα/REV-ERBα* locus does not have an important contribution to variation in BMD, fracture risk and bone geometry in the elderly population.

INTRODUCTION

Thyroid hormone (TH) is essential for normal bone development and the maintenance of adult bone mass (1). Childhood hypothyroidism leads to growth retardation, delayed bone age and short stature, whereas hyperthyroidism accelerates growth and advances bone age. In adults, hyperthyroidism leads to osteoporosis and increased fracture risk (1).

TH action is mediated via nuclear TH receptors (TRs), thereby regulating gene expression. TRα is the predominant TR in bone (1). No human patients with mutations in *TRα* have been described. Mice with different mutations in the ligand-binding domain of *TRα* (2-5) show signs of a hypothyroid skeleton, characterized by a delay in bone development. In adulthood, osteosclerosis is seen.

The circadian clock gene *REV-ERBα* is located on the opposite chromosomal strand of *TRα*. The *TRα* and *REV-ERBα* genes partially overlap and *REV-ERBα* expression has been shown to influence splicing and expression of *TRα* (6, 7).

To date, limited data exist on the role of the *TRα/REV-ERBα* locus in human bone physiology. Therefore, we studied the effects of genetic variation in the *TRα/REV-ERBα* locus on human bone health. The effects on bone mineral density (BMD) were studied in a large consortium including 19,195 subjects. The effects on BMD change, fracture risk and bone geometry were studied in 5,974 subjects from the Rotterdam Study.

MATERIALS AND METHODS

Study populations

Associations with BMD were studied in aggregate results from 5 populations of the GEFOS consortium. Associations with BMD change, bone geometry and vertebral fractures were studied in the Rotterdam Study (RS1). Associations with osteoporotic fractures were studied in RS1 and RS2 (which is an extension of RS1).

GEFOS

The Genetic Factors for Osteoporosis (GEFOS) consortium is a coalition of investigators working on the genetics of osteoporosis. Analyses described in this study were based on 19,195 Caucasians (74.4% women, age: 60.9 (11.9) yrs). Details have been described previously (8). The study includes the deCODE Genetics Study (n = 6,743), the Framingham Osteoporosis Study (n = 3,503), the Twins UK Study (n = 2,734), the Erasmus Rucphen Family Study (n = 1,228) and RS1 (n = 4,987).

RS1 and RS2

RS1 is a prospective population-based cohort study in 7,983 Caucasians aged ≥ 55 years. Details have been described previously (9). At baseline (1989-1993) blood was drawn, and BMD measurements and radiographs were taken. In the present study, 5,974 subjects (59.4% women, age: 69.4 (9.1) yrs) from RS1 were included. In 1999, RS1 was expanded (RS2) with 3,011 subjects who had become 55 years of age or moved into the study district. In the present study, 2,157 subjects (54.4% women, age: 64.8 (8.0) yrs) from RS2 were included.

Outcome assessment

Thyroid hormone measurements:

In RS1, serum TSH (TSH lumitest, Henning, Berlin, Germany) and FT4 levels (Vitros, ECI Immunodiagnostic System, Ortho-Clinical Diagnostics, Amersham, UK) were determined in 1,350 subjects of whom DNA was available.

BMD and BMD change:

In GEFOS, femoral neck and lumbar spine BMD were measured in 19,195 subjects with available genotype data with similar methods using DXA (8). In RS1, femoral neck BMD measurements were performed at baseline and at the second follow-up visit (follow-up: 6.51 (0.38) yrs) (9). Rates of BMD loss were calculated as annual percentages of change in femoral neck BMD in 2,366 subjects with available genotype data.

Vertebral fractures:

In RS1, lateral radiographs of the thoracolumbar spine were obtained at baseline and at the second follow-up visit (follow-up: 6.43 (0.38) yrs). All second follow-up visit radiographs from 2,994 subjects with available genotype data were scored for the presence of vertebral fractures (9). If the vertebral fracture was present at baseline, it was considered a baseline prevalent fracture (n = 220), otherwise the fracture was defined to be incident (n = 151).

Incident osteoporotic fractures:

Information on incident osteoporotic fractures was retrieved from computerized records of the general practitioners (GPs), and by research physicians from GP patient records. For the classification of osteoporotic fractures (n = 900 in RS1; n = 131 in RS2), foot, hand, face, skull and pathological fractures were excluded. Information on incident osteoporotic fractures was collected in 5,974 RS1 subjects with genotype data from baseline until December 31st 2001 (follow-up: 7.79 (3.04) yrs), and in 2,157 RS2 subjects with genotype data from baseline until December 31st 2004 (follow-up: 3.95 (0.84) yrs) (9).

Bone geometry:

In RS1, hip bone geometry was measured from DXA scans at baseline (10). Four geometric outcomes measured at the narrow-neck region in 4,131 subjects with available genotype data were used: the narrow-neck width, narrow-neck cortical thickness, buckling ratio and section modulus. The buckling ratio is an index of bone instability and the section modulus an index of bending strength (10).

Selection and genotyping of polymorphisms

Based on linkage disequilibrium (LD) analysis (http://www.hapmap.org) and previous sequencing results (11), a tagging set of 14 polymorphisms with a minor allele frequency (MAF) above 5% were selected to cover most of the genetic variation in the *TRα/REV-ERBα* locus and the 10 kb upstream and downstream regions (Fig.1).

For GEFOS, (imputed) genotypes were available from various genotyping platforms (8). For RS1 and RS2, (imputed) genotypes were extracted from the Illumina HumanHap 550K (Duo) array. Imputation procedures have been described previously (8).

Using genotype data from 5,974 subjects from RS1, the LD structure of the *TRα/REV-ERBα* locus was analyzed using Haploview 4.1 (12).

Statistical methods

Genotype and allele frequencies were tested for Hardy-Weinberg equilibrium. Linear regression was used to compare baseline characteristics between genotype groups.

All GEFOS populations studied the association with femoral neck and lumbar spine BMD using sex-specific, age- and weight-adjusted standardized residuals analyzed under an additive (per allele) genetic model. Inverse-variance weighted fixed-effects meta-analyses were conducted using METAL (http://www.sph.umich.edu/csg/abecasis/Metal).

In RS1, the association with BMD change and bone geometry was analyzed by linear regression. For vertebral fractures, odds ratios (OR) with 95% confidence intervals (95%CI) were calculated using logistic regression (considering time-to-event was unknown). For incident osteoporotic fractures, hazard ratios (HR) with 95%CI were assessed by a Cox proportional-hazards model (taking time-to-event into account), using pooled data from RS1 and RS2. All analyses were adjusted for age, gender, height and weight. SPSS 15.0 for Windows (SPSS, Chicago, IL, USA) was used for all analyses.

The number of independent polymorphisms tested was calculated in RS1 using PLINKv1.07 (13), for which an LD threshold of $r^2 > 0.2$ was used. To control for multiple testing, a p-value threshold to declare statistical significance was calculated by dividing $P = 0.05$ by the number of independent tests. Consequently, the p-value thresholds were $P = 0.005$ (lumbar spine BMD, BMD change, narrow-neck width, narrow-neck cortical

thickness and section modulus), $P = 0.006$ (buckling ratio) and $P = 0.007$ (femoral neck BMD, vertebral and osteoporotic fractures).

Power calculations for detectable effect sizes were performed at $\beta = 0.80$ and α-values corresponding to the respective p-value thresholds.

RESULTS

Allele and genotype frequencies of all polymorphisms were in Hardy Weinberg equilibrium with similar frequencies as reported in literature (11) and established databases, such as dbSNP (http://www.ncbi.nlm.nih.gov/projects/SNP/) and HapMap (http://www.

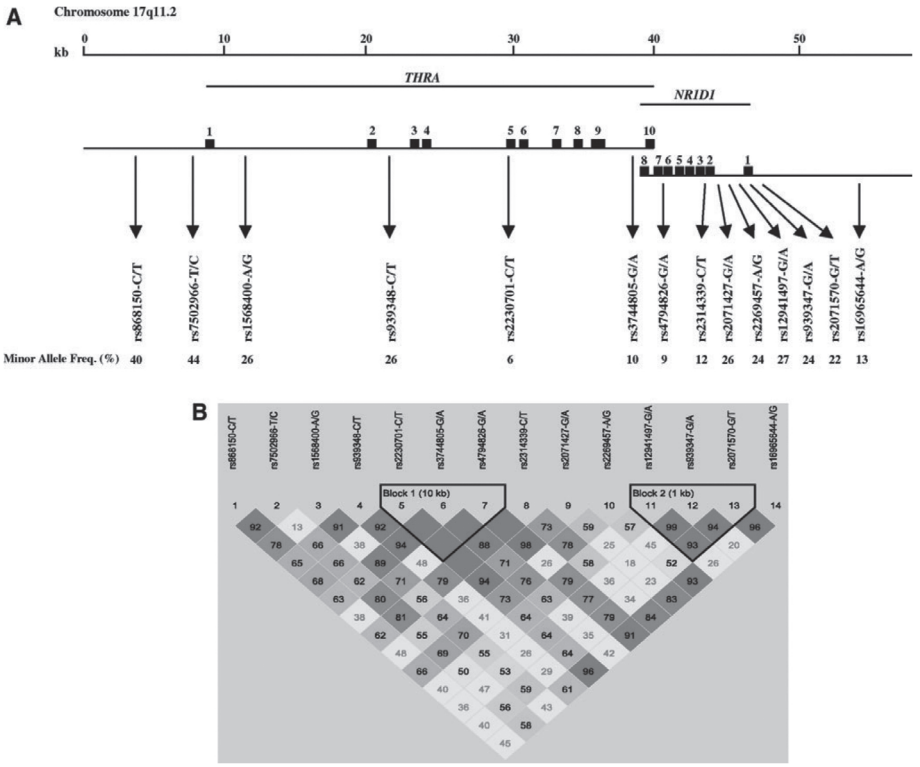

Figure 1. A. The genomic organization of the *TRα/REV-ERBα* locus is shown in the upper part of the figure. Exons are indicated by boxes. Selected polymorphisms are shown in the lower part of the figure, together with the minor allele frequencies in RS1. B. LD structure of the *TRα/REV-ERBα* locus based on 5974 subjects from RS1, calculated by Haploview 4.1. LD values (D') are shown. In case of maximum LD (i.e., D' = 100), the value is not shown. The higher the LD, the more reddish the boxes. Two blocks of high LD can be identified (i.e., 'Block 1' and 'Block 2'). A color figure is available at: http://online.liebertpub.com/doi/abs/10.1089/thy.2011.0245

Table 1. Polymorphisms in the *TRα/REV-ERBα* locus in relation to bone mineral density and fracture risk

		BMD						Fractures			
Gene	Polymorphism	Femoral Neck BMD (n = 19,195)		Lumbar Spine BMD (n = 19,195)		BMD Change (n = 2,366)		Vertebral Fractures (n = 2,994)		Osteoporotic Fractures (n = 8,131)	
		β (S.E.M.)	*P*	β (S.E.M.)	*P*	β (S.E.M.)	*P*	OR (95%CI)	*P*	HR (95%CI)	*P*
TRα	rs868150-C/T	0.01 (0.01)	0.30	0.02 (0.01)	0.09	-0.01 (0.03)	0.70	1.02 (0.86-1.21)	0.84	1.00 (0.92-1.10)	0.96
	rs7502966-T/C	0.01 (0.01)	0.60	>-0.01 (0.01)	0.83	-0.01 (0.03)	0.66	0.96 (0.81-1.13)	0.62	1.02 (0.93-1.11)	0.68
	rs1568400-A/G	-0.01 (0.01)	0.37	-0.03 (0.01)	0.02	-0.01 (0.03)	0.73	0.96 (0.80-1.15)	0.66	0.93 (0.84-1.03)	0.17
	rs939348-C/T	0.02 (0.01)	0.20	0.01 (0.01)	0.44	0.01 (0.03)	0.89	0.97 (0.81-1.17)	0.76	1.00 (0.90-1.11)	1.00
	rs2230701-C/T	0.02 (0.03)	0.63	0.04 (0.03)	0.26	-0.10 (0.06)	0.12	1.04 (0.73-1.47)	0.85	1.08 (0.89-1.30)	0.45
	rs3744805-G/A	0.01 (0.02)	0.72	0.01 (0.02)	0.78	0.02 (0.05)	0.65	1.09 (0.84-1.41)	0.52	1.00 (0.87-1.16)	0.98
REV-ERBα	rs4794826-G/A	-0.01 (0.02)	0.70	0.03 (0.02)	0.28	-0.01 (0.05)	0.87	1.00 (0.75-1.34)	1.00	0.95 (0.80-1.11)	0.49
	rs2314339-C/T	<0.01 (0.02)	0.88	-0.01 (0.02)	0.52	0.03 (0.05)	0.49	1.25 (0.98-1.59)	0.07	1.00 (0.87-1.15)	0.99
	rs2071427-G/A	<0.01 (0.01)	0.88	<0.01 (0.01)	0.92	-0.03 (0.03)	0.45	0.99 (0.82-1.20)	0.94	1.08 (0.98-1.19)	0.12
	rs2269457-A/G	0.01 (0.01)	0.58	0.01 (0.02)	0.34	-0.02 (0.04)	0.49	1.03 (0.85-1.25)	0.80	1.10 (0.99-1.22)	0.06
	rs12941497-G/A	0.01 (0.02)	0.58	0.01 (0.02)	0.46	-0.06 (0.03)	0.08	1.15 (0.96-1.38)	0.12	1.07 (0.97-1.19)	0.16
	rs939347-G/A	<0.01 (0.02)	0.77	0.02 (0.02)	0.33	-0.07 (0.04)	0.06	1.11 (0.93-1.34)	0.25	1.08 (0.97-1.19)	0.15
	rs2071570-G/T	<0.01 (0.02)	0.89	0.03 (0.02)	0.07	-0.09 (0.04)	0.01	1.02 (0.84-1.24)	0.83	1.05 (0.95-1.17)	0.34
	rs16965644-A/G	-0.02 (0.02)	0.47	-0.03 (0.02)	0.24	-0.03 (0.05)	0.57	1.04 (0.82-1.33)	0.73	0.97 (0.85-1.11)	0.63

Femoral neck and lumbar spine BMD are expressed in SD, corrected for gender, age and weight. BMD change is expressed as % loss/year. For vertebral and osteoporotic fractures the odds ratios (OR) and hazard ratios (HR) with 95% confidence intervals (95%CI) are shown. Analyses were corrected for gender, age, height and weight. Multiple testing corrected p-value thresholds were $P = 0.005$ (lumbar spine BMD, BMD change) and $P = 0.007$ (femoral neck BMD, vertebral and osteoporotic fractures).

hapmap.org) (Fig.1). The LD structure of the *TRα/REV-ERBα* locus, with 2 blocks of high LD, is shown in Fig.1.

None of the studied polymorphisms were associated with baseline characteristics, including serum TSH and FT4.

As shown in Table 1, none of the studied *TRα* or *REV-ERBα* polymorphisms were associated with BMD, BMD change, vertebral or incident osteoporotic fractures below the multiple testing corrected p-value threshold. None of the studied polymorphisms were associated with narrow-neck width, narrow-neck cortical thickness, buckling ratio or section modulus either (data not shown).

For polymorphisms with a minor allele frequency of 10%, 20% and 30%, we had power to detect differences in femoral and lumbar spine BMD of 0.06, 0.04, 0.04 SD, and 0.06, 0.05, 0.04 SD respectively. Similarly, we had power to detect differences in BMD change of 0.18, 0.13, and 0.12 SD (1 SD = 1.04% / yr). For vertebral and osteoporotic fractures, we had power to detect relative risks of 1.56, 1.42, 1.37, and 1.31, 1.23, 1.20, respectively. For the geometric outcomes, we had power to detect differences of 0.13, 0.10 and 0.09 SD.

DISCUSSION

In this study, we investigated the effects of genetic variation in the *TRα/REV-ERBα* locus on BMD (change), fracture risk and geometric parameters, thereby covering various aspects of bone (patho)physiology. Despite a very large sample size, no effects of genetic variation in the *TRα/REV-ERBα* locus on bone were identified. This was unexpected, considering the essential role of TH in bone physiology (1). *TRα*, the predominant TR in bone, is expressed in both osteoblasts and chondrocytes. Mouse models with different *TRα* mutants display, depending on the mutation, a modest or severe delay in bone development and osteosclerosis (2-5). *TRα*$^{0/0}$ mice, lacking all *TRα* transcripts, show a similar bone phenotype (14, 15). In addition, patients with thyroid hormone resistance due to mutations in *TRβ* have increased TH serum levels and an increased risk of osteoporosis, which is thought to result from overstimulation of TRα leading to increased bone turnover (1).

It has been shown that core circadian clock transcription factors (including REV-ERBα) and multiple metabolic bone homeostasis pathways display a circadian expression profile in bone (16), and that mice lacking circadian clock components display abnormal bone remodeling (17). Nevertheless, polymorphisms in the circadian clock gene *REV-ERBα*, which has been shown to influence splicing and expression of *TRα* (6, 7), were not associated with bone physiology either. Recently, genetic variation in *REV-ERBα* has been associated with bipolar disorder (18).

Strength of this study is that, due to the large sample size, we were fully powered to detect even small differences in BMD. For fractures, BMD change and geometric outcomes, we were powered to detect at least moderate effects. To our knowledge, the role of *REV-ERBα* in bone has not been studied in humans, and previous studies analyzing *TRα* in relation to BMD had limited sample sizes and analyses were restricted to a subgroup of the population (i.e., older men) (19, 20). In this study, we analysed a substantial number of polymorphisms in the *TRα/REV-ERBα* locus, thereby tagging most of the genetic variation in this locus (Fig.1). However, we cannot exclude potential (small) effects of low frequency polymorphisms, which will have a minor contribution to the variation in these parameters in the general population. Finally, it is important to note that the apparent absence of (common) functional variation in this locus does not negate its importance in bone.

Although TRα is the major TR in bone, mediating important effects of thyroid hormone on bone development and turnover, our study excludes an important contribution of common genetic variation in the *TRα/REV-ERBα* locus to variation in BMD, fracture risk and bone geometry in the elderly population.

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Chapter 11

The thyroid hormone receptor α locus and white matter lesions: a role for the clock gene *REV-ERBα*.

M. Medici, M.A. Ikram, F. van der Lijn, T. den Heijer, M. Vernooij, A. Hofman, W. Niessen, T.J. Visser, M.M. Breteler, R.P. Peeters

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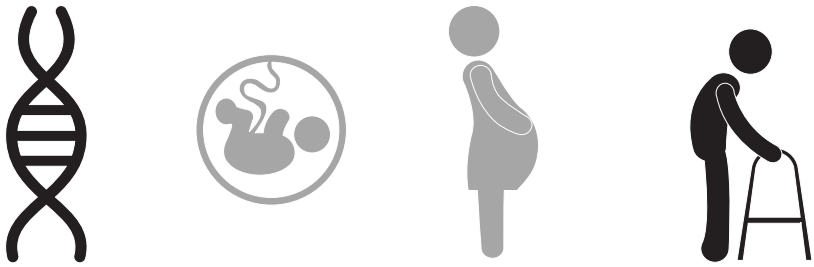

ABSTRACT

Background: Thyroid disorders are associated with an increased risk of cognitive impairment and Alzheimer's Disease. Both small vessel disease and neurodegeneration have a role in the pathogenesis of cognitive impairment and Alzheimer's Disease. Thyroid hormone receptor alpha (TRα) is the predominant TR in brain. The circadian clock gene *REV-ERBα* overlaps with the *TRα* gene and interferes with *TRα* expression. Limited data are available on the role of the *TRα/REV-ERBα* locus in small vessel disease and neurodegeneration. We therefore studied genetic variation in the *TRα/REV-ERBα* locus in relation to brain imaging data, as early markers for small vessel disease and neurodegeneration.

Methods: Fifteen polymorphisms, covering the *TRα/REV-ERBα* locus, were studied in relation to white matter lesion (WML), total brain and hippocampal volumes in the Rotterdam Study I (RS-I, n=454). Associations that remained significant after multiple testing correction were subsequently studied in an independent population for replication (RS-II (n=607)).

Results: No associations with total brain or hippocampal volumes were detected. A haplotype block in *REV-ERBα* was associated with WML volumes in RS-I. Absence of this haplotype was associated with larger WML volumes in women (0.38±0.18% (β±SE), P=0.007), but not in men (0.04±0.11%, P=0.24), which was replicated in RS-II (women: 0.15±0.05%, P=0.04; men: 0.05±0.07%, P=0.80). Meta-analysis of the two populations showed that women lacking this haplotype have a 1.9 times larger WML volume (P=0.001).

Conclusion: Our results suggest a role for REV-ERBα in the pathogenesis of WMLs.

INTRODUCTION

Thyroid hormone (TH) plays an essential role in the mature human brain. Its importance is illustrated by the effects of thyroid disorders in the elderly, including cognitive impairment and Alzheimer's Disease (1-3).

The actions of the active TH T3 (3,5,3'-triiodo-L-thyronine) are mediated through binding to nuclear TH receptors (TRs), thereby regulating gene expression. TRα is the predominant receptor in the brain (4). In mice, a knock-in mutation in *TRα* leading to a lower affinity to T3 results in, besides a bone and metabolic phenotype, memory impairment in adulthood (5).

On the opposite chromosomal strand of *TRα*, the circadian clock gene *REV-ERBα* is located. These genes partially overlap and *REV-ERBα* expression has been shown to influence splicing of *TRα* (6-8). Given that circadian rhythm abnormalities have been associated with cognitive impairment and Alzheimer's Disease (9), it is of interest to study genes involved in the circadian clock.

To date, limited data are available on the role of the *TRα/REV-ERBα* locus in the mature human brain, and in cognitive impairment and Alzheimer's Disease in particular. In recent years, it has been shown that both neurodegeneration and small vessel disease have a role in the pathogenesis of cognitive impairment and Alzheimer's Disease (10-13). Therefore, we studied genetic variation in the *TRα/REV-ERBα* locus in relation to (early) markers of small vessel disease and neurodegeneration derived from MR brain imaging data. White matter lesion (WML) volume was used as a marker for small vessel disease (14), and hippocampal and total brain volumes were used as markers for neurodegeneration (15-18).

The associations of the *TRα/REV-ERBα* locus with WML, hippocampal and total brain volumes were studied in a population-based cohort study. Associations that remained significant after multiple testing correction were tested in an independent population for replication.

MATERIALS AND METHODS

2.1 Participants

The Rotterdam Study I (RS-I) is a prospective population-based cohort study from 1990 onwards in 7,983 Caucasians aged ≥ 55 years, aimed at investigating determinants of various chronic diseases among elderly persons (19). In 1995, a structured interview, physical examination, blood drawing and brain magnetic resonance imaging (MRI) were performed in a random subset of 536 nondemented subjects of RS-I.

In 1999, RS-I was expanded (RS-II) with 3,011 subjects who had become 55 years of age or moved into the study district. In 2005, a structured interview, physical examination, blood drawing and brain MRI scans were performed in a random subset of 895 nondemented subjects of RS-II.

The medical ethical committee of the Erasmus MC, University Medical Center, Rotterdam, approved both studies and all participants gave written informed consent.

2.2 MRI measures

2.2.1 Rotterdam Study I (RS-I)

Brain scans were performed on a 1.5 T MRI System (VISION MR, Siemens AG, Erlangen, Germany). In 490 participants we obtained a proton-density, a T2-weighted, and a high-resolution inversion-recovery double contrast 3D HASTE sequence for multi-spectral volumetry (15, 20). Image preprocessing and automated measurements of WML and total brain volume have been described in detail previously (15). Hippocampal volumes were measured based on manual segmentations (15).

2.2.2 Rotterdam Study II (RS-II)

Brain scans were performed in 895 participants on a 1.5 T MRI System (General Electric Healthcare, Milwaukee, WI, USA) (21). For all participants a T1-weighted, proton-density and FLAIR sequence were acquired. Preprocessing of these images and the automated measurement of WML, total brain and hippocampal volume have been described in detail previously (21, 22).

2.3 Thyroid hormone measurements

In RS-I, blood samples were collected at the time of MRI. Serum TSH (Thyroid-Stimulating Hormone), FT4 (Free 3,5,3',5'-tetraiodo-L-thyronine) and T3 levels (n=470) were measured with chemoluminescence assays (Vitros ECI Immunodiagnostic System, Ortho-Clinical Diagnostics, Rochester, USA).

2.4 Selection and genotyping of polymorphisms

Based on linkage disequilibrium (LD) analysis (http://www.hapmap.org) and previous sequencing results (23), a tagging set of 15 polymorphisms with a minor allele frequency (MAF) above 5% was selected to cover most of the genetic variation in the *TRα/REV-ERBα* locus and the 10 kb upstream and downstream regions (Fig. 1). As no rs number has yet been assigned to A2390G, we named it by its (*TRα* 3' UTR) nucleotide substitution (23).

For RS-I and RS-II, genotypes were extracted from the Illumina HumanHap 550K (Duo) array. Genotypes for rs2230701 and A2390G were determined with Taqman Allelic

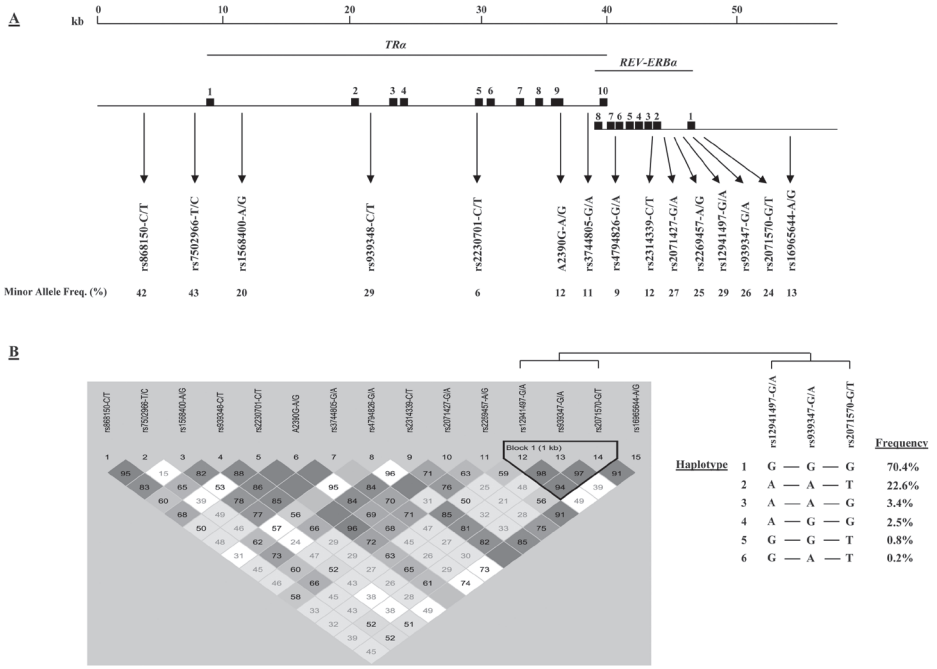

Figure 1. A. The genomic organization of the *TRα/REV-ERBα* locus is shown in the upper part of the figure. Exons are indicated by boxes. Selected polymorphisms are shown in the lower part of the figure, together with minor allele frequencies in RS-I. B. LD structure of the *TRα/REV-ERBα* locus based on 470 subjects from RS-I, calculated by Haploview 4.1. LD values (D′) are shown. In case of maximum LD (i.e., D′ = 100), the value is not shown. The higher the LD, the more reddish the boxes. Blue boxes indicate high D′ but low logarithm of odds (LOD) scores. Frequencies of the haplotypes defined by *REV-ERBα*-rs12941497, -rs939347 and –rs2071570 are shown for RS-I. A color figure is available at: http://online.liebertpub.com/doi/abs/10.1089/thy.2012.0198

Discrimination (Applied Biosystems, Nieuwerkerk a/d IJssel, the Netherlands). Genotype data were available in 454 (RS-I) and 607 (RS-II) subjects with imaging data.

Using genotype data from 470 subjects from RS-I, the LD structure of the *TRα/REV-ERBα* locus was analyzed using Haploview 4.1 (24).

2.5 Statistical analysis

Genotype and allele frequencies were tested for Hardy-Weinberg equilibrium. Linear regression was used to compare baseline characteristics between genotype groups. WML, total brain and hippocampal volumes were expressed as percentage of total intracranial volume to adjust for head size differences. WML volume was additionally natural log transformed because of skewness of the untransformed measure. The associations with WML, total brain and hippocampal volumes were tested using linear regression. All analyses were adjusted for age and gender. To minimize the risk of false-positive findings,

multiple testing correction by permutation analysis was performed, thereby taking the LD structure between these polymorphisms into account. Results were obtained after 10,000 permutations, using PLINKv1.07 (25). As REV-ERBα is a circadian clock gene and gender-related differences in circadian rhythm regulation have long been recognized (26-29), we investigated the gender-specific effects of REV-ERBα polymorphisms that remained significant after multiple testing correction at $P = 0.05$.

Associations that remained significant after multiple testing correction at $P = 0.05$ in RS-I, were tested in RS-II for replication. Meta-analyses were conducted using the METAL software package applying inverse-variance weighted fixed-effects methodology (http://www.sph.umich.edu/csg/abecasis/Metal). SPSS 15.0 for Windows (SPSS, Chicago, IL, USA) was used for all analyses, unless stated otherwise. Haplotypes were determined by indirect haplotyping using PHASE (30).

Power calculations for detectable effect sizes in RS-I and RS-II combined, and in RS-I alone were performed at $\beta = 0.80$ and α-values corresponding to the multiple testing corrected p-value thresholds. In RS-I and RS-II, we had power to detect differences in WML, total brain and hippocampal volumes of 0.27, 0.20 and 0.17 SD, for polymorphisms with a minor allele frequency of 10 %, 20 %, and 30 % respectively. Similarly, in RS-I alone, we had power to detect differences of 0.41, 0.31 and 0.27 SD. 1 SD WML volume equals 1.53 % and 0.66 % in RS-I and RS-II, respectively. 1 SD hippocampal volume equals 0.10 % and 0.05 % in RS-I and RS-II, respectively. Similarly, 1 SD total brain volume equals 3.66 % and 3.41 %.

RESULTS

Allele and genotype frequencies of all polymorphisms were in Hardy-Weinberg equilibrium with similar frequencies as reported in literature (23) and established databases, such as HapMap (http://www.hapmap.org) and dbSNP (http://www.ncbi.nlm.nih.gov/projects/SNP/) (Fig. 1). The LD structure of the *TRα/REV-ERBα* locus is shown in Fig. 1.

Both RS-I and RS-II consisted of 51% women. Mean ages were 73.4±7.9 (mean±SD) and 67.5±5.5 years, respectively. None of the studied polymorphisms were associated with baseline characteristics, including serum TSH, FT4 and T3 levels.

In RS-I, *REV-ERBα*-rs939347-A was associated with larger WML volumes ($\beta = 0.26\pm0.10$ % (mean±SE), $P = 0.002$), which remained significant after multiple-testing correction ($P = 0.021$) (Table 1). As this polymorphism is located in a region of high LD, haplotypes defined by *REV-ERBα*-rs12941497, -rs939347 and –rs2071570 were created ('Block 1' in Fig. 1). Absence of haplotype 1 was associated with larger WML volumes ($\beta = 0.20\pm0.10$ %, $P = 0.007$). We additionally investigated the gender-specific effects of haplotype 1 on WML volumes. This effect was largely driven by women (women: $\beta = 0.38\pm0.18$ %, $P = 0.007$; men: $\beta = 0.04\pm0.11$ %, $P = 0.24$) (Fig. 2). This effect was replicated in RS-II, which

also showed a significant association with larger WML volumes in women ($\beta = 0.15 \pm 0.05$ %, $P = 0.041$), but not in men ($\beta = 0.05 \pm 0.07$ %, $P = 0.795$) (Fig. 2). Meta-analysis of the two populations resulted in $\beta = 0.17 \pm 0.05$ % ($P = 0.001$) in women, and in $\beta = 0.05 \pm 0.06$ % ($P = 0.42$) in men. None of the other studied polymorphisms were associated with WML (Table 1) or total brain volumes (data not shown).

Table 1. Effects of polymorphisms in *TRα/REV-ERBα* on white matter lesion volumes in men and women from RS-I

Gene	Polymorphism	β (mean (SE))*	*p (uncorrected)*	*p (corrected)***
TRα	rs868150-C/T	-0.07 (0.10)	0.241	0.932
	rs7502966-T/C	0.13 (0.10)	0.080	0.560
	rs1568400-A/G	0.04 (0.11)	0.645	0.999
	rs939348-C/T	0.07 (0.09)	0.669	0.999
	rs2230701-C/T	-0.11 (0.18)	0.886	0.999
	A2390G-A/G	0.28 (0.13)	0.677	0.996
	rs3744805-G/A	0.32 (0.14)	0.208	0.897
REV-ERBα	rs4794826-G/A	0.23 (0.16)	0.338	0.982
	rs2314339-C/T	0.32 (0.13)	0.124	0.727
	rs2071427-G/A	0.10 (0.11)	0.527	0.999
	rs2269457-A/G	0.06 (0.11)	0.135	0.757
	rs12941497-G/A	0.22 (0.10)	0.006	0.069
	rs939347-G/A	0.26 (0.10)	0.002	0.021
	rs2071570-G/T	0.21 (0.11)	0.005	0.059
	rs16965644-A/G	-0.10 (0.14)	0.087	0.591

* Expressed as white matter lesion volume percentage. Volume is expressed as percentage of intracranial volume to adjust for head size differences. Effects are adjusted for age and gender.
** Obtained after multiple testing correction by permutation analysis (10,000 permutations).

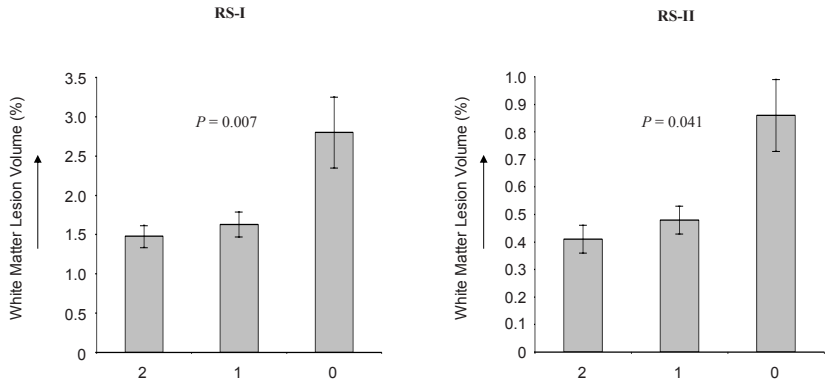

Figure 2. White matter lesion volumes in women by number of *REV-ERBα* haplotype 1 copies
White matter lesion volumes by number of *REV-ERBα* haplotype 1 copies in 218 women from RS-I and 293 women from RS-II. Volume is expressed as percentage of intracranial volume to adjust for head size differences. Meta-analysis of the two populations resulted in $\beta = 0.17 \pm 0.05$ % ($P = 0.001$).

In RS-I, *TRα*-A2390G-G was associated with smaller hippocampal volumes (β = -0.03±0.01 %, P = 0.002), which remained significant after multiple-testing correction (P = 0.027). However, this effect could not be replicated in RS-II (β = 0.01±0.01 %, P = 0.28). Meta-analysis of the two populations resulted in β = -0.02±0.05 % (P = 0.63). None of the other studied polymorphisms were associated with hippocampal volumes (data not shown).

DISCUSSION

In the present study, we investigated the effects of genetic variation in the *TRα/REV-ERBα* locus on WML, total brain and hippocampal volumes. A haplotype block covering exon 1 of the *REV-ERBα* gene was associated with larger WML volumes. REV-ERBα is a nuclear hormone receptor with a key role in the regulation of the circadian rhythm, which is generated by feedback loops of gene expression (31). In this system, REV-ERBα acts as a constitutive repressive transcription factor, as it has an atypical ligand-binding domain lacking the carboxy-terminal activation function-2, required for recognition of co-activators (32).

WMLs, presumed to result from cerebral small vessel disease, range from reduced myelination and edema to gliosis and complete axonal destruction (14). WMLs are associated with a substantial increased risk of cognitive decline, dementia, stroke and death (33). In the present study, the association of the *REV-ERBα* haplotype was exclusively driven by its association in women. We show that women lacking *REV-ERBα* haplotype 1 have a 1.9 times larger WML volume compared to women with 1 or 2 copies of this haplotype (Fig. 2).

Gender differences in circadian rhythm regulation have long been recognized (26-29). Barger et al. found differences in the circadian timing system of body temperature, heart rate, physical activity, and feeding between male and female rhesus monkeys (26). In humans, others have shown gender differences in the circadian rhythms of body temperature and sleep regulation as well (27). Also at the level of the individual clock components, a number of studies have shown gender differences in circadian rhythm regulation. For example, the type of depression in relation to variants in the clock gene *TIMELESS* is dependent on gender (34). Recently, Hadden et al. studied the effects of circadian disruption on mouse lung mechanics, and demonstrated that the effects on the lungs, as well as the changes in *REV-ERBα* expression patterns, were different between men and women (35).

Taken together, various studies have shown that the regulation of circadian clock genes, as well as the effects of dysregulation of those genes, including *REV-ERBα*, can differ between genders. However, no studies are available on the gender-specific effects

of REV-ERBα on the pathogenesis of white matter lesions. The exact mechanism behind the gender-specific effects of REV-ERBα on white matter lesions therefore needs to be clarified in future studies.

The associated haplotype block in *REV-ERBα* covers exon 1 and the promoter region of the gene, and may therefore influence splicing or the transcriptional level of *REV-ERBα*. In addition to a direct effect of the *REV-ERBα* haplotype, the effects of this haplotype on WML volumes may also be mediated via *TRα*. As can be expected from the genomic organization of the *TRα/REV-ERBα* locus (see Fig. 1), *REV-ERBα* transcription also influences splicing of TRα (6-8). There are two major TRα isoforms, the T3-binding TRα1 and the non-T3-binding TRα2, which has an antagonistic function. Basepairing with REV-ERBα mRNA blocks splicing of TRα2 mRNA, thereby favoring formation of TRα1 mRNA. In this way, *REV-ERBα* expression influences the TRα1/ TRα2 ratio, thereby regulating local T3 action (6-8).

Recently, the first three patients with a mutation in *TRα* have been described (36, 37). Patients suffered from growth retardation, as well as from motor and cognitive dysfunction. However, no brain imaging data were available in these patients.

Little is known about the exact role of circadian clock components in the pathogenesis of WMLs. Our results suggest a role for the circadian system, and for REV-ERBα in particular, in the pathogenesis of WMLs, the exact molecular mechanism of which needs to be clarified in future studies. In this context it is interesting to note that circadian rhythm disturbances are frequently observed in patients with Alzheimer's Disease, and even in non-demented patients with the earliest signs of Alzheimer's neuropathology (9).

Genetic variation in *TRα* has previously been studied in relation to Alzheimer's disease, which did not reveal significant associations (38). This is in line with the results of the present study, which do not show an association of genetic variation in *TRα* with early markers of neurodegeneration or small vessel disease.

Strengths of our study include the high coverage of genetic variation in the studied locus. In addition, due to the relatively large sample size, we were powered to detect at least moderate differences in WML, total brain and hippocampal volumes. However, we cannot exclude other potential (small) effects of low frequency polymorphisms.

A point for concern in genetic association studies is the risk of false-positive findings. To minimize this risk, we applied both a multiple testing correction and replicated significant results in an independent population. Furthermore, the relation between the *REV-ERBα* haplotype 1 and WML volume was similar in RS-I and RS-II: absence of both haplotype 1 copies was associated with higher WML volumes, whereas carriage of only one haplotype 1 copy was not (Fig. 2). It is therefore highly unlikely that these observed effects are false-positive findings.

In conclusion, we have shown that genetic variation in the circadian clock component *REV-ERBα* is associated with WML volumes in women. Future studies are needed

to clarify the exact role of the *TRα/REV-ERBα* locus, and the circadian rhythm system in general, in the pathogenesis of WMLs. Given the close relation between *TRα* and *REV-ERBα*, these studies should identify the independent contributions of *REV-ERBα* and *TRα* to the observed effects on WMLs.

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Chapter 12

Thyroid function within the normal range and the risk of depression: a population-based cohort study.

M. Medici, N. Direk, W.E. Visser, T.I.M. Korevaar, A. Hofman, T.J. Visser, H. Tiemeier, R.P. Peeters

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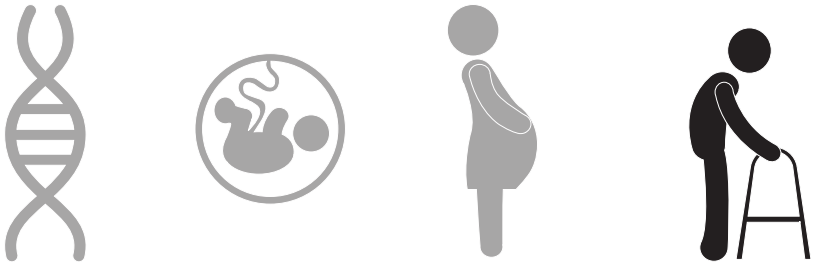

ABSTRACT

Context: Overt hypo- and hyperthyroidism are associated with an increased risk of depression. Little is known about the effects of variation in thyroid function within the normal range on the risk of depression.

Objective: To examine the association between normal range thyroid function and the risk of depression.

Design, Setting and Participants: Cohort study in 1503 Dutch men and women, aged 70.6 (7.3) years. At baseline, serum TSH, TPO-antibody levels, and depressive symptoms (Center for Epidemiologic Studies Depression Scale (CES-D)) were assessed. A CES-D ≥16 is indicative of a depressive disorder. During follow-up (mean 8.0 years), participants were continuously monitored for the occurrence of incident depressive syndromes (n=156).

Results: Cross-sectionally, persons in the lowest TSH tertile (0.3-1.0 mU/L) had more depressive symptoms (CES-D score (mean): 7.95 vs 6.63, *P*=0.014), as well as an increased risk of a CES-D ≥16 (10.7 vs 5.0 %, OR (95% CI) = 2.22 (1.18-4.17)), compared to persons in the highest normal range TSH tertile (1.6-4.0 mU/L). In the prospective analyses, persons in the lowest TSH tertile who were depression-free at baseline had a higher risk of incident depressive syndromes (12.3 vs 7.6 %, OR (95% CI) = 1.85 (1.10-3.11)).
Thyroid autoimmunity (TPOAb-positivity) was not associated with CES-D scores or incident depressive syndromes.

Conclusions: Elderly persons with low-normal TSH levels have more concurrent depressive symptoms, as well as a substantially increased risk of developing a depressive syndrome in the subsequent years. This study identifies low-normal TSH as an important risk factor for depression in the elderly.

INTRODUCTION

Thyroid abnormalities are associated with the occurrence of psychiatric diseases, including depression (1, 2). Classically, hypothyroidism is associated with an increased risk of depression. However, a number of studies have also shown an increased risk of depression in patients with hyperthyroidism (1, 3-5). In this context it is remarkable to note that only limited and mainly cross-sectional data are available on the effects of variation in thyroid function within the normal range on the risk of depression.

Thyroperoxidase antibodies (TPOAb) are antibodies against thyroperoxidase, which plays a key role in thyroid hormone (TH) synthesis. TPOAb-positive persons have an increased risk of developing hypothyroidism. Various autoimmune diseases have been associated with an increased risk of depression (6, 7). Although TPOAb-positivity is a common finding in the general population, the relation between depression and TPOAb-positivity has only been studied in a limited number of studies with conflicting results (8-12).

Although severe thyroid dysfunction has been shown to influence the risk of depression, depression itself may also have an effect on thyroid parameters (1, 2). Decreased food intake and chronic illness can cause important changes in thyroid function tests, known as the non-thyroidal illness syndrome (13, 14). It is therefore of importance to study the relation between thyroid function and depression not only cross-sectionally but also in a prospective study design.

For these reasons, we studied the effects of variation in thyroid function within the normal range on depression, both cross-sectionally and prospectively, in a population-based cohort study. In addition, the relation between thyroid autoimmunity and depression was studied.

MATERIALS AND METHODS

Study Population

The Rotterdam Study is a prospective population-based cohort study in 7,983 Caucasians aged ≥ 55 years from Rotterdam, the Netherlands. Depressive symptoms and syndromes were assessed from the second examination round (September 1993 - December 1995) onward, which constituted the baseline of the present study (15).

The Medical Ethics Committee of the Erasmus Medical Center, Rotterdam, approved the study, and written informed consent was obtained from all adult participants.

Population for Analysis

For the thyroid-stimulating hormone (TSH) analyses, data on CES-D scores and incident depressive syndromes were complete for 1093 and 1369 persons with available TSH data. TPOAb-positives, those on thyroid therapy, or with abnormal TSH levels (see statistical analyses section) were excluded. Persons with dementia were also excluded, as depression is difficult to assess in demented persons, and thyroid dysfunction has been associated with dementia (16). In the incident depressive syndrome analyses, only persons who were depression-free at baseline were included. Thus, in total, 943 and 1110 persons were included in the cross-sectional and prospective analyses, respectively.

For the TPOAb analyses, data on CES-D scores and incident depressive syndromes were complete for 1273 and 1503 dementia-free persons, respectively.

Assessment of Thyroid Function

In 2009, serum TSH (TSH Lumitest; Henning, Berlin, Germany) and TPOAb (ELISA; Milenia, DPC, Los Angeles, USA) levels were determined in a random subset of the baseline serum samples. TSH and TPOAb levels were available in 1110 and 1503 persons, respectively. TPOAb levels > 10 IU/mL were regarded as positive.

In an examination round after baseline of the current study (4.27 (0.44) yrs), serum TSH (TSH Lumitest; Henning, Berlin, Germany) and FT4 (Vitros, ECI Immunodiagnostic System, Ortho-Clinical Diagnostics, Amersham, UK) levels were determined in 1071 samples.

As shown in Table 1, the use of thyroid medication was almost 10-fold higher in women compared to men, whereas the prevalence of TPOAb-positivity was only 2.7 times higher in women. This may (in part) be explained by the fact that not only Hashimoto's thyroiditis, but also other thyroid diseases are more prevalent among women. These include for example Graves' disease and thyroid cancer, which are respectively 7 and 3 times more common in women than in men (17, 18). We did not have specific data on these diseases, but it is unlikely that this has affected our analyses, as we restricted our TSH analyses to TPOAb-negative persons with normal-range TSH levels, and excluded all persons using thyroid medication.

Assessment of Depression

At baseline, assessment of depressive symptoms was performed using the validated Dutch version of the Center for Epidemiologic Studies Depression Scale (CES-D) (15). The CES-D is a 20-item self-report measure of depressive symptoms experienced in the last week. Items are scored on a scale of 0 to 3 points. A score of ≥16 is considered indicative of a depressive disorder (15, 19).

During follow-up, from baseline until October 2005 (mean: 8.0 yrs), depressive episodes were identified using different methods, as has been described in detail

Table 1. Population characteristics

	Total (n = 1503)	Men (n = 576)	Women (n = 927)	*P*
Age (y, mean (SD))	70.6 (7.3)	70.2 (7.1)	70.8 (7.4)	0.133
BMI (kg/m^2, mean (SD))	26.5 (3.6)	26.0 (2.9)	26.7 (4.0)	< 0.001
Smoking status (%)				
Current	19.9%	23.6%	17.5%	0.004
Past	44.0%	65.8%	30.1%	< 0.001
Never	36.1%	10.6%	52.4%	< 0.001
Dementia (%)	0.9%	1.2%	0.7%	0.23
TSH (mU/L, median (IQR))	1.30 (0.90-2.00)	1.30 (0.90-1.90)	1.40 (0.90-2.00)	0.032
TPOAb-positives (%)	5.1%	2.5%	6.8%	< 0.001
Thyroid therapy (%)	3.3%	0.5%	4.9%	< 0.001
CES-D score (mean (SD))	7.63 (6.98)	6.52 (6.21)	8.42 (7.38)	< 0.001
Incident depressive syndromes (%)	9.2%	6.6%	10.9%	0.005

Abbreviations: BMI, body mass index (calculated as weight in kilograms divided by height in meters squared); IQR, interquartile range; TSH, Thyroid-Stimulating Hormone; TPOAb, Thyroid Peroxidase Antibody; CES-D, Center for Epidemiologic Studies Depression Scale.
TPOAb-positives were defined as subjects with TPOAb >10 IU/mL.

previously (15). During the 2 follow-up examination rounds (March 1997 - December 1999 and January 2002 - July 2004), participants were screened with the CES-D, and screen-positive participants were invited for a clinical psychiatric interview to diagnose depression. A psychiatrist, psychogeriatrician, or clinical psychologist, each with extensive clinical experience, conducted the interview using the Dutch version of the Present State Examination (15). This is a semi-structured psychiatric interview included in the Schedules for Clinical Assessment in Neuropsychiatry. Scoring of items is conservative and relies on clinical judgment instead of the participant's answer only. Each interviewer was trained in the certified Dutch World Health Organization center. With a computerized diagnostic algorithm based on the item scores, major and minor depressive disorders and dysthymia were classified according to *DSM-IV* (Diagnostic and Statistical Manual of Mental Disorders version 4) criteria. Additionally, a medical history was taken to assess if depressive episodes had occurred between follow-up rounds. From baseline onward, trained research assistants systematically scrutinized all information contained in the medical records of the general practitioners (GPs), for instance, hospital discharge letters, specialist reports, and notes of the GP, for a number of predefined cues such as symptoms of depression, prescriptions of psychiatric medication, the occurrence of major life events, and psychosocial problems. Next, 2 physicians and a research psychologist independently read all information and categorized each depression accord-

ing to a predefined protocol. All discordant categorizations were discussed in consensus meetings (15).

Using the above mentioned methods, we recorded depression that fulfilled *DSM-IV* criteria, as well as depressive episodes that were clinically relevant but did not fulfill *DSM* criteria. The GPs frequently diagnosed depression without using or documenting the formal *DSM* criteria. Depressive syndromes therefore included *DSM-IV* major depressive disorder and dysthymia, as well as depression recorded by a GP or a physician, self-reported depression for which the participant consulted a GP or mental health professional, and *DSM-IV* minor depression. Grief, adjustment disorder, and burnout, characterized by emotional exhaustion and reduced satisfaction in personal accomplishment, were not regarded as depression.

Covariates

Information on smoking status and thyroid therapy were obtained by questionnaires at baseline and during follow-up examination rounds. Smoking was categorized as never, past and current. Height and weight were measured to calculate body mass index (BMI, kg/m^2). The presence of dementia was assessed at baseline and during follow-up, as has been described in detail previously (20).

Statistical Analyses

The TSH reference range was 0.3 - 4.0 mU/L, defined as the range between the 2.5^{th} and 97.5^{th} percentiles after exclusion of subjects with thyroid therapy or TPOAb-positivity. The group with normal-range TSH levels was divided in tertiles. Due to the skewed distribution of TSH and high clustering of TSH levels around 1.0 mU/L, it was not possible to make 3 equal-sized groups. At baseline, the cross-sectional relations between TSH tertiles, continuous CES-D scores, and the risk of a CES-D score ≥16 were studied using AN(C)OVA and logistic regression analyses, respectively. In the prospective analysis, logistic regression was used to study the relation between TSH tertiles and the incidence of depressive syndromes.

Similarly, TPOAb-status was studied in relation to CES-D using AN(C)OVA. Logistic regression was used to study the relation between TPOAb-status, the risk of a CES-D score ≥16, and the incidence of depressive syndromes.

Analyses were additionally corrected for gender, age, BMI and smoking status. All analyses were repeated in men and women separately and gender*TSH tertile interaction terms were calculated to investigate the presence of gender-specific effects.

IBM SPSS Statistics for Windows, Version 20.0 (IBM Corp, Armonk, New York, USA) was used for all analyses.

Power calculations

Power calculations for detectable effect sizes were performed at $\beta = 0.80$ and $\alpha = 0.05$. For the TSH tertiles analyses, we had power to detect differences in CES-D of 0.15 SD (1 SD = 6.98), and to detect ORs of 1.79 and 1.72 for CES-D scores ≥16 and depressive syndromes, respectively. For the TPOAb-positivity analyses, we had power to detect differences in CES-D of 0.26 SD (1 SD = 6.98), and to detect ORs of 2.37 and 2.29 for CES-D scores ≥16 and depressive syndromes, respectively.

RESULTS

Characteristics of the studied population are shown in Table 1. Compared to men, women had a higher BMI, smoked less, had a higher median TSH level, as well as more TPOAb-positivity and thyroid therapy. In addition, women had a higher mean CES-D score, as well as a higher incidence of depressive syndromes. All further analyses were performed in non-demented persons who were not on thyroid therapy.

Normal Range TSH Levels and Depression

Table 2 shows the cross-sectional relations between normal range TSH tertiles, continuous CES-D scores and the risk of a CES-D score ≥16, as well as the longitudinal relation between normal range TSH tertiles and the incidence of depressive syndromes. At baseline, persons with lower normal range TSH levels had higher CES-D scores, also after correction for gender, age, BMI and smoking status. Persons in the lowest normal range TSH tertile had a higher risk of a CES-D score ≥16 (OR = 2.09 (95% CI 1.16-3.76), $P = 0.015$), compared to persons in the highest tertile. These effects remained similar after correction for gender, age, BMI and smoking status (OR = 2.22 (1.18-4.17), $P = 0.013$).

In the prospective analyses of persons free of depression at baseline, those in the lowest normal range TSH tertile had a higher incidence of depressive syndromes during follow-up (OR = 1.75 (1.06-2.88), $P = 0.029$). This association remained significant after correction for gender, age, BMI and smoking (OR = 1.85 (1.10-3.11), $P = 0.020$).

No gender-specific effects were observed in the TSH tertile vs CES-D score ≥16, and incident depressive syndrome analyses, with gender*TSH tertile interaction term *P*-values of 0.28 and 0.82, respectively. The association between TSH tertiles and CES-D scores was mainly driven by women (gender*TSH tertile interaction term $P = 0.012$): CES-D scores in the low, middle and high TSH tertiles were 6.36 (0.44) (mean (SE)), 6.11 (0.51), and 6.58 (0.51) in men ($P = 0.75$) and 9.49 (0.52), 8.22 (0.60), and 7.00 (0.55) in women ($P = 0.002$).

Table 2. Normal range TSH levels, CES-D scores and the risk of Incident Depressive Disorders

		TSH Tertiles				
		Tertile 1 (0.30-1.00 mU/L)	**Tertile 2 (1.01-1.60 mU/L)**	**Tertile 3 (1.61-4.00 mU/L)**	**OR (95% CI)**	***P***
Cross-sectional analyses						
CES-D score (continuous)	**N**	365	273	305		
	Model 1 * (mean (SE))	8.03 (0.35)	7.24 (0.41)	6.83 (0.39)	-	0.025
	Model 2 † (mean (SE))	7.95 (0.36)	7.37 (0.42)	6.63 (0.39)	-	0.014
CES-D ≥16	**N**	365	273	305		
	Model 1 * (% (SE))	11.0 (1.4)	6.7 (1.6)	5.6 (1.5)	2.09 (1.16 – 3.76)	0.015
	Model 2 † (% (SE))	10.7 (1.5)	7.6 (1.7)	5.0 (1.6)	2.22 (1.18 – 4.17)	0.013
Prospective analyses						
Incident Depressive Syndromes	**N**	416	327	367		
	Model 1* (% (SE))	12.0 (1.4)	8.3 (1.6)	7.6 (1.6)	1.75 (1.06 – 2.88)	0.029
	Model 2 † (% (SE))	12.3 (1.5)	8.9 (1.7)	7.6 (1.6)	1.85 (1.10 – 3.11)	0.020

Analyses were performed in TPOAb-negative non-demented persons who were not on thyroid therapy. Abbreviations: TSH, Thyroid-Stimulating Hormone; CES-D, Center for Epidemiologic Studies Depression Scale.

Normal range TSH tertiles vs CES-D scores, the risk of a CES-D score ≥16, and the risk of incident depressive syndromes (in baseline depression-free subjects). Subjects receiving thyroid therapy, TPOAb-positives, subjects with abnormal TSH levels, and dementia cases were excluded. The OR comparing low-normal with high-normal TSH levels is indicated.

* Model 1 No adjustments.

† Model 2 Adjusted for gender, age, BMI and smoking status.

In the examination round after baseline of the current study, TSH and FT4 levels were negatively correlated (r = -0.19, $P = 1.5 \times 10^{-8}$; $\beta = 0.06$ (0.01) mU/pmol, $P < 0.001$).

Thyroid Autoimmunity (TPOAb-positivity) and Depression

As shown in Table 3, TPOAb-status did not show any associations with continuous CES-D scores, and the risk of a CES-D score ≥16. Neither were there associations with the risk of incident depressive syndromes. No gender-specific effects were observed (data not shown).

Table 3. TPOAb-status, CES-D scores and the risk of Incident Depressive Disorders

		TPOAb-negatives	TPOAb-positives	OR (95% CI)	*P*
Cross-sectional analyses					
CES-D score (continuous)	**N**	1201	72		
	Model 1 * (mean (SE))	7.55 (0.20)	8.10 (0.82)	-	0.51
	Model 2 † (mean (SE))	7.52 (0.20)	7.52 (0.82)	-	0.99
CES-D ≥16	**N**	1201	72		
	Model 1 * (% (SE))	8.6 (0.8)	12.5 (3.3)	1.52 (0.74 – 3.15)	0.26
	Model 2 † (% (SE))	8.7 (0.8)	11.4 (3.4)	1.31 (0.63 – 2.75)	0.47
Prospective analyses					
Incident Depressive Syndromes	**N**	1427	76		
	Model 1 * (% (SE))	9.2 (0.8)	9.2 (3.3)	1.12 (0.49 – 2.56)	0.79
	Model 2 † (% (SE))	9.4 (0.8)	8.5 (3.4)	1.04 (0.45 – 2.44)	0.92

Analyses were performed in non-demented persons who were not on thyroid therapy.
Abbreviations: TPOAb, Thyroid Peroxidase Antibody; CES-D, Center for Epidemiologic Studies Depression Scale.
TPOAb-status vs CES-D score, the risk of a CES-D score ≥16, and the risk of incident depressive syndromes (in baseline depression-free subjects). Dementia cases were excluded. TPOAb-positives were defined as TPOAb > 10 IU/mL.
* Model 1 No adjustments.
† Model 2 Adjusted for gender, age, BMI and smoking status.

DISCUSSION

In the current study, we investigated the effects of variation in thyroid function within the normal range on the risk of depression, as well as the relation between thyroid autoimmunity and depression. Various studies have shown an increased risk of depression in both hypo- and hyperthyroidism, but little is known about the effects of normal range thyroid function on depression (1-5). This is the first individual study to demonstrate a relation between low-normal TSH levels and depression, and this relation was demonstrated both cross-sectionally and longitudinally.

The only other study that has previously reported a relation between high-normal thyroid function and depression concerned a meta-analysis of 6 studies (21), but the included studies in this meta-analysis differed substantially in age ranges (e.g., 17-39 vs 85-89 yrs), type and sensitivity of depression scale and assessment methods used, and no data on thyroid autoimmunity were available (21-26). Furthermore, only two of the included studies investigated the associations between thyroid status and the risk of depression prospectively (21, 24), whereas the other studies were cross-sectional

(22, 23, 25, 26). These prospective studies were limited by the fact that they were either restricted to men (21) or included a smaller sample size with a shorter follow-up period (599 participants with follow-up of 3.7 years) (24). In the current study, we show an increased risk of depressive syndromes in persons with low-normal TSH levels by intensively monitoring a large group of elderly persons for eight years for the occurrence of depressive episodes, additionally taking the effects of potentially interfering factors such as TPOAbs, age, gender, dementia and smoking into account. In addition, persons with low-normal TSH levels had more concurrent depressive symptoms, and were more likely to have a CES-D score ≥16, which is considered indicative of a depressive disorder (15, 19). These results show that even minor variation in thyroid function within the normal range can have important effects on affective behaviour.

Based on the fact that both hypo- and hyperthyroidism have been associated with an increased risk of depression, one could expect a U-shaped relation between thyroid function within the normal range and depression. However, such a relation was not observed, as only low-normal TSH levels were found to be associated with an increased risk of depression, both cross-sectionally and longitudinally. A number of conditions could underlie the observed low-normal TSH levels. Illness in general can lead to changes in thyroid parameters via a wide range of mechanisms (13, 14). This condition is called the non-thyroidal illness syndrome (NTIS), and is characterized by low serum T3 levels. In addition, this condition can lead to a decrease in TSH and FT4 levels (13, 14). Therefore, the low-normal TSH levels in the current study could be a reflection of NTIS. Serum FT4 levels were not determined at baseline, but both TSH and FT4 levels were available 4.3 yrs after baseline. The fact that we observed an inverse correlation between TSH and FT4 at this timepoint, makes NTIS a less likely explanation for the observed low-normal TSH levels, and could point towards mild autonomous thyroid function, which is common in the elderly (27). In addition, in the previously discussed meta-analysis by Williams et al., higher normal range FT4 levels were found to be positively associated with depression (21). Taken together, our data suggest a high-normal thyroid function in these subjects, and in this context it is interesting to note that TH regulates neuronal cell survival, and interferes with serotonergic neurotransmission, which play a key role in affective behaviour (28, 29). However, the exact biological mechanism behind the association between a higher thyroid function, both outside and within the normal range, and depression remains to be clarified in future studies.

Various studies have found a higher prevalence of depression in women, which is in line with the results from the current study (30). Gender-related differences have been observed in various aspects of the pathophysiology of depression (31), and we therefore investigated the gender-specific effects of TSH levels and TPOAb status on depression. The association between a low-normal TSH and more depressive symptoms was found to be driven by women. However, these gender-specific differences were only observed

for mean CES-D scores, and not for the risk of CES-D scores ≥16 or incident depressive syndromes, which therefore seems to be of less clinical relevance.

Thyroid autoimmunity (TPOAb-positivity) is a common finding in the general population, especially in the elderly, with a prevalence in the current population of 5.1%. Autoimmune diseases, such as systemic lupus erythematosus and Sjogren's syndrome, have been associated with an increased risk of depression (7, 32), but little is known about the effects of TPOAb-positivity on the risk of depression. A few cross-sectional studies have investigated the relation between TPOAb-positivity and depression, with conflicting results (8-12). The largest of these studies did not find an association between TPOAb-positivity and depression in men and women (9). This is in line with the results from the current study, in which we did not find an association of TPOAb-positivity with concurrent depressive symptoms. We did not find an association with the risk of incident depressive syndromes either.

Our study has some limitations. As mentioned, no serum FT4 levels were available at baseline. Both serum TSH and FT4 levels were available 4.3 yrs after baseline, and their inverse correlation helped us to exclude NTIS as an important explanation for the observed low-normal TSH levels. However, we cannot exclude the possibility that there were persons with subclinical forms of pituitary insufficiency present in our analyses. Also, although we excluded persons using thyroid medication and there were no persons using amiodarone, we cannot exclude the possibility that our analyses still included subclinical persons using other thyroid interfering medication, such as glucocorticoids, as we did not have complete data on other medication use in our cohort.

Finally, we have not studied if an increase of the low-normal TSH levels with antithyroid drugs reduces the risk of depression. This should be clarified in future studies.

The fact that the current study identifies low-normal TSH levels as an important risk factor for developing a depressive syndrome will likely be of clinical importance for various reasons. The treatment of depression with conventional therapies such as antidepressants and psychotherapy is suboptimal, as 70% of treated depressed patients have residual symptoms, and 20% is treatment-resistant (33, 34). To exclude underlying thyroid disease, clinical guidelines advise to measure serum TSH in persons with a new-onset depressive disorder (35). When TSH levels fall within the reference ranges, a thyroidal origin of the depression is excluded, and conventional depression treatment is started. Our results show that a low-normal TSH level results in an increased risk of depression, and it is tempting to speculate if these persons may benefit from additional treatment with antithyroid drugs.

Furthermore, thyroid disorders are common in the general population and the target of treatment of both hypo- and hyperthyroidism is to maintain TSH within the normal range (36). However, it has been shown that patients on thyroxine therapy with a TSH within the normal range have a significant impairment in psychological well-being

compared to controls of similar age and sex (37). As the TSH reference range is wide (generally around 0.4 – 4.0 mU/L), it is remarkable to note that little data are available on the benefits of targeting treatment on low-normal vs high-normal TSH levels, especially with respect to the risk of affective complaints. Walsh et al. performed a double-blind randomized cross-over trial in thyroxine treated hypothyroid patients to investigate the effects of adjustments in thyroxine dose (38). No differences in well-being and quality of life were found between patients with low-normal and high-normal TSH levels. However, this trial included only a limited number of patients (i.e., 56 patients), no depression-specific questionnaires were used, and treatment and follow-up periods were short (i.e., 8 wks). Saravanan et al. studied the relation between TH parameters and well-being in 697 patients on thyroxine therapy (39). A positive correlation between serum TSH levels and continuous depression scores, as measured by the Hospital Anxiety and Depression Scale (HADS), was found. However, this relation was not seen when HADS depression score was used as a categorical variable, and no data on the incidence of depressive episodes were available. In a large study of more than 1000 women with thyroid disease who were taking thyroxine, Panicker et al. found that higher TSH levels were associated with more depression and anxiety (40). However, these results were only based on cross-sectional (and not prospective) analyses, and the study was unfortunately not powered to investigate these relations in men. As this is the first individual study to demonstrate a relation between low-normal TSH levels and depression in men and women, results should be first replicated in an independent study. If replicated, large randomized controlled trials should investigate the psychological well-being and risk of depressive syndromes when targeting patients on low-normal vs high-normal TSH levels.

In conclusion, this study identifies low-normal TSH as an important risk factor for depression in the elderly, which is independent of thyroid autoimmunity.

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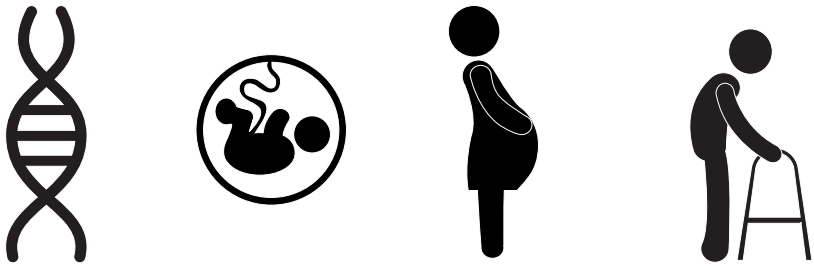
General discussion



GENERAL DISCUSSION

The studies described in this thesis consist of three major parts. In the first part, we studied determinants and effects of thyroid function during pregnancy. We calculated population-based TSH and FT4 reference ranges in the Generation R Study, and showed that a substantial part of the women with normal range TSH levels had a TSH level above the fixed cut-offs recommended by international guidelines. We also found that even minor variation in thyroid function within the normal range can have substantial effects on the risk of maternal and child complications, including the risk of preeclampsia and low-birth-weight newborns. The results of these studies are put into perspective in Chapter 13, in which we review the available literature on thyroid function during pregnancy, as well as the effects of thyroid dysfunction during pregnancy on maternal and child complications.

In the second part of this thesis, we searched for new genetic determinants of thyroid function and autoimmunity. In our GWAS we identified new loci associated with serum TSH, FT4 and/or TPOAbs, part of which was also associated with clinical thyroid disease. A few of these variants were tested in relation to thyroid function during pregnancy. The loci that have been detected in these and other recent thyroid GWAS are discussed in Chapter 14. This chapter aims to provide a comprehensive overview of the genetic basis of the HPT-axis by discussing both monogenic and polygenic causes of thyroid dysfunction and altered thyroid function. Furthermore, new techniques are discussed which will further unravel the genetic basis of thyroid (dys)function in the near future.

The results of our studies on the effects of common genetic variation in the thyroid hormone receptor alpha (*THRA*) locus on human bone and brain are also put into perspective in Chapter 14, in a separate section on the effects of various thyroid hormone pathway genes on clinical endpoints. Finally, in the last study of this thesis, we showed that in the elderly even variation in thyroid function within the normal range is associated with an increased risk of developing a depressive syndrome.

A logical bridge between the different parts of this thesis would be to study the effects of all identified genetic loci in relation to thyroid function in pregnancy, as well as on the risk of adverse pregnancy outcomes and/or depression, as many of these loci were also associated with thyroid function within the normal range. Besides the effects of the individual polymorphisms, it would be especially interesting to study the risk of depression when carrying multiple risk alleles, by applying the genetic risk scores that we calculated in our studies. However, these effects may very well be different in pregnancy, as pregnancy induces many changes in thyroid hormone synthesis and metabolism. Furthermore, there can be many interfering factors in the relation between thyroid function and pregnancy complications, such as hCG levels, iodine status and smoking behaviour. Future studies should therefore first investigate the effects of these loci on thyroid function during pregnancy, before investigating their effects on the risk of pregnancy complications.




Chapter 13

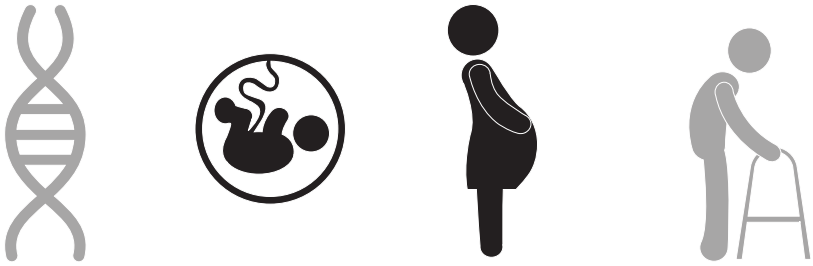
Determinants and effects of thyroid function during pregnancy

Based on:
Thyroid function in pregnancy: What is normal?

M. Medici*, T. Korevaar*, W.E. Visser, T.J. Visser, R.P. Peeters

Submitted

**Both authors contributed equally to this work.*



INTRODUCTION

As discussed in the introduction of this thesis, reference ranges for TSH and FT4 are different in the pregnant state. For this reason, the guidelines of the Endocrine Society and American Thyroid Association recommend to calculate trimester-specific reference ranges per center (1, 2). If these calculated ranges are not available in the laboratory, TSH reference ranges of 0.1–2.5 mU/liter for the first trimester and of 0.2–3.0 mU/liter for the second trimester are recommended (1, 2). These fixed reference ranges are also the recommended ranges for the diagnosis and treatment of thyroid disorders during pregnancy. The importance of calculating trimester specific RRs per centre is illustrated by the fact that in the Generation R Study 8.6 and 4.9 % of the TPOAb-negative women with normal range TSH levels had a TSH level above 2.5 mU/L and 3.0 mU/L in the first and second trimesters, respectively (Figure 1).

Overt hypothyroidism has clearly been linked to obstetrical complications, adverse pregnancy outcomes and impaired neurocognitive development (3-6). An increased risk of these complications has been published for subclinical hypothyroidism as well, but different studies show conflicting results. However, comparison of these studies is difficult since several studies combine patients with hypothyroidism and subclinical hypothyroidism in the analyses (7). In addition, different cut-offs for TSH are used in the different studies (non-pregnant cut-offs, fixed upper limits, or calculated reference ranges).

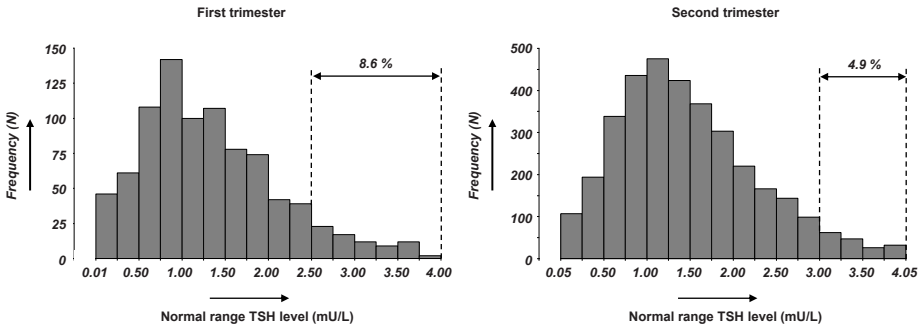


Fig. 1 Distribution of normal range serum TSH levels in the first and second trimester in a Dutch cohort of TPOAb negative pregnant women

Distribution of normal range serum TSH levels (2.5th – 97.5th percentiles) in the first and second trimester in 5186 Dutch women, after exclusion of women with TPOAb positivity, known thyroid disease, thyroid (interfering) medication usage, twin pregnancies, and pregnancies after fertility treatment. In the first trimester, 8.6 % of the women with normal range TSH levels had a TSH level > 2.50 mU/L. In the second trimester, 4.9 % of the women with normal range TSH levels had a TSH level > 3.00 mU/L.

The fixed TSH cut-offs of 2.5 and 3.0 mU/L as advocated in the current guidelines are based on 6 studies in less than 6,000 pregnant women (8-13). These cut-offs are recommended to be used for the diagnosis of (subclinical) hypothyroidism during pregnancy, and a TSH < 2.5 mU/L has been defined as the treatment goal for these conditions (1, 2). Since the publication of these guidelines, additional studies in >30,000 pregnant women have been published (14-16). In the current review we therefore discuss the definition of normal serum thyroid parameter reference ranges during pregnancy, different factors that contribute to these reference ranges, as well as the association of subclinical thyroid disease and the risk of maternal and child complications in relation to these reference ranges.

STUDIES ON THYROID FUNCTION REFERENCE RANGES DURING PREGNANCY

The distribution of serum thyroid parameters in pregnant populations is the first thing to be studied when trying to define limits for a normal thyroid function during pregnancy. It is currently well recognized that pregnancy reference ranges for TSH and FT4 largely differ from non-pregnancy reference ranges (11, 17). However, pregnancy-specific reference ranges for TSH and FT4 also differ considerably worldwide (10, 11, 16). Furthermore, substantial differences may even occur when reference ranges are stratified *e.g.* for ethnicity and body mass index (BMI). Given the substantial differences in reported thyroid reference ranges between various pregnancy cohorts, the guidelines of the Endocrine Society and American Thyroid Association advocate the use of pregnancy-specific population-based reference ranges (P2.5-97.5) (18, 19). This is in accordance with recommendations by the International Federation of Clinical Chemistry (20). It is important to perform these analyses in a sufficiently sized population which consists of 'healthy' reference subjects. A basic rule of thumb is that a minimum of 400 individual measurements is required (21). Although the term "healthy subjects" can be interpreted in many ways for TSH and FT4 reference range determinations, this at least means a population free of major known thyroid function inhibiting or stimulating factors. Preferably, such a population would consist of TPO-antibody (TPOAb) negative women without pre-existing thyroid disease or other thyroid interfering factors (such as medication use, twin pregnancies etc.).

Table 1 shows reference ranges for TSH and FT4 during early pregnancy calculated according to the international guidelines in sufficiently sized cohorts amongst TPOAb-negative women (8, 13-16, 22-30). For both hormones, a wide range of normal values has been reported. These data underline the importance of calculating population-based pregnancy-specific thyroid parameter reference ranges.

Table 1. Reference ranges for TSH and FT4 during early pregnancy in various populations.

			TSH in mU/L		FT4 in pmol/L (ng/dl)			
Author, Country (reference) (assay)	**N**	**Gestation (week)**	**Median**	**P2.5-P97.5**	**Median**	**P2.5-P97.5**	**(Median, P2.5-P97.5)**	**Iodine insufficiency**
Gilbert *et al.*, Australia (22)[a] (Architect, Abbott Diagnostics)	1817	9-13	0.74	0.02 - 2.15	13.5	10.4 - 17.8	(1.05, 0.81 - 1.39)	Borderline
Bocos-Terraz *et al.*, Spain (8) (Architect, Abbott Diagnostics)	481	<14	0.94	0.41- 2.63	13.9	10.8 – 17.8	(1.08, 0.84 - 1.38)	Mild
Lambert-Messerlian *et al.*, USA (23)[b]	8351	T1	1.00	0.12 - 3.37	14.2	10.4 – 17.8	(1.10, 0.81 - 1.38)	Mild
(Immulite 2000)	8415	T2	1.19	0.35 - 3.35	13.0	9.3 – 16.2	(1.01, 0.72 - 1.26)	
Bestwick *et al.* UK (14) (Advia Centaur)	16,334	<16	1.11	0.06 - 3.50	13.9	10.9 - 17.9	(1.08, 0.85 - 1.40)	Moderate-Mild
Bestwick *et al.* Italy (14) (AutoDELFIA)	5505	<16	1.07	0.04 - 3.19	9.3	7.4 - 12.2	(0.73, 0.58 - 0.95)	Moderate-Mild
Li *et al.*, China (24) (Cobas Elesys 601)	640	7-12	1.47	0.10 - 4.34	15.8	12.3 - 20.9	(1.23, 0.96 - 1.63)	Proven sufficient[e]
Männisto *et al.*, Finland (15)	4333	T1	1.11	0.08 – 3.54	15.3	11.7 – 22.8	(1.12, 0.86 - 1.58)	Sufficient
(Architect i2000)	747	T2	1.37	0.11 – 4.24	14.6	11.2 – 23.4	(1.13, 0.87 – 1.82)	
Medici *et al.*, the Netherlands (16) (Vitros ECI)	5186	8-18	1.40	0.03 - 4.04	14.7	10.4 - 22.0	(1.15, 0.81 - 1.72)	Proven sufficient[e]
Pearce *et al.*, USA (25) (Advia Centaur)	585	<14	1.1	0.04 - 3.60	2.1[*]	1.5 - 2.9[*]	-	Borderline
Quinn *et al.*, Russia (26)	380	T1	1.66	0.09- 4.67	-	- -	-	Moderate
(Abbott AxSYM)	549	T2	2.00	0.20- 4.68	-	- -	-	
Springer *et al.*, Czech Republic (27)[c] (ADVIA Centaur)	4337	9-11	1.21	0.06 - 3.67	-	- -	-	Mild
Vaidya *et al.* UK (28) (Modular E 170)	1089	<12	1.08	0.14 - 3.19	14.6	10.7 - 19.4	(1.12, 0.83 - 1.59)	Mild-moderate
Stricker *et al.*, Switserland (13)	575	6-12	0.95	0.07 - 2.82	13.9	10.5 - 18.5	(1.08, 0.82 - 1.44)	Sufficient
(Architect i2000SR)	528	T2	1.02	0.20 - 2.79	12.2	9.5 - 15.7	(0.95, 0.74 - 1.22)	
La'ulu *et al.*, U SA (29,30)[d]	2172	10-13	0.94	0.02 - 2.69	14.7	11.4 - 18.6	(1.15, 0.89 - 1.45)	Mild
(Architect i2000SR)	2683	14-20	1.14	0.15 - 3.11	12.0	9.3 - 15.2	(0.94, 0.73 - 1.19)	

[a] Reported FT4 level is the mean; [b] Limits are P5 and P98 for TSH and P2 and P95 for FT4; [c] High hCG levels excluded; [d] FT4 determined in normal-range TSH only; [e] Iodine measurement in study population; [*] Free T4 index (normal range 1.0-4.0). T1, first trimester; T2, second trimester.

Studies were selected according to the following criteria: eligible N≥500, exclusion of TPOAb-positive women and availability of required information from the published manuscript or via personal communication. Iodine status was estimated based on references from the published manuscript, WHO iodine status reports or Vitamin and Mineral Nutrition Information System (VMNIS).

FACTORS INFLUENCING THYROID FUNCTION REFERENCE RANGES DURING PREGNANCY

Various commercial TSH and FT4 assays have been used to evaluate thyroid function during pregnancy. While previous studies have shown that the inter-assay differences for TSH are relatively small (r=0.91-0.98), FT4 measurements seem much more prone to interference and have larger inter-assay differences (r=0.68-0.89) (31, 32). These differences are mostly attributed to differences in susceptibility for interference with pregnancy-specific proteins or antibodies per assay. However, considering the large differences depicted in Table 1, it is unlikely that use of different assays fully explains the differences between the studies. As recently suggested by Bestwick *et al.*, TSH and FT4 values can be expressed as multiple of medians (MoM), in order to interpret and compare the upper and lower limits obtained via different assays (14). Table 2 shows the lower and upper limits expressed as MoM values for the same studies as Table 1. It is noticeable that the large inter-study differences in TSH remain, whereas the numbers are much more uniform for FT4. As such, these data suggest that especially TSH is subject to change by non-analytical factors.

Severe iodine deficiency has well known adverse effects on maternal thyroid function and child (neuro)development (33). More recently, an increased risk of child neurocognitive impairment has also been found in moderately to mildly iodine deficient populations (34, 35). Unfortunately, there is only limited data on the effects of iodine fortification programs on population based FT4 and TSH reference ranges (36, 37). A number of studies have found that thyroid antibodies, thyroid size, multinodular goiter, baseline TSH level, baseline urinary iodine level and the increase in urinary iodine level are all factors associated with changes in population TSH levels after iodine supplementation (36, 38, 39). Taken together, it is very likely that population iodine status is a major determinant of pregnancy-specific thyroid function reference ranges, and alteration in iodine status may shift the serum thyroid parameter distribution curves, especially for TSH. However, there are too little data to quantify the extent and/or timing of these changes.

A group of major determinants that are much better quantifiable are differences in population characteristics. Characteristics such as ethnicity, BMI and smoking have all been associated with differences in serum thyroid parameters (14, 15, 25, 29, 30, 40-47). As is in accordance with Table 2, TSH is most often affected, although specific associations for FT4 and TPOAb-positivity have also been reported.

La'ulu et al. found that both upper and lower limits for a wide range of serum thyroid function tests differ according to ethnic background in the first and second trimester. Once again, especially upper limits for TSH differed, ranging from 2.73 in blacks to 3.64 mU/L in Asians (29, 30). We have shown similar results in the Generation R Study (Chapter

Table 2. Reference ranges for TSH and FT4 during early pregnancy in various populations expressed as multiple of medians (MoMs)

	MoM TSH	MoM FT4	
Author, Country (reference)	**P2.5-P97.5**	**P2.5-P97.5**	**Iodine insufficiency**
Gilbert *et al.*, Australia (22)	0.03 - 2.91	0.77 - 1.32	Borderline
Bocos-Terraz *et al.*, Spain (8)	0.44 - 2.80	0.78 - 1.28	Mild
Lambert-Messerlian *et al.*, USA (23)	0.12 - 3.37	0.73 - 1.52	Mild
	0.29 - 2.82	0.70 - 1.45	
Bestwick *et al.* UK (14)	0.05 - 3.15	0.78 - 1.29	Moderate-Mild
Bestwick *et al.* Italy (14)	0.04 - 2.98	0.80 - 1.31	Moderate-Mild
Li *et al.*, China (24)	0.07 - 2.95	0.78 - 1.32	Proven sufficient [a]
Männisto *et al.*, Finland (15)	0.07 - 3.19	0.76 - 1.49	Sufficient
	0.08 - 3.09	0.77 - 1.60	
Medici *et al.*, the Netherlands (16)	0.01 - 2.84	0.71 - 1.53	Proven sufficient [a]
Pearce *et al.*, USA (25)	0.04 - 3.27	- -	Borderline
Quinn *et al.*, Russia (26)	0.05 - 2.81	- -	Moderate
	0.10 - 2.34	- -	
Springer *et al.*, Czech Republic (27)	0.05 - 3.03	- -	Mild
Vaidya *et al.* UK (28)	0.13 - 2.95	0.73 - 1.33	Mild-Moderate
Stricker *et al.*, Switserland (13)	0.07 - 2.97	0.76 - 1.33	Sufficient
	0.20 - 2.74	0.78 - 1.29	
La'ulu *et al.*, USA (29,30)	0.02 - 2.86	0.78 - 1.27	Mild
	0.13 - 2.73	0.78 - 1.27	

[a] According to iodine measurements in study population.
MoM values were calculated by dividing each individuals' TSH or FT4 level by the (trimester-specific) median level.

3), and also found that these ethnic differences in thyroid parameter reference ranges may lead to considerable misclassification of thyroid disease in up to 18% of cases (43).

BMI has been associated with both TSH and FT4 levels during pregnancy (14, 15, 41, 45). Männisto *et al.* found that the upper limit (P95) for TSH increased from 2.86 mU/L in women with a BMI <20 kg/m^2, to 3.50 mU/L amongst women with a BMI >30 kg/m^2. For the same groups, they also showed that the lower limit for FT4 (P5) decreased from 12.3 pmol/L to 11.6 pmol/L, respectively (15). Bestwick *et al.* expressed these values in MoMs and found an increase in TSH of 0.025 MoM, and a decrease in FT4 of 0.009 MoMs per 10 kg increase in body weight (14).

Different effects of smoking on thyroid function during pregnancy have been described. If any effect is present, smoking is associated with both lower mean TSH and FT4 levels (14, 25, 40, 44, 46, 48). In line with this, we found in the Generation R study that TSH (1.46 *vs.* 1.38 mU/L; p=0.07), FT4 (15.1 *vs.* 14.6 pmol/L; p<0.01) and T4 (151 *vs*. 148 pmol/L; p<0.05) were all lower in smoking *vs.* non-smoking TPOAb-negative euthyroid

women (unpublished results). However, given the described effect sizes, it seems unlikely that population differences in smoking prevalence have any noteworthy influence on TSH and FT4 reference ranges.

THYROID FUNCTION DURING PREGNANCY AND THE RISK OF MATERNAL AND CHILD COMPLICATIONS

When defining a normal thyroid function during pregnancy, one should not only consider the distribution of serum thyroid parameters in the healthy pregnant population of interest, but also the potential adverse effects of variations in thyroid function on both the mother and child. For long, it has been known that both overt hypo- and hyperthyroidism during pregnancy are associated with pregnancy complications, and also subclinical thyroid abnormalities have been associated with pregnancy complications. In the last few years it has become clear that also minor variations in thyroid function can have negative effects on both the mother and the child. The interpretation of these studies is complicated by the fact that most did not use population-based reference ranges and used different serum thyroid parameter reference ranges limits. Furthermore, many of these studies did not take potentially interfering factors into account, such as antibody status, hCG levels, BMI, smoking and parity, which may confound the associations between the studied thyroid parameters and pregnancy complications. We will provide an overview of the effects of thyroid dysfunction during pregnancy on

Table 3. Overview of the effects of thyroid dysfunction during pregnancy on the risk of maternal and child adverse outcomes

Thyroid (dys) function group	Pregnancy loss	Prematurity	Hypertensive disorders	Low birth weight	Neurodevelopmental delay
Overt hypothyroidism	↑ (+++)	↑ (+++)	↑ (+++)	? (+++)	↑ (+++)
Subclinical hypothyroidism	↑ (+++)	? (+++)	↑ (++)	? (+++)	↑ (+++)
Hypothyroxinemia	↔ (+)	↔ (+++)	↔ (++)	↔ (+)	↑ (+++)
Overt hyperthyroidism	↑ (+++)	↑ (+++)	↑ (+++)	↑ (+++)	↔ (+)
Subclinical hyperthyroidism	-	? (+++)	↔ (++)	-	↔ (+)
TPOAb-positivity	↑ (+++)	↑ (+++)	↔ (++)	↔ (+++)	↑ (++)
Normal-range FT4 levels	-	↔ (+)	? (++)	↑ (++)*	-

*Compared to low-normal FT4 levels (10.38-12.80 pmol/L), high-normal FT4 levels (17.01-22.00 pmol/L) were associated with a lower birth weight (98).

the risk of a number of important and well-studied maternal and child complications, including pregnancy loss, prematurity, hypertensive disorders, low birth weight and neurodevelopmental delay, as summarized in Table 3.

Pregnancy loss

It has clearly been demonstrated that both overt hypo- and hyperthyroidism are associated with an increased risk of pregnancy loss (4, 49, 50). Later studies have shown that the risk of pregnancy loss is also higher in women with subclinical hypothyroidism during pregnancy (51-54). In a Dutch cohort of 2497 pregnant women, it was shown that the incidence of child loss increased by 60% by every doubling of the maternal TSH concentration (53). In 2002, Abalovich *et al.* followed 35 pregnancies with subclinical hypothyroidism (> 5 mU/L; non-pregnancy specific TSH cut-off) that were treated with levothyroxine and found that inadequately treated women had a 71.4% risk of an abortion, while none of the adequately treated women had an abortion (51). To study the effects of thyroid antibodies on the risk of pregnancy loss, independent of subclinical or overt hypothyroidism, Thangaratinam *et al.* performed a meta-analysis including 12,126 women and showed that euthyroid women with thyroid antibodies had a 1.8 to 3.9-fold increased risk of pregnancy loss (55). Negro *et al.* studied these relations in a group of TPOAb-negative pregnant women and concluded that women with serum TSH levels of 2.5-5.0 mU/L had a 6.1% risk of pregnancy loss, compared to 3.6% in women with a TSH level below 2.5 mU/L (54), showing that the association between subclinical hypothyroidism and pregnancy loss is only partly driven by thyroid autoimmunity.

Premature delivery

Premature delivery has been identified as the most important direct cause of child death in almost all high- and middle- income countries and is associated with psychiatric, metabolic, cardiovascular, and renal disease later in life (56-59). Although the pathophysiological mechanism remains poorly understood, thyroid dysfunction during pregnancy has been associated with an increased risk of a premature delivery. This has been shown for both hypo-and hyperthyroidism in various studies, the largest of which is a study by Mannisto *et al.* in 223,512 pregnancies, which concluded that hypo- and hyperthyroidism during pregnancy were associated with a 1.34- and 1.81-fold increased risk of prematurity (5). However, the effects of more subtle variations in thyroid function are less clear. Subclinical hypothyroidism has been described as a risk factor for premature deliveries, while other studies did not find any associations (51, 54, 60-67). This could be partly explained by the fact that different TSH cut-off values were used in the different studies. We therefore studied the association between increased TSH levels and the risk of premature deliveries using the population-based P97.5 (4.0 mU/L) or the fixed 2.5 mU/L cut-off. While no associations were seen with a TSH >2.5 mU/L, a 1.9-2.5 times

increased risk of prematurity was seen among women with a TSH >4.0 mU/L (68). This association no longer persisted after exclusion of TPOAb-positive women and women with comorbidities, suggesting that these factors underlie the observed association between thyroid dysfunction and prematurity. These results underline the importance of performing in-depth analyses in a detailed cohort, taking the interfering role of various confounders into account, when analyzing the effects of thyroid dysfunction on pregnancy outcomes.

Furthermore, limited data are available on the effects of hypothyroxinemia on the risk of premature deliveries, and most of the available studies did not find any effects (61, 62, 68, 69). TPOAb-positivity during pregnancy is generally considered to be associated with an increased risk of premature deliveries. This is supported by a meta-analysis of He *et al.*, which showed a 1.69 fold increased risk of prematurity among TPOAb-positive euthyroid women, which has been suggested to be independent of thyroid function (68, 70).

Hypertensive disorders

Hypertensive disorders, including pregnancy-induced hypertension (PIH) and (pre) eclampsia, are common during pregnancy and are a major cause of maternal and fetal morbidity and mortality (71-76). Both hypo- and hyperthyroidism have been shown to have important vascular effects, including endothelial cell dysfunction (77-80), and therefore a number of studies have investigated the effects of thyroid dysfunction on the risk of hypertensive disorders during pregnancy (5, 62-64, 81-87). While various studies have shown conflicting results on the associations between overt thyroid dysfunction and hypertensive disorders (62, 64, 83-86), the largest study by Mannisto *et al.* showed that both hypo- and hyperthyroidism were significantly associated with a 1.5-3.6 fold increased risk of (superimposed) preeclampsia (5). Unfortunately, this study lacked information on treatment of thyroid disease during pregnancy and the authors were not able to investigate the effects of more subtle alterations in thyroid function, such as subclinical hypo- and hyperthyroidism. In a prospective cohort study in nearly 25,000 pregnancies by Wilson *et al.*, subclinical hypothyroidism was associated with a 1.6-fold increased risk of severe pre-eclampsia, even after correction for confounding factors (87). Subclinical hypothyroidism was defined as a serum FT4 between P2.5 and P97.5 (0.9-2.0 ng/dL) and a TSH above P97.5 (>4.13 mU/L).

Low birth weight

Birth weight is frequently used as a proxy for fetal growth and development. A low birth weight can either be due to intrauterine growth retardation (SGA; small size for gestational age) or prematurity, and has been associated with an increased risk of perinatal

mortality and the development of various cardiovascular, renal and psychiatric diseases in later life (88-92).

Overt hyperthyroidism during pregnancy increases the risk of low-birth weight children (83, 85, 93). In a pregnant US population, Millar *et al.* found that hyperthyroidism was associated with a 9.2-fold increased risk of low birth weight (LBW; <2500 g) newborns, while a study from Thailand found a 2.7-fold increased risk of LBW newborns (85, 93). These substantial differences in risk estimates are likely caused by the limited number of cases included in these studies (N<60).

Conflicting results have been published on the relation between subclinical and overt hypothyroidism and birth weight. A few studies with a limited number of hypothyroid cases suggested an increased risk of LBW and SGA children in hypothyroid pregnancies (63, 86, 94). However, the largest study on this relation has been performed by Mannisto *et al.* in a cohort of 5805 pregnancies, in which population-based reference ranges for TSH and FT4 were calculated. No effects of overt hypothyroidism (TSH >P95 and FT4 <P5; n=54) and subclinical hypothyroidism (TSH >P95 and FT4 P5-P95; n=224) were detected on birth weight (67). This is line with results from the study by Karagiannis *et al.*, who measured early-pregnancy serum thyroid parameters in pregnancies that subsequently delivered SGA neonates and did not find any differences compared to a control cohort of uncomplicated pregnancies (95). While Mannisto *et al.* found that TPOAb-positive women had an increased risk of a high-birth weight newborn, this finding could not be replicated in other large population-based cohorts, and may therefore be due to a chance finding (67, 92, 96).

In Chapter 5, we studied the birth weights of children from 4464 mothers with normal-range TSH and FT4 levels during pregnancy (92). Compared to low-normal FT4 levels (10.38-12.80 pmol/L), high-normal FT4 levels (17.01-22.00 pmol/L) during early pregnancy were associated with a 2.8-times increased risk of LBW children, while no effects on the risk of prematurity were found. Similar effects were detected in the study by Haddow *et al.* (97).

Neurodevelopmental delay

The detrimental effects of low maternal TH levels on fetal brain development have been appreciated for long (98). In 1999, Pop *et al.* showed that children born to mothers with low early-pregnancy FT4 levels (<P10, n = 22) have an impaired psychomotor development (99). A later study by Henrichs *et al.* in 3700 mother-child pairs showed associations between early-pregnancy severe and mild hypothyroxinemia (FT4 <P5 and <P10, respectively) and an increased risk of expressive language delay at 18 and 30 months (OR = 1.80). In addition, severe hypothyroxinemia was also associated with an increased risk of nonverbal cognitive delay at 30 months of age (OR = 2.03) (100). These results were also confirmed by other studies (101-103). As most of these studies investigated

the effects of hypothyroxinemia, less is known about the effects of overt and subclinical hypothyroidism on child neurocognitive development. In a hallmark study published in 1999, Haddow *et al.* studied the IQ scores of 7 to 9 year old children born to mothers with an early-pregnancy TSH >P99.7, and showed that their IQs were on average 7 points lower compared to 124 controls born to mothers with a normal TSH during pregnancy (3). A later study in a Chinese pregnancy cohort confirmed that mothers with subclinical hypothyroidism (population-based TSH >P95 and FT4 P5 – P95) have an increased risk of newborns with neurodevelopmental delay (OR = 10.4) (94). Limited data are available on the role of TPOAbs in these associations, but the few available studies suggest that maternal TPOAb-positivity is associated with an increased risk of child neurocognitive delay (104, 105).

Treatment of thyroid disorders during pregnancy

Although treatment of overt hypo- and hyperthyroidism during pregnancy is universally accepted, this is less clear for the subclinical forms. A study on the benefits of universal screening versus case-finding of thyroid disorders during pregnancy suggested that levothyroxine (LT4) treatment of TPOAb-positive pregnant women with a TSH > 2.5 mU/L results in less pregnancy complications (106). Lepoutre *et al.* showed that LT4 treatment of TPOAb-positive pregnant women with a TSH > 1.0 mU/L was associated with less miscarriages (107). However, these studies did not use population-based reference ranges, results were based on a limited number of treated cases, and analyses were not restricted to only women with subclinical hypothyroidism. Therefore, large RCTs are needed to clarify if subclinical hypo- and hyperthyroidism during pregnancy should be treated. Finally, Negro *et al.* showed that LT4 treatment of pregnant TPOAb-positive euthyroid women is associated with a decreased risk of pregnancy loss and premature births (108). As this has not been studied in other pregnancy cohorts, large RCTs are currently investigating the benefits of treating TPOAb-positive euthyroid women with LT4, including the TABLET (Thyroid AntiBodies and LEvoThyroxine) trial (UK) and the T4 Life trial (The Netherlands).

CONCLUSIONS

In the last decade a large number of studies have been published on thyroid reference ranges during pregnancy. These studies show substantial differences in serum TSH and FT4 reference ranges between populations, which can be partly explained by the use of different assays, as well as by population-specific characteristics such as ethnicity, iodine status and BMI. Given these substantial differences between studies (Table 1), it is crucial that institutions calculate their own population-based ranges, and do not rely

on reference ranges from other populations or fixed universal TSH cut-off values. Consequently, clinicians should also use these population-based ranges for the diagnosis and treatment of thyroid diseases, instead of using the fixed serum TSH cut-off levels of 2.5 and 3.0 mU/L.

The use of MoMs illustrated that part of the differences in thyroid parameter reference ranges between populations can be explained by the use of different assays. Provided that institutions determine their own population-based ranges, there is no direct need for using MoMs in daily clinical practice. However, the use of MoMs could be useful in clinical studies on the effects of thyroid dysfunction during pregnancy. In this way, results from studies using different assays could be easily compared and combined. Furthermore, by reporting MoMs, the individual clinician could more easily interpret published results in light of his/her patient population and assay used.

Besides data on reference ranges, these pregnancy cohorts also provided insight into the effects of maternal thyroid dysfunction on both maternal and child outcomes. The interpretation of part of these studies is hampered by the fact that different cut-offs were used to define thyroid dysfunction; while some cohorts calculated population-based pregnancy-specific reference ranges, others used the fixed guideline cut-offs, or non-pregnancy ranges. Despite this, these studies have shown that, besides overt hypo- and hyperthyroidism, also more subtle variations in thyroid function are associated with an increased risk of various pregnancy complications. This especially holds true for subclinical hypothyroidism, which has been associated with pregnancy loss, hypertensive disorders, and neurodevelopmental delay, while conflicting effects have been reported on the risk of prematurity and birth weight. These studies underline the importance of not only maintaining serum FT4 but also serum TSH levels within the normal range. Less is known about the effects of subclinical hyperthyroidism, or even variation in normal-range TSH and F4 levels. This should therefore be the focus of future studies, while large RCTs are needed to clarify if subclinical hypo- and hyperthyroidism during pregnancy should be treated.

In conclusion, given the substantial differences in pregnancy-specific TSH and FT4 reference ranges between populations, it is essential that institutions calculate their own pregnancy-specific populated-based reference ranges. These should also be used for the diagnosis and treatment of thyroid disorders during pregnancy.

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Chapter 14

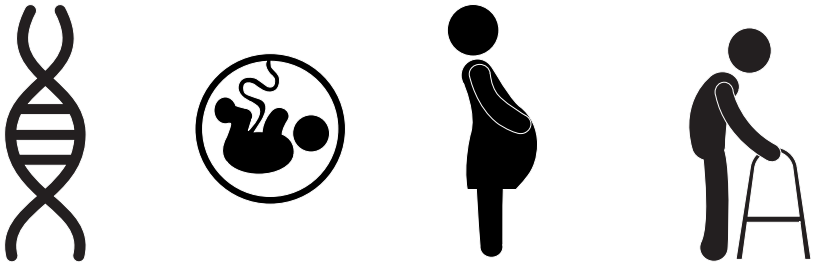
Genetic determinants of thyroid function and autoimmunity

Based on:

Genetic determination of the hypothalamic-pituitary-thyroid axis: where do we stand?

M. Medici, W. E. Visser, T.J. Visser, R.P. Peeters

Submitted



OUTLINE

I. Background
II. Single gene disorders causing thyroid disease
    A. Hypothyroidism
    B. Hyperthyroidism
III. Single gene disorders affecting the HPT-axis due to defects in TH signaling
IV. Common genetic variants associated with thyroid (dys)function (pre-GWAS)
    A. Linkage studies
    B. Candidate gene analyses for HPT-axis setpoint
    C. Candidate gene analyses for clinical thyroid related endpoints
    D. Linkage and candidate gene studies in autoimmune thyroid disease
V. Common genetic variants associated with thyroid (dys)function (GWAS)
    A. GWAS on hypothyroidism
    B. GWAS on hyperthyroidism
    C. GWAS on TPO-antibodies
    D. GWAS on HPT-axis setpoint
VI. Discussion and future perspectives

I. INTRODUCTION

In the past 5 years, advances in genetic research have led to the identification of a large number of new candidate genes for thyroid function and autoimmunity. The genetic architecture of the HPT axis is similar to other complex traits, with contributions from several genes, most of which have small effects, but some of which have large effects, as illustrated in Figure 1. Therefore, this review will first discuss monogenic causes of thyroid dysfunction and altered thyroid function tests (i.e., rare variants, large effects). We will then discuss the polygenic causes of thyroid (dys)function (i.e., common variants, small effects), including the new candidate genes identified by GWAS, and what insights these genes provide about the genetic basis of thyroid (dys)function. Finally, we will discuss new techniques which will help to further unravel the genetic basis of thyroid (dys)function in the near future, which will likely lead to a better understanding of disease identification and treatment.

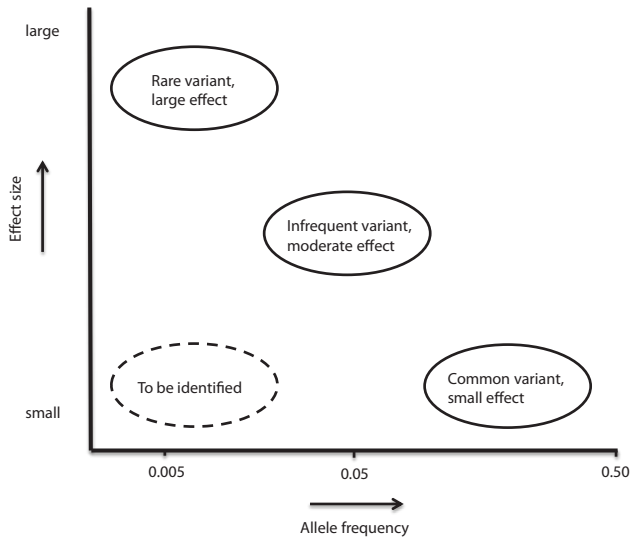


Figure 1 Adapted from Ralston and Uitterlinden, Endocr Rev 2010. The genetic architecture of the HPT axis, with contributions from various genetic variants with different frequencies and effects. Most of the common variants have small effects, while some rare variants have large effects. Common variants with large effects have not been found, and probably do not exist. Rare variants with small to moderate effects are likely to be identified by future sequencing efforts.

II. SINGLE GENE DISORDERS CAUSING THYROID DISEASE

A. Hypothyroidism

The incidence of congenital hypothyroidism (CH) has been estimated to be around 1 in 3500 live-born infants, and is two times more frequent in women than in men (1-4). 75-85% of the cases are due to thyroid dysgenesis, 15-20% are due to abnormalities in TH synthesis (dyshormonogenesis), and only a minor fraction has a central origin (5, 6). Up to 15% of the CH cases have a hereditary basis, while the others are considered sporadic forms. An overview of the monogenic causes of CH is shown in Table 1, together with the monogenic causes of hyperthyroidism and altered thyroid function tests.

Single gene disorders causing central hypothyroidism

Congenital CH is rare, with an estimated prevalence of 1 in 50,000 live-born infants. No human mutations in the *TRH* gene have yet been identified. So far, only one patient with a compound heterozygote TRH receptor gene mutation has been described, presenting with severe CH, short stature and mental retardation (7). However, a number of patients with loss-of-function mutations of the *TSHβ* gene have been identified (8-14). The phenotypes of these patients ranged from mild to severe CH. Most of these patients had a developmental delay and were identified by a very low TSH, which could not be stimulated by TRH.

Mutations in transcription factors which are important for pituitary development can lead to various forms of combined pituitary hormone deficiency. These patients present with impaired secretion of one or more pituitary hormones, frequently including TSH deficiency.

POU1F1 (PIT1) plays an important role in pituitary development and hormone secretion. Mutations in *POU1F1* lead to a clinical picture in which CH is the leading symptom, with a delayed manifestation of growth hormone and prolactin deficiencies (15-18). The mode of inheritance can be recessive or dominant, depending on the location of the mutation.

The expression of *PROP1* precedes *POU1F1* expression. Various families with *PROP1* mutations have been described, frequently leading to not only growth hormone, TH, and prolactin deficiency, but also to LH and FSH deficiency (19-22). These patients therefore do not enter puberty spontaneously.

LHX3 plays an important role in the development of the anterior pituitary, and mutations in *LHX3* have been described for the first time in 2000 in two families (23). Affected individuals had deficiencies of all pituitary hormones, except for ACTH, and displayed rigidity of the cervical spine.

Finally, Sun et al. more recently identified *IGFS1* mutations as a cause of X-linked central hypothyroidism (24). IGSF1 is a membrane glycoprotein highly expressed in the

Table 1. Monogenic causes of thyroid dysfunction and altered thyroid function tests

	Subtype	Gene	Affected process	Inheritance	Serum thyroid parameters
Hypothyroidism	Central hypothyroidism	*TRHR*	TRH signaling	Compound heterozygous	Normal TSH, low T4
		β-TSH	TSH signaling	AR	Low TSH, T4 and T3
		POU1F1	Thyrotrophic pituitary cell development	AR, AD	Low TSH and T4 (also GH and prolactin deficiencies)
		Prop-1	Thyrotrophic pituitary cell development	AR	Low TSH and T4 (also GH, prolactin, LH and FSH deficiencies)
		LHX3	Thyrotrophic pituitary cell development	AR	Low TSH and T4 (also GH, prolactin, LH and FSH deficiencies)
		IGSF1	unknown	AR (X-linked)	Low TSH and T4, especially in males (also prolactin deficiency)
	Complete TSH resistance	*TSHR*	TSHR *(nactivating mutations)*	AR	High TSH, low T4
	Thyroid dysgenesis	*PAX8*	Thyroid development	AD	High TSH, low T4
		FOXE1	Thyroid development	AR	High TSH, low T4, athyreosis
		NKX2.1	Thyroid development	AD	Ranging from hyperthyrotropinemia to CH
	Thyroid dyshormogenesis	*NIS*	Iodide trapping	AR	High TSH, low T4, low or absent radioiodide uptake
		SLC26A4	Intrathyroidal iodide transport	AR	Ranging from hyperthyrotropinemia to CH (depending on iodine status)
		Tg	Tg	AR	High TSH, low Tg, T3 disproportionately high compared to T4
		TPO	Iodide organification defect	AR	Ranging from transient to permanent CH, increased radioiodine uptake and discharge >90%
		DUOX2	Iodide organification defect	AR	Permanent CH, increased radioiodine uptake and discharge 10-90%

Table 1. (continued)

Subtype	Gene	Affected process	Inheritance	Serum thyroid parameters
	DUOXA2	Iodide organification defect	AD, compound heterozygous	Permanent mild CH, increased radioiodine uptake and discharge 10-90%
	DEHAL	MIT and DIT deiodination	AR	CH (varying time of onset in childhood)
	GLIS3	unknown	AR	CH with varying thyroid anatomy (ranging from athyreosis to normal thyroid)
Hyperthyroidism	*TSHR*	TSHR (activating mutations)	AD	Overt or subclinical hyperthyroidism, with varying age of onset
Altered thyroid function tests	*DUOX2*	Iodide organification defect	AD	Transient CH, potential risk of (subclinical) hypothyroidism in later life
	TSHR	TSHR (inactivating mutations)	AD, AR	High TSH, normal T4
	SBP2	Deiodinase synthesis	AR, compound heterozygous	Normal to slightly elevated TSH, high T4 and rT3, low to low-normal T3
	MCT8	TH transport	AR (X-linked)	High-normal TSH, low to low-normal T4, high T3 (males)
Resistance to TH	*THRA*	TH receptor alpha	AD	Normal TSH, low T4 and rT3, high T3
	THRB	TH receptor beta	AD (most cases)	Normal TSH, high T4 and T3

CH, congenital hypothyroidism; AR, autosomal recessive; AD, autosomal dominant

anterior pituitary. In males, clinical characteristics include CH, testicular enlargement, hypoprolactinemia, delayed puberty and increased body weight, while in females only a subset of carriers exhibits CH or hypoprolactinemia (25, 26).

TSH receptor mutations causing resistance to TSH

Mutations in *TSHR* can lead to TSH resistance. This has been extensively reviewed by Persani *et al.* and Cassio *et al.* (27, 28). Depending on the type of mutation, the clinical presentation may vary considerably, ranging from severe CH to only limited elevations in TSH with a normal T4 level in the absence of clinical symptoms of hypothyroidism. Complete resistance to TSH was first described by Abramowicz *et al.* in 1997, who found a homozygous loss-of-function mutation in *TSHR* in a brother and sister from consanguineous parents (29). These patients suffered from severe CH, and were initially diagnosed with thyroid agenesis at scintigraphy due to the severe hypoplasia and greatly impaired iodine uptake. However, the presence of thyroid tissue was ensured by detectable Tg levels. Since then, various loss-of-function mutations in *TSHR* have been described in patients with resistance to TSH (27).

Single gene disorders causing thyroid dysgenesis

In patients with CH, mutations have also been identified in genes involved in thyroid development, including *PAX8*, *FOXE1* and *NKX2.1*. PAX8 is a paired domain transcription factor involved in thyroid development and the expression of the *TPO* and *Tg* genes. Several patients with heterozygous *PAX8* mutations have been identified. This disorder has a variable penetrance, ranging from ectopy and hypoplasia of the thyroid associated with severe CH to an eutopic thyroid associated with mild hypothyroidism (30-32). The molecular mechanisms underlying this phenomenon remain to be elucidated.

FOXE1, also known as thyroid transcription factor 2 (TTF-2), is an intronless transcription factor that binds DNA through a forkhead domain. During development, FOXE1 is expressed in the thyroid anlage, Rathke's pouch, pharyngeal structures and hair follicles. Mutations in *FOXE1* are the first described genetic causes of thyroid agenesis in humans, leading to the so-called Bamforth-Lazarus syndrome (33). Besides CH, characteristics include developmental delay, cleft palate, choanal atresia, bifid epiglottis and spiky hair.

Belonging to the family of homeobox domain containing transcription factors, NKX2.1 (also known as thyroid transcription factor 1 (TTF-1)) has a wide expression pattern, including thyroid, forebrain, basal ganglia, pituitary and lung. Human *NKX2.1* mutations can lead to a wide spectrum of thyroid and pulmonary manifestations ranging from hyperthyrotropinaemia to CH due to thyroid agenesis, and from severe neonatal respiratory distress syndrome to a slightly increased sensitivity to pulmonary infections (34-36). Neurological complications may include global developmental delay, hypotonia, ataxia, microcephaly and choreoathetosis.

Single gene disorders causing thyroid dyshormogenesis

In addition to the genetic defects leading to central hypothyroidism and thyroid dysgenesis, mutations have also been identified in the various steps involved in TH synthesis. These include the substrate for TH synthesis (Tg), iodide trapping (NIS), iodide efflux across the apical membrane (Pendrin), organification of iodide (TPO, DUOX2, DUOXA2), and the ability to recover and retain intrathyroidal iodide (DEHAL).

NIS mutations have been identified in patients with CH, but the onset of hypothyroidism varies from birth to childhood and correlates with the residual activity of the mutant transporter (37-40). A radioactive iodide scan typically shows blunted or absent uptake in a normally located thyroid gland. Besides its expression in the apical thyrocyte membrane, the SLC26A4 transporter is furthermore expressed in the inner ear and kidney. Patients with biallelic *SLC26A4* mutations (Pendred syndrome) typically present with deafness, and only part of these patients suffer from an impaired thyroid function (41-43). Iodine status is an important modifier of the thyroid phenotype in this syndrome, as 90% of patients with sufficient iodine intake are both biochemically and clinically euthyroid (44).

Since the first description of a mutation in the *Tg* gene in a patient with congenital goiter and hypothyroidism by Ieri *et al.* in 1991, over 50 other *Tg* mutations have been described (45, 46). Although phenotypes of *Tg* gene mutants can vary depending on the mutation, patients typically present with a large goiter and are biochemically characterized by a high serum TSH and a low Tg level. In addition, serum T3 levels are disproportionately high compared to the T4 level, which is thought to be due to increased intrathyroidal D2 activity (47).

TPO gene mutations are the most frequent cause of inherited dyshormonogenesis with permanent CH and homozygous mutations are characterized by a total iodide organification defect, as extensively reviewed by Ris-Stalpers *et al.* (48). These patients therefore require lifelong thyroxine treatment. Heterozygous *TPO* mutations are not a direct cause of permanent hypothyroidism. However, a study in 260 Chinese children with neonatal transient hypothyroidism and 1000 controls showed that a common *TPO* founder mutation was 16 times more common in the children with neonatal transient hypothyroidism, suggesting that these mutations may play a role in the susceptibility for neonatal transient hypothyroidism (49).

Various monoallelic *DUOX2* mutations have been identified in newborns with CH, but follow-up studies revealed the only transient nature of this hypothyroidism, as no thyroxine substitution was needed to maintain euthyroidism in later life (50, 51). However, biallelic *DUOX2* mutations are characterized by permanent CH (38, 51). In contrast to *TPO* mutants, these patients have only a partial iodide organification defect. This residual organification capacity is likely explained by the expression of DUOX1 in thyrocytes.

It was not until 2008 that the first mutation in *DUOXA2* has been identified in a patient with mild permanent CH and a partial iodide organification defect (52). Since then, one other *DUOXA2* mutation has been identified in a Chinese patient with mild CH (53).

Genetic defects in intrathyroidal iodine recycling can also lead to hypothyroidism. *DEHAL* mutations have been identified in a number of patients that tested normal at CH screening, but presented with clinical signs of hypothyroidism in early childhood (54, 55). It is interesting to note that, besides the classical clinical and biochemical signs of hypothyroidism, these patients also had higher urinary MIT and DIT concentrations, which could be a useful future diagnostic tool.

Finally, in 2003 Taha *et al.* described a syndrome characterized by neonatal diabetes, CH and facial anomalies, which was later found to be caused by mutations in the *GLIS3* gene (56, 57). GLIS3 is a transcription factor highly expressed in the thyroid and pancreas, and later studies showed that the phenotype can furthermore include hepatic fibrosis, polycystic kidney disease, glaucoma, osteopenia, bilateral sensorineural deafness and pancreatic exocrine insufficiency (56, 58). Until now, only 5 families with this syndrome have been described.

B. Hyperthyroidism

Whereas inactivating *TSHR* mutations can lead to CH, activating *TSHR* mutations can lead to constitutive activation of the cAMP cascade and induce growth and hyperfunctioning of the thyroid follicular cells, leading to thyroid autonomy. This has previously been reviewed by Gozu et al. and Hébrant et al (59, 60).

The first activating *TSHR* mutation leading to this so-called familial non-autoimmune hyperthyroidism was detected in a French family, which had already been described in 1982 by Thomas *et al.* (61) Since then, various activating *TSHR* mutations have been identified in families with familial non-autoimmune hyperthyroidism (59, 60). The degree of hyperthyroidism varies from subclinical hyperthyroidism (62) to overt hyperthyroidism with severe complications, including facial hypoplasia, advanced bone age, neurodevelopmental delay, jaundice and cerebral ventriculomegaly (63). Also the age of presentation varies greatly, ranging from the neonatal period to 60 years. Besides a positive family history for hyperthyroidism and the absence of clinical signs of thyroid autoimmunity, it is characterized by a clinical course with multiple relapses, even after radioiodine therapy or partial thyroidectomy.

Activating *TSHR* mutations have also been detected in sporadic forms of non-autoimmune hyperthyroidism, as first described by Kopp *et al* (64). The sporadic form is characterized by an earlier and more severe onset compared to the hereditary form. Part of these patients presents with complications of fetal hyperthyroidism, such as prematurity, advanced bone age, craniosynostosis or mental retardation. Giving the relapsing

nature of the hyperthyroidism, even after subtotal thyroidectomy, some patients need combined treatment with near-total thyroidectomy and radioiodine therapy (59, 60).

Finally, although also mutations in the *Gsα* gene have been detected in patients with non-autoimmune hyperthyroidism, the prevalence and exact functional significance of these mutations still remain to be established (65, 66).

III. SINGLE GENE DISORDERS AFFECTING THE HPT-AXIS DUE TO DEFECTS IN TH SIGNALING

Not all single gene disorders affecting TH pathway genes lead to hypo- or hyperthyroidism. This is clearly illustrated by various monogenetic disorders in which mutations in TH pathway genes lead to alterations in thyroid function tests, without a classical phenotype of hyper- or hypothyroidism. The HPT-axis can be affected at different levels of TH signaling. In addition to a decreased capacity of the thyroid to produce TH, variations in response of the thyroid to TSH, in the peripheral metabolism of TH, in the cellular uptake of TH, and variations in the intracellular response to TH may all contribute to a thyroid function setpoint that is different for each individual. These single gene disorders suggest that common polymorphic variants with even more subtle consequences in the same genes affect serum TSH and FT4 levels within the normal range, and may lead to subtle defects in TH signaling in specific tissues as well, depending on the gene involved.

Decreased thyroid reserve due to mutations in genes involved in TH synthesis

Most inborn errors of TH synthesis are caused by defects in iodide organification. As discussed, mutations in the genes encoding TPO, Tg, Pendrin, NIS and DUOX2 (DUOXA2) have been demonstrated in patients with organification defects, as discussed in section II (38, 50, 51). One could speculate that less detrimental defects in these genes involved in TH synthesis may lead to a decreased thyroid reserve. This has been shown for the DUOX2 gene. Whereas bi-allelic inactivating mutations lead to a severe and permanent CH (50), mono-allelic mutations lead to a milder, transient hypothyroidism. This is caused by insufficient capacity to produce TH at the beginning of life, when there is an increased TH requirement. These patients have a decreased thyroid reserve and may be at risk for subclinical or overt hypothyroidism, goiter, or both in adolescence and adulthood, especially during pregnancy, when the need for TH increases (50).

Decreased sensitivity to TSH due to mutations in the TSH receptor

Complete TSH resistance due to bi-allelic loss of function mutations in the TSH receptor can lead to non-syndromic CH or severe thyroid hypoplasia (section II). However, depending on the severity of the mutation and on the number of mutated alleles, the

clinical phenotype may be much more subtle (27, 28). In these cases elevated serum TSH levels with a very variable pattern may compensate for the mild functional impairment of the mutant receptor, leading to FT4 and T3 concentrations within the normal range. Whereas early substitution treatment with LT4 is mandatory in all patients with CH due to complete and uncompensated TSH resistance, the benefit of LT4 therapy is less clear in patients with partial TSH resistance and subclinical hypothyroidism. In these cases, careful long-term follow-up can be recommended as well. When the TSH resistance is not fully compensated, LT4 treatment should aim to normalize serum TSH levels, since these individuals have normal responsiveness to TH.

Interestingly, mutations in the *PAX8* gene, which is essential for the formation of T4-producing follicular cells, and defects in the long arm of chromosome 15 can cause TSH resistance as well. Patients often are indistinguishable clinically and by thyroid tests from those with loss-of-function mutations in the *TSHR* gene (67-69). An impaired association of the mutant PAX8 with other transcription factors regulating the *TSHR*, *Tg* and/or *TPO* genes appear to be responsible for the TSH resistance. The exact mechanism of how the defect in chromosome 15 causes TSH resistance is not yet known.

Decreased sensitivity of the pituitary to T4 due to a defect in TH metabolism

TH metabolism is predominantly controlled by the iodothyronine deiodinases. All three deiodinases contain the rare amino acid selenocysteine (Sec) in their catalytic center, which is essential for normal enzyme function. The incorporation of a Sec requires the presence of a Sec insertion sequence (SECIS), which is recognized by SECIS-binding protein SBP2. Subsequently, various factors are recruited ultimately leading to the incorporation of Sec.

So far, no mutations in these deiodinases have been reported. However, a novel clinical syndrome of abnormal thyroid parameters and growth retardation due to mutations in SBP2 was described in 2005 (70). Patients with SBP2 mutations have elevated serum (F)T4 and rT3 levels, low to low-normal serum T3 levels and normal to slightly elevated serum TSH levels. This is very similar to what is observed in *Dio1* and *Dio2* knock-out (KO) mice. In skin fibroblasts of SBP2 patients it was shown that D2 activity was significantly decreased. Similar to *Dio2* KO mice, patients with SBP2 mutations require much higher LT4 doses to suppress serum TSH, whereas the response to LT3 treatment is normal. This is in line with an important role of pituitary D2 in the regulation of the HPT-axis.

Until now, a total of 8 families with mutations in SBP2 have been identified (71). Growth retardation is the most prominent clinical feature, but in addition to these altered thyroid function tests and growth retardation, patients may also suffer form (mild) mental and motor retardation, muscle weakness, hypoglycaemia, impaired hearing and infertility (72). SBP2 mutations will result in impaired synthesis of all selenoproteins (about 30

have been identified so far). At this point, it is unknown which part of the phenotype can be attributed to each of the deiodinases. For a recent excellent review see reference 71.

Decreased sensitivity to TH due to a defect in cellular uptake of TH

The only clinical syndrome due to a defect in TH transport that is known so far, is the Allan-Herndon-Dudley syndrome (AHDS), caused by mutations in the *MCT8* gene. The *MCT8* gene is located on the X-chromosome. Affected males suffer from severe psychomotor retardation and abnormal thyroid function tests (73-75). Serum FT4 concentrations are low or low-normal, whereas serum T3 levels are markedly elevated. In particular during childhood serum T3 levels are far above the upper reference limit. TSH levels vary from normal to elevated. Female carriers do not exhibit neurological features. However, they have serum FT4 levels in between those in affected males and unaffected relatives (76).

The mechanisms behind the altered serum thyroid function tests in patients with AHDS are only partially understood. The identified MCT8 mutations largely or completely impair TH uptake suggesting that this is the basis of the disease (77). Although FT4 levels are low, TSH levels appear inappropriately high in the context of the high serum T3 concentrations pointing towards a disturbed negative feedback of TH at the level of the MCT8 expressing pituitary and/or hypothalamus (76). It was recently shown that TH secretion is disturbed in *MCT8* KO mice (78, 79). This accumulation of T4 within the thyroid gland of patients with AHDS may subsequently lead to an increased intrathyroidal conversion to T3. This will result in an increased T3/T4 ratio in the thyroid and a net increase in T3 secretion via other efflux pathways. Furthermore, kidney T4 levels are increased in *MCT8* KO mice despite the low serum T4 levels, suggesting that T4 is accumulated in the kidney (79). Liver and kidney D1 activity are markedly increased, further increasing the T4 to T3 conversion. The low serum rT3 levels are due to a reduced availability of T4 as well due to the elevated D1 activity, for which rT3 is the preferred substrate.

The neurological phenotype of AHDS patients is much less understood, but a disturbed TH homeostasis in the brain is likely to be the mechanism since neuronal differentiation and myelination are TH-dependent processes (80).

Resistance to TH due to mutations in TRβ (RTHβ)

The *THRB* gene has two splice variants, TRβ1 and TRβ2. Secretion of TSH and TRH is downregulated via binding of T3 to TRβ2 in the hypothalamus and pituitary. More than 20 years ago it was demonstrated that heterozygous mutations in the ligand-binding domain (LBD) of TRβ1 or TRβ2, impairing their hormone binding and/or transcriptional activity, result in elevated serum TH levels and a non-suppressed TSH (81). The mutant TRβ interferes with the function of wild-type (WT) TRβ, resulting in a dominant-negative effect and dominant inheritance (82). Diffuse goiter and sinus tachycardia are the most

common clinical findings in this syndrome of resistance to TH (RTH) due to inactivating mutations in *THRB* (RTHβ). RTHβ patients treated by thyroidectomy and/or radioiodine therapy and substituted with different doses of LT4 show a negative log-linear TSH-FT4 relationship with a slope lower than non-RTHβ patients. This is in agreement with the decreased affinity of the mutated TRβ receptor for T3 (83). Homozygous mutations in TRβ are rare and result in a more severe phenotype (84). The high serum levels of T4 and T3 in patients with RTHβ are accompanied by relatively few symptoms. Symptoms are due to a combination of low TH action in predominantly TRβ-expressing tissues, and TH overexposure in TRα-expressing tissues (85). Until now, more than 1000 patients with mutations in *THRB* have been described, belonging to more than 350 families. See references (71, 86, 87) for a more detailed discussion of the clinical phenotype and underlying pathophysiological mechanism.

Resistance to TH due to mutations in TRα (RTHα)

More recently, the first human patients with heterozygous inactivating mutations in TRα1 were identified (88-90). Because TRβ (and more specifically TRβ2) is the predominant isoform expressed in the pituitary and hypothalamus, patients with TRα1 mutations were expected to have normal levels of TH. However, patients with RTHα have clear alterations of the HPT-axis as well. Despite normal TSH levels, these patients have low (F)T4, high T3, and low rT3 levels. The high T3/T4 ratio as well as the low rT3 levels in all four patients with TRα1 mutations identified so far suggest an altered metabolism of TH. TRα1-PV mutant mice, with a similar frame-shift mutation in TRα1 as two of the three patients, have increased levels of hepatic D1, resulting in an increased T4 to T3 conversion and degradation of rT3 (91). TRα1-/- mice have an impaired regulation of D3, leading to a reduced production of rT3 and degradation of T3 (92, 93). Both an increased D1 and decreased D3 expression may contribute to the particular TH changes in patients with TRα1 mutations. However, this remains to be established in future studies. In addition to the altered HPT-axis setpoint, patients suffer from growth retardation, delayed bone development, delayed motor and mental development, and constipation (88-90, 94). This is in line with an important role of TRα1 in bone, brain, and intestine. All mutations identified so far result in a complete lack of T3 affinity of the receptor but it can be expected that less severe mutations will be identified in the near future. Whether these more subtle mutations in TRα1 will lead to a similar biochemical profile remains to be determined. Please see the original publications for a more detailed description of the phenotype (88-90, 94).

IV. COMMON GENETIC VARIANTS ASSOCIATED WITH THYROID (DYS)FUNCTION (PRE-GWAS)

In the previous chapters we have discussed monogenic causes of thyroid diseases and altered thyroid function tests (i.e., rare variants with large effects). Below, we will discuss the polygenic causes of thyroid diseases and altered thyroid function tests (i.e., common variants with small effects), as identified in linkage and candidate gene studies.

A. Linkage studies

Linkage studies use microsatellite markers spread across the entire genome to detect co-segregation with the phenotype of interest. This technique has been very successful in identifying rare causative variants with large effects for monogenic diseases. To date, only one linkage study on serum TH parameters has been published (95). This study by Panicker *et al.* was performed in 613 dizygotic female twin pairs, and linkage peaks were detected on chr 2q36, 4q32, and 9q34 for TSH, on chr 14q13 and 18q21 for FT4, and on chr 7q36, 8q22, and 18q21 for FT3. No further mutational screening was done to identify the causative variants within the detected linkage region. Furthermore, Liu *et al.* performed a linkage scan in a Chinese family with non-autoimmune hyperthyroidism and detected a linkage peak on chr 14q24.2–31.3 (96). Further mutational screening in this region led to the identification of a new mutation in the *TSHR* gene.

B. Candidate gene analyses on HPT-axis setpoint

Candidate gene association studies have been widely used to study the genetics of complex diseases in the last 10-15 years. Regarding the HPT-axis setpoint they involve the analysis of polymorphic variants in candidate genes (i.e., genes with a role in the regulation of TH production and/or activity) in relation to serum thyroid function tests, thyroid disease and/or TH related endpoints. These studies are relatively easy to perform and can be powered to detect small effects of specific alleles, but replication in independent cohorts is mandatory to avoid false-positive results. Causes of false-positive findings may be small sample size, lack of standardized phenotyping and genotyping, and population stratification when insufficient care has been paid to matching cases and controls (97). However, this can usually be avoided by careful study design and statistical correction for confounding factors (98). Although most studies nowadays take a genome-wide approach by genotyping large numbers of polymorphisms across the genome instead of focusing on a single candidate gene, candidate gene analyses can still be very useful. This is especially the case for variants in which the effects on gene function have been demonstrated *in vitro*.

The first candidate gene study analyzing the effect of genetic variation in relation to the HPT axis studied several TH pathway genes, i.e. all 3 deiodinases, TSHR, THRB, and MCT8 (99). Since then, multiple studies have been published analyzing the association between polymorphisms in candidate genes and the HPT-axis setpoint. Studies vary in quality, and not all studies have been replicated by independent cohorts. Below we aim to give an overview of the consistent findings in literature, as well as the controversies for the different candidate genes that have been analyzed. For genetic variants in genes that were initially identified by GWAS, and that were subsequently confirmed or studied in specific populations by candidate gene analysis, the reader is referred to section V of this review.

Deiodinases

Peripheral TH metabolism is mediated importantly by the 3 deiodinases that catalyze the inner ring and/or outer ring deiodination of the different iodothyronines (100-102). Outer ring deiodination is regarded as an activating pathway, whereas inner ring deiodination is an inactivating pathway. D1 is present in liver, kidney, and thyroid, and plays a key-role in the production of serum T3 from T4 and in the breakdown of the metabolite rT3. Deiodination of rT3 is the most efficient reaction catalyzed by D1. D2 is present in brain, anterior pituitary, brown adipose tissue, thyroid, skeletal muscle, and D2 mRNA has also been detected in the human heart. D2 only has outer ring deiodinase activity and catalyzes the conversion of T4 to T3 and of rT3 to 3,3'-diiodothyronine (3,3'-T2). In tissues such as the brain, D2 is important for local production of T3, whereas D2 in skeletal muscle may also contribute to plasma T3 production. D3 is present in brain, skin, placenta, pregnant uterus, and various fetal tissues. D3 has only inner ring deiodinase activity, and catalyzes the inactivation of T4 and T3 by deiodination to rT3 and 3,3'-T2, respectively. It is the major T3 and T4 inactivating enzyme and contributes to TH homeostasis by protecting tissues from excess TH.

DIO1

Candidate gene analyses of *DIO1* have predominantly focused on 3 polymorphisms, 2 located in the 3'-UTR (DIO1-C785T (rs11206244) and DIO1-A1814G (rs12095080)) and one located in intron 3 of *DIO1* (rs2235544). Initial studies focused on the 2 SNPs in the 3'UTR. In general, The DIO1-785T allele is associated with higher serum (F)T4 and rT3 levels in combination with lower serum (F)T3 concentrations (99, 103-110). As a consequence, this variant allele is associated with lower T3/rT3 and T3/T4 ratios. These data suggest a negative effect of the DIO1-785T variant on D1 activity, since liver D1 plays a key role in the production of serum T3 from T4 and in the breakdown of rT3. The DIO1-A1814G polymorphism has been studies in fewer studies, in which it showed opposite results from the DIO1-785T allele. The DIO1-1814G allele was associated with a higher

T3/rT3 ratio in 2 independent cohorts, suggesting that this variant is associated with an increased D1 activity (99, 103). Based on stronger effects of these variants on serum T3 levels in elderly subjects, it has been proposed that the relative contribution of D2 to serum T3 production decreases with an increase in age (111), but this hypothesis remains to be demonstrated. No effects on mRNA levels, mRNA decay rate or enzyme activity have been demonstrated for any of these two variants in the 3'-UTR of *DIO1* (110).

A candidate gene analysis using a set of 9 tagging polymorphisms to capture the majority of common variation across the *DIO1* gene demonstrated that the intronic SNP rs2235544 is associated with decreased levels of FT4 and rT3 and an increased FT3/FT4 ratio (105). This suggests an increased D1 activity as well in carriers of the variant allele. A similar tagging approach in a large-scale candidate gene analysis of 68 genes also showed a significant association of rs2235544 with FT4 in the same direction (104). This association was recently confirmed in a meta-analysis of GWAS for TSH and FT4 (see section VI (112)). None of the *DIO1* SNPs is associated with serum TSH levels, which is likely due to the fact that circulating T4 and T3 are affected in opposite directions.

DIO2

Candidate gene analyses of *DIO2* have predominantly focused on 2 polymorphisms, located in exon 1 (DIO2-ORFa-Glu3Asp, rs12885300) and exon 2 (DIO2-Thr92Ala, rs225014). DIO2- Thr92Ala, the best studied polymorphism in DIO2 *in vitro* as well as *in vivo*, is not associated with any change in circulating TH and/or TSH levels. This has been demonstrated in multiple populations with and without thyroid disease (99, 103, 105, 109, 113-117). The DIO2-Thr92Ala polymorphism is located in a part of the protein that is important for stability (118), but *in-vitro* studies have produced inconsistent results about its functionality. DIO2-92Ala was associated with decreased D2 activity in skeletal muscle and thyroid samples of homozygous patients with type 2 diabetes mellitus (119). In contrast, cells transiently expressing the Thr92Ala form of D2 display similar kinetic properties with either T4 or rT3 as substrate as compared to wild-type D2 (99, 119). This discrepancy might be explained by linkage to a functional variant elsewhere in the genome.

In contrast, the DIO2-ORFa-Gly3Asp polymorphism was associated with an increased T3/T4 ratio in one study, suggesting an increase in deiodinase activity (117). However, this finding has not been replicated in other cohorts (103). This could be due to differences in population characteristics such as age (111, 117), or could be due to a chance finding.

DIO2-ORFa-Glu3Asp is located in a short open reading frame (sORF) within the 5'-UTR. This sORF is considered to be primarily responsible for the inhibitory effect of the 5'-UTR on DIO2 translation, since mutation of the start codon of the sORF completely abolished this inhibitory effect (120). *In vitro* analysis of the DIO2-ORFa-Glu3Asp polymorphism

showed that the minor Asp variant was associated with an increased gene transcription and increased D2 activity (121), suggesting that the observed associations with the T3/T4 ratio may very well be true effects.

Although none of the D2 variants were associated with serum TSH levels, there is evidence that the HPT axis setpoint is affected. Hoftijzer *et al.* studied the relations between serum TSH and FT4 levels in patients treated for differentiated thyroid carcinoma and showed that the negative feedback of FT4 on TSH was weaker in homozygous carriers of the DIO2-ORFa-3Asp allele (122). Furthermore, homozygous subjects showed a delayed rise in serum T3 for DIO2-92Ala and a blunted rise in FT4 for DIO2-ORFa-3Asp, respectively, indicating subtle alterations in intrathyroidal conversion of T4 into T3 (123, 124). Finally, there is one study in patients with differentiated thyroid cancer after thyreoidectomy reporting that carriers of the DIO2-92Ala allele need a higher dose of LT4 to suppress TSH (125). However, the fact that serum FT4 and FT3 levels were not different between the genotype groups in this study, and the fact that the findings could not be replicated in a similar cohort of patients with DTC, does not support an altered pituitary setpoint in these patients (126).

DIO3

The *DIO3* gene is an imprinted gene (127), hampering candidate gene analysis studies. The few candidate studies that have studied the *DIO3* gene in relation to the HPT-axis setpoint, did not find any association (99, 104, 105).

TSH receptor

A variety of activating as well as inactivating mutations have been identified within the *TSHR* gene (128), as discussed in sections II and III. One of the best studied polymorphisms within the *TSHR* gene is a C-to-G transition at position 2281 resulting in a Asp727Glu substitution in the cytoplasmic tail of TSHR. Several candidate gene analyses have shown that this genetic variant is significantly associated with serum TSH levels (99, 129, 130). A study analyzing the effect of this particular SNP in a healthy population of twins demonstrated that, although the effect on TSH was clearly significant, the proportion of genetic variation that could be accounted for by this Asp727Glu polymorphism was very small (0.91% of the overall variation in TSH) (129). No statistically significant evidence was found for interaction between the genotype and environmental factors such as iodine intake and cigarette smoking. This polymorphism is associated with lower TSH levels but normal FT4 levels in all 3 studies (99, 129, 130), a finding we were able to replicate in another independent Dutch population of more than 1,000 subjects (unpublished data by van der Deure, Peeters and Visser). A study in preeclamptic women also showed lower levels of TSH but no data on FT4 were provided in this study (131). These results suggest that the setpoint of the HPT axis is affected by this particular poly-

morphism, due to an altered sensitivity of the receptor. An increased activity of TSHR in carriers of the Glu727 allele would require less TSH to produce normal FT4 levels. There is indeed one *in vitro* study showing that the TSHR-Glu727 variant results in an increased cAMP response of the receptor to TSH (132). However, others have not been able to replicate this finding (133, 134). This suggests that the Asp727Glu polymorphism might also be linked to another functional polymorphism elsewhere in the *TSHR* gene.

It should be noted, however, that these findings were not confirmed in a large-scale association analysis of 68 TH pathway genes nor in several GWAS (see section V) (104, 112, 135-137).

Thyroid hormone receptors

While many studies have been published on associations between clinical endpoints and polymorphisms in other nuclear receptors, such as the estrogen and glucocorticoid receptors, relatively little is known about functional polymorphisms in TRs. Since patients with mutations in TRα and TRβ have clear alterations in serum thyroid function tests (71, 87, 138), it could be expected that polymorphisms in these receptors would also be associated with alterations in the HPT axis setpoint.

By sequencing all *THRA* and *THRB* exons and their flanking regions in more than 100 alleles, 8 SNPs were identified in *THRA* and 7 in *THRB* (99, 139). The SNPs in *THRA* were not associated with serum thyroid parameters. One SNP in *THRB* was associated with higher levels of TSH in one population, but this could not be replicated in a second, older population. Genetic variation in these receptors did not show a significant association in the large-scale association analysis of 68 TH pathway genes or the GWAS performed so far (104, 112, 136, 137).

Thyroid hormone transporters

The first TH transporter identified at the molecular level was organic anion transporter subtype 3 (OATP3) (140). In subsequent years, it has been demonstrated that thyroid hormones are transported by various types of transporters, including the Na^+/taurocholate cotransporting polypeptide (NTCP, SLC10A1) (141, 142), the heterodimeric L-type amino acid transporters LAT1 and LAT2 (143), various members of the OATP family (144, 145), and MCT8 (SL10A2) and MCT10 (SLC16A10) (146, 147). Most of these transporters accept a variety of ligands, with MCT8, and to a lesser extent MCT10 and OATP1C1 as exceptions. Very little is known about the possible effects of genetic variation in NTCP, LAT1 and LAT2 (148). For this reason we will only discuss MCT8, MCT10, and several members of the OATP family.

MCT8 and MCT10

Only few studies exist on the relationship between polymorphisms in *MCT8*, located on the X-chromosome, and serum TH levels (104, 148-150). The largest and most recent study by Roef *et al.* found that 2 SNPs in *MCT8* were related to circulating TH levels in men but not in women. The rs5937843 polymorphism (G/T) in intron 5 of the *MCT8* gene was inversely associated with FT4 levels (150). This is in line with a previous, smaller study in which carriers of another polymorphism in intron 5 of the *MCT8* gene (rs5937843) had lower FT4 levels than WT male subjects. This finding could not be replicated in the homozygous female carriers in the same population (148). Roef *et al.* also found that a nonsynonymous polymorphism (Ser107Pro; rs6647476) was significantly associated with lower serum FT3 levels in males (150), which is in contrast to previous smaller studies (148, 149). No *in vitro* effects of this polymorphism could be demonstrated so far (148, 149).

The few studies of genetic variation in the *MCT10* gene failed to show any significant association with serum thyroid parameters (104, 148, 149).

OATPs

The OATPs are a large family of transporters responsible for Na^+-independent transmembrane transport of amphipathic organic compounds, including bile salts, bromosulfophthalein (BSP), steroid conjugates, and numerous drugs (145). In humans, 11 OATPs have been identified, all containing 12 transmembrane domains. Whereas most OATPs proteins are expressed in multiple tissues, some members show a more tissue-specific distribution (151). OATP1B1 and OATP1B3 are exclusively expressed in liver (152, 153), whereas OATP1C1 is only present in the brain and in the Leydig cells of the testis (154).

Several members of the large OATP family facilitate uptake of TH, including members of the OATP1 subfamily: OATP1A2 (155), OATP1B1 (156, 157), OATP1B3 (157), and OATP1C1 (154). This family has best been studied in relation to genetic variation and thyroid function.

Although the OATP1A2-Glu172Asp (rs11568563) variant showed decreased transport activity *in vitro*, this variant was not associated with serum thyroid parameters in 2 Caucasian populations (145).

Polymorphisms in the *OATP1B1* and *OATP1B3* genes have been extensively studied as they impact on the interindividual variability of drug disposition and drug response (158). To date, only one study has focused on associations between a polymorphism in the *OATP1B1* gene, OATP1B1-Val174Ala, and serum TH levels. OATP1B1 preferentially transports sulfated hormones, i.e. T4S, T3S, rT3S, and E1S and the OATP1B1-Ala174 variant shows 40% lower transport of these substrates than OATP1B1-Val174 (159). This is in line with a decreased activity of this variant in transporting other substrates as well (160). These *in vitro* data are supported by population-based data, showing that this

polymorphism is associated with higher serum T4S and E1S levels (159). No studies on associations between genetic variation in the *OATP1B*3 gene and serum TH levels have yet been published.

OATP1C1, which is capable of T4, T4S, and rT3 transport, is almost exclusively expressed at the blood–brain barrier, suggesting a critical role for T4 uptake into the brain. Polymorphisms in the OATP1C1 gene are not consistently associated with serum TH levels (150, 161).

None of the SNPs in these transporters showed a significant association in the previously mentioned large-scale association analysis of 68 TH pathway genes or the GWAS performed so far (104, 112, 135-137).

C. Candidate gene analyses of TH related clinical endpoints

Since TH is such a pleiotropic hormone, with effects on almost all tissues and organ systems, it can be expected that polymorphic variants affecting local TH action will have consequences for a variety of clinical phenotypes (111, 162). Although the effects of common variants are usually very small, they exert their effects throughout life. In recent years, multiple studies have studied the association between genetic variation and TH pathway genes (especially *DIO1* and *DIO2*) and a large variety of clinical endpoints, varying from osteoporosis to vulnerability to disease. Interestingly, most of the effects of genetic variation were seen independently of serum TH levels, highlighting the importance of local regulation of TH in tissues.

Neurocognitive function

In euthyroid patients

The brain is particularly sensitive to relatively small changes in TH, as is illustrated by the increased risk of cognitive complaints and depression in patients with clinical and subclinical thyroid disease (163, 164). In two relatively small studies of patients treated for depression (n=96 and n=64 respectively), D2 polymorphisms were not associated with response to paroxetine (165) or potentiation of sertraline by T3 addition (166). However, genetic variation associated with a lower D1 activity was associated with a better response to potentiation of sertraline by T3 addition in depressed patients (166). In a large study of 3 cohorts with high-risk subjects (total N>1300 subjects), DIO1-785T (rs11206244) was associated with increased FT4 levels, as well as with increased risk of lifetime major depression in white females (107). In a Chinese population, D2 polymorphisms were associated with an increased risk of bipolar disorder (167), but these data have not been replicated in a separate cohort.

Thyroid disorders have also been associated with dementia (168, 169). However, studies using MRI did not find any association between *DIO1*, *DIO2*, or *THRA* polymorphisms

and markers of early Alzheimers'dementia (170, 171). Interestingly, the circadian clock gene *REV-ERBα*, which overlaps with the *THRA* gene and interferes with *THRA* expression, was associated with white matter lesions (Chapter 11) (171).

In patients with TH replacement therapy

Although no clear effects of polymorphisms in TH pathway genes have yet been described on cognition and depression in euthyroid subjects, effects may be more clear in patients on TH substitution therapy (172, 173). A small but significant proportion of thyroidectomized patients on LT4 replacement have low serum T3 despite normal TSH and high-normal FT4 levels (174). These patients may be more vulnerable to genetic variants affecting local T3 production. Perhaps in these patients a reduced D2 activity cannot fully compensate for the absence of the thyroidal T3 production (162). This might explain why a subgroup of patients who receive TH replacement have decreased wellbeing (175). Two studies analyzed the effects of genetic variation in deiodinases in hypothyroid patients (113, 172). Both studies were secondary analyses of prospective trials comparing LT3/LT4 combination therapy to LT4 alone in primary hypothyroidism. The largest study showed that genetic variation in *DIO2* (i.e. Thr92Ala, rs225014) was associated with impaired psychological wellbeing at baseline (172). It should be noted that although 16 polymorphisms were tested in this study, no multiple testing correction was applied because the study was underpowered to detect all but very large differential gene-treatment effects. Therefore, these results need replication in an independent cohort. Interestingly, this polymorphism was also associated with the response to combination LT3/LT4 treatment in this study of 552 patients. This is in contrast with the second study (in 141 patients), which did not find an association of D2 polymorphisms with measures of well-being, neurocognitive functioning or treatment preference (113). However, the DIO2-Thr92Ala polymorphism did show a similar but non-significant trend with impaired wellbeing.

The third study analyzed OATP1C1, a T4 transporter expressed at the blood brain barrier (see above), in the same 141 patients (173). Polymorphisms in this transporter were associated with fatigue and depression, but did not explain differences in neurocognitive functioning or preference for LT3/LT4 combination therapy. It should be realized that all these studies retrospectively genotyped the participants. Therefore, these results have to be confirmed in randomized prospective studies.

Osteoporosis and other bone related phenotypes

TH is crucial for bone development and maintenance. Hyperthyroidism results in bone loss, osteoporosis and an increased risk of fractures, whereas hypothyroidism has been reported to result in increased cortical thickness (176). Both subclinical hyper- and

hypothyroidism have been related to fracture risk as well, although conflicting results have been reported (163, 177).

Several studies have investigated variation in TH pathway genes and osteoporosis. The D2-Thr92Ala polymorphism was associated with a decreased bone mineral density (BMD) as well as markers of bone turnover in 154 patients with differentiated thyroid cancer (178), suggesting a role in bone homeostasis. This is in line with data from *Dio2* KO mice, which have increased fracture susceptibility due to an essential role for D2 in osteoblasts in reaching optimal bone strength and mineralization (179). Polymorphisms in *TSHR* have been related to bone formation as well. Carriers of the TSHR-727Glu allele had 2.3% higher femoral neck BMD in almost 5000 subjects (130). This association with BMD persisted when TSH was added to the regression model. This finding was recently confirmed in a smaller population of 150 males with osteoporosis (180).

Genetic variation in the deiodinases has also been associated with osteoarthritis. A genomewide linkage scan identified an association between the DIO2-Thr92Ala polymorphism and generalized osteoarthritis, which was replicated in three independent cohorts with a total of more than 4000 subjects (181). The same authors later demonstrated increased D2 protein in cartilage of patients with osteoarthritis, as well as allelic imbalance of the DIO2 mRNA. In heterozygous carriers, mRNA from the variant allele was more abundant than from the WT allele (182).

Also genetic variation in *DIO3* has been implicated in osteoarthritis. The minor allele of DIO3-rs945006 showed suggestive evidence for a protective association in a meta-analyses of 4 European populations containing more than 3000 cases of osteoarthritis (183), but these findings still require independent replication. As discussed in Chapter 10, we did not find any associations of common genetic variation in the *THRA* locus with BMD, bone geometry or fracture risk.

Metabolic syndrome

The first study that associated a TH pathway gene with a clinical endpoint concerned the DIO2-Thr92Ala polymorphism in relation to insulin resistance (184). Since then, various studies have analyzed polymorphisms in different TH pathway genes in relation to insulin resistance and other parameters of the metabolic syndrome.

Diabetes and insulin resistance

The initial study by Mentuccia *et al.* analyzed a population of 135 non-diabetic women undergoing euglycemic-hyperinsulinemic clamps to determine insulin sensitivity (184). In these women, a consistent strong relationship between the Thr92Ala polymorphism and lower glucose disposal rate was observed, pointing towards an increased risk for insulin resistance. Subsequent studies in larger populations showed conflicting results in populations with different characteristics. In multiple non-diabetic cohorts of varying

size but with a total of more than 10,000 subjects, no association of this polymorphism with diabetes or insulin resistance could be demonstrated (115, 116, 185-189). However, in one case-control study of 1057 type 2 diabetic (DM2) and 516 non-diabetic subjects, the DIO2-THr92Ala variant was associated with a significantly increased risk of DM2 (190). A subsequent meta-analysis of the available case-control studies in 2010 resulted in a significantly increased risk as well, with a pooled odds ratio of 1.18 (95% CI 1.03-1.36, *P*=0.02) (190).

Three studies analyzed insulin sensitivity in patients with DM2, using HOMA-IR and fasting insulin. All 3 studies showed an increased insulin resistance in homozygous carriers of the DIO2-Thr92Ala variant allele (119, 190, 191). Altogether, these data suggest that genetic variation in *DIO2* is indeed associated with a mild increase in insulin resistance. So far, however, large GWAS of diabetes have not identified the *DIO2* gene as a susceptibility locus. One study found an association between the TSHR-Asp727Glu polymorphism (TSHR is known to be expressed in adipose tissue) and insulin resistance in 349 non-diabetic elderly men, but this finding has not yet been confirmed in an independent replication cohort (189).

Blood pressure

Studies analyzing the association of the *DIO2*-Thr92Ala variant in relation to blood pressure show conflicting results as well. Two studies in relatively small populations (n=372-590) showed a positive association between the *DIO2*-Thr92Ala variant and blood pressure (115, 185), but this finding was not replicated in 4 larger studies of non-diabetic and diabetic patients (190, 192-194).

One study has reported on the association of genetic variation in the *TRHR* gene and hypertension, but this study has not been replicated either (195). In a population-based study of normotensive subjects, we did not find any associations of these *TRHR* SNPs with blood pressure (van der Deure, unpublished results).

Dyslipidemia

Hypothyroidism results in a marked increase in total and LDL cholesterol levels, and several (but not all) cross-sectional studies have suggested an association of subclinical hypothyroidism with total and LDL cholesterol levels as well (163). As a consequence, several studies analyzing TH pathway genes (more specifically *DIO2*) in relation to insulin resitance also studied its relation with total, HDL and LDL cholesterol as well as triglycerides, but none of these studies found significant associations (116, 119, 185, 186, 191). However, considering the fact that D2 is not expressed in liver and *DIO2*-Thr92Ala does not affect circulating TH levels, a direct effect of this polymorphism on cholesterol metabolism would not be expected either.

To the best of our knowledge, no candidate gene studies have been published on the association between genes involved in TH uptake or metabolism in the liver, such as *NTCP, OATP1B1* and *DIO1*, and dyslipidemia. Interestingly, OATP1B1 is also involved in liver uptake of statins. A GWAS in patients using statins revealed that a polymorphism in this transporter (rs4363657) is associated with statin induced myopathy (196). This is variant is in almost complete linkage disequilibrium with rs4149056 (R^2=0.97), which is associated with increased serum T4S levels (159).

Body composition

The only study reporting on the association between genetic variation in *DIO1* and BMI was negative (106). However, *DIO1* polymorphisms putatively associated with decreased D1 activity were also associated with higher serum free IGF-1 levels in 2 independent populations (106). The pathophysiological significance of this association with IGF-1 was supported by increased muscle strength and muscle mass in elderly carriers of this variant allele. However, these data have not yet been replicated, nor have other studies been pulished to date on IGF-1 related endpoints such as body length. Almost all studies of the *DIO2*-Thr92Ala polymorphism in relation to body composition showed a lack of association with BMI (116, 119, 125, 126, 185-187), except for one small study (n = 139) which showed that treated Hashimoto's thyroiditis patients homozygous for the Ala allele have an increased BMI (126).

Susceptibility to disease

Considering the importance of TH for development and functioning of almost all tissues and organ systems, including the immune system, one could speculate that genetic variation in TH pathway genes might also be associated with susceptibility to disease. It has for example been shown that D3 is highly expressed in infiltrating neutrophilic granulocytes in response to acute bacterial infection and that *Dio3* KO mice have an impaired bacterial clearance (197, 198). Furthermore, increased lung D2 expression has been observed in a mouse model of acute lung injury, with expression directly increasing with the extent of lung injury (199). Mice with reduced levels of D2 expression (by silencing RNA) showed increased bronchoalveolar lavage protein and leukocytes pointing to increased lung injury, suggesting a protective role of D2 in severe sepsis-associated acute lung injury. In the same study it was also reported that the DIO2-92Ala allele was protective in patients with severe sepsis and severe sepsis-associated acute lung injury. These findings, suggesting that increased *DIO2* expression may dampen the acute lung injury inflammatory response are in contrast with *in vitro* studies suggesting that the DIO2-92Ala variant is associated with a decreased activity (119). No data on genetic variants of *DIO3* with regard to inflammatory response are yet available.

TH and its essential trace element iodine are crucial for normal brain development. Two studies investigated if the risk of mental retardation was associated with genetic variation in *DIO2*, both of which were conducted in an iodine-deficient area (200, 201). Several SNPs were associated with mental retardation, but DIO2-Thr92Ala was only investigated in the study by Zhang *et al*., and was not associated with mental retardation (201). Genetic variation in the *TSHR* was also studied in one of these populations, but no associations with mental retardation were found (202).

Both hypo- and hyperthyroidism can have important vascular consequences, such as endothelial cell dysfunction (203), and thyroid dysfunction during pregnancy is associated with hypertensive disorders during pregnancy (204). A small case-control study of 50 cases of preeclampsia showed an association of TSHR-Asp727Glu and DIO1-C785T with severity of preeclampsia (108, 131). However, these results first need to be replicated in larger independent cohorts.

D. Linkage and candidate gene studies in autoimmune thyroid disease

Since the 1970s, a number of loci have been consistently associated with the risk of AITD, including *HLA Class I and II*, *CTLA4*, *PTPN22*, *IL2RA*, *TSHR* and *FCRL3*. The discussion of these studies is beyond the scope of this review and we therefore refer to a number of comprehensive reviews published over the years (205-210). For the results of a large AITD candidate gene analysis using the ImmunoChip, please see the original publication by Cooper *et al.* and the review by Simmonds *et al.* (208, 211). The new loci more recently discovered by GWAS are covered in section V.

V. COMMON GENETIC VARIANTS ASSOCIATED WITH THYROID (DYS)FUNCTION (GWAS)

As discussed above, linkage and candidate gene studies have identified only a limited number of genes consistently associated with thyroid function or dysfunction. More recently, GWAS have had much more success in identifying genetic variants associated with thyroid-related traits. These studies have been made possible by advances in genotyping techniques, in which 100,000 to 500,000 variants are genotyped across the whole genome, and tested against the phenotype of interest. A stringent p-value threshold of $P < 5x10^{-8}$ is used to prevent false-positive results due to multiple testing. As can be expected for 'common' variants with an allele frequency > 1-5%, effect sizes are small and therefore large populations and often meta-analyses of populations are needed to reach sufficient statistical power. In this way, in the last 7 years GWAS have identified many genetic variants associated with thyroid-related traits, the results of which are discussed below.

A. GWAS on hypothyroidism

Only two GWAS on hypothyroidism have been published. Using electronic medical records for case identification, Denny *et al.* published the first GWAS on hypothyroidism in 2011 (212). A drawback of the second hypothyroidism GWAS by Eriksson *et al.* was that the identification of hypothyroidism cases was based on web-based questionnaires (213). The five GWAS significant hits in these studies are discussed below.

Loci with an established role in autoimmunity

Four of the GWAS significant hypothyroidism loci had an established role in autoimmunity and include the *HLA class I region*, *PTPN22, SH2B3* and *VAV3* loci. The *HLA class I region* emerged as a possible candidate region in the hypothyroidism GWAS by Eriksson *et al* (213). The detected rs2517532 SNP is located between the *HLA-E* and *HLA-C* genes. Variation in *HLA-C* has previously been also associated with Graves' disease (214). These HLA class I region molecules play an important role in antigen presentation, including viral antigens, which have been suggested to play an important role in triggering AITD (208, 215). However, the exact effects of these SNPs on antigen presentation remain unclear at present.

As discussed, early candidate gene studies had already associated genetic variation in *PTPN22* with AITD (206, 208, 209). The Eriksson *et al.* hypothyroidism GWAS also detected a significant association with rs6679677, which is located near *PTPN22* (213). This SNP is in high LD with the missense mutation R620W, which has been previously associated with Hashimoto's thyroiditis (216). The results of a study by Menard *et al.* suggested that this mutation results in impaired removal of autoreactive B cells, as well as the upregulation of genes such as *CD40*, *TRAF1*, and *IRF5*, which encode proteins that promote B cell activation and have been identified as susceptibility genes also associated with other autoimmune disorders (217). Furthermore, variations in *PTPN22*, and specifically R620W, have been associated with various autoimmune disorders, such as type 1 diabetes, rheumatoid arthritis, and systemic lupus erythematosus (218-221).

SH2B3 encodes the adaptor protein LNK, a key negative regulator of T-cell cytokine signaling, which plays a critical role in hematopoiesis (222). Furthermore, LNK has been shown to play an important role in the expansion and function of early hematopoietic progenitors (223). Eriksson *et al.* were the first to find an association of genetic variation in *SH2B3* with hypothyroidism (213). The identified variant rs3184504 causes a Trp262Arg substitution, and had already been associated with other autoimmune diseases, such as celiac disease, DM1, vitiligo, and rheumatoid arthritis (218, 224-226). It remains to be determined how this mutation affects the protein structure and its function.

Finally, the *VAV3* locus emerged as a potential candidate locus in the hypothyroidism GWAS by Eriksson *et al* (213). VAV3 is a guanine nucleotide exchange factor for Rho and Rac family GTPases. VAV3 is expressed in the thyroid and has been shown to be down

regulated in some subtypes of thyroid tumors (227). However, there is no clear role for VAV3 in human thyroid physiology or autoimmunity. However, Fujikawa *et al.* has shown in mice that the VAV family proteins, including VAV3, play an important role in lymphocyte development and activation (228). Mouse *VAV3* has furthermore been suggested as a candidate gene for type 1 diabetes (229). Future human studies should investigate the potential role of VAV3 in human thyroid autoimmunity.

FOXE1

As discussed in section II, FOXE1 is a transcription factor essential in thyroid development, mutations in which can lead to CH. *FOXE1* was also identified as a candidate gene for hypothyroidism in the GWAS by Denny *et al.* (212). Associations were detected with 4 SNPs which were in strong LD, located 58-71 kb upstream from the *FOXE1* gene. The strongest association was with rs7850258, which was replicated in an independent set. One of the 4 SNPs (rs925489) was also the strongest hit in the hypothyroidism GWAS by Eriksson *et al* (213). The identified SNPs in these studies have also been associated with other types of thyroid diseases. Several studies have detected associations between *FOXE1* SNPs and the risk of follicular and papillary thyroid cancer (230-232). In this context it is interesting to note that higher TSH levels have been associated with an increased risk of thyroid cancer and advanced-stage disease (233). As a second part of the hypothyroidism GWAS of Denny *et al.*, a so-called phenome-wide association analysis was performed of the identified top *FOXE1* SNP (rs7850258) (212). In this way, associations were found with thyroiditis, nodular and multinodular goiters, and thyrotoxicosis. However, the associations in this phenome-wide approach need replication in independent cohorts and the underlying biological mechanisms need to be clarified in future studies.

B. GWAS on hyperthyroidism

Two GWAS have been published for Graves' disease. Chu *et al.* performed the first Graves' disease GWAS in the Chinese Han population (234), and an extension of this study was published in 2013 by Zhao *et al* (235). These studies confirmed previously identified candidate genes, including HLA class II region genes, *TSHR*, *CTLA4*, and *FCRL3*, but also identified new candidates, which are discussed below.

Loci with an established role in autoimmunity

Similar to the hypothyroidism associated loci, most of the GWAS significant loci for hyperthyroidism have a known role in autoimmunity or immunity in general, including *Tg, GPR174-ITM2A,* the *C1QTNF6-RAC2* locus, *SLAMF6*, and the 6q27 and 14q32.2 loci.

Various studies have investigated the relation between genetic variation in the Tg locus and AITD, which have led to conflicting results (209, 210, 236). Zhao *et al.* was the first to report an association between Tg and Graves' disease (GD) in a GWAS setting

(235). The authors additionally showed that the top SNP rs2294025 influenced Tg splicing, skipping exon 46. Tg is located on chrom. 8q24.22, and is a key auto-antigen in the pathogenesis of GD with 40-70 % of the GD patients having Tg-antibodies (TgAbs) (237). The role of Tg in AITD is underlined by the fact that Jacobsen *et al.* generated an AITD mouse model by immunizing mice with human Tg (238). Furthermore, Nielsen *et al.* have shown that TgAbs promote the formation of complement-activating complexes, binding of immune complexes to B-cells, and the proliferation of B- and T-cell subsets (239).

The *GPR174-ITM2A* was the most significant new hit in the GD GWAS by Zhao *et al* (235). It is located on chrom. Xq21.1, which is of interest as GD is more prevalent among women. The top SNP, rs5912838, lies between the *GPR174* and *ITM2A* genes. *ITM2A* has been shown to escape X-chromosome inactivation, and is induced during thymocyte selection and T-cell activation (240-242). By then, little was known about GPR174, encoding a protein belonging to G-protein coupled receptor family. However, a X-chromosome specific follow-up study on the GWAS by Chu *et al.*, showed that a non-synonymous SNP located in *GPR174* (rs3827440) was significantly associated with GD and affected GPR174 mRNA levels (243). GPR174 was furthermore shown to be widely expressed in immune related tissues such as spleen, lymph nodes, thymus, bone marrow, and leucocytes, with a moderate expression in the thyroid. Therefore, future studies should investigate the distinct roles of GPR174 and ITM2A in the pathogenesis of GD.

Also genetic variation in the *C1QTNF6-RAC2* locus has been associated with GD in the GWAS by Zhao *et al.* (235). This locus has previously been associated with various autoimmune diseases, such as Crohn's disease, DM1 and multiple sclerosis (244, 245). RAC2 has been shown to play an important role in both T- and B-cell development and signaling (246-249) and *RAC2* mutations have been detected in human neutrophil immunodeficiency syndrome (250). However, the role of *C1QTNF6* in (thyroid) autoimmunity remains to be established.

It is already known for several years that the signaling lymphocytic activation molecule (SLAM) pathway members, including SLAMF6, have an important role in T-cell stimulation as well as in the pathogenesis of lupus in mice (251, 252). Recently, it has also been shown by Menard *et al.* that this pathway also influences B-cell tolerance in humans, which has an important role in autoimmunity (253). *SLAMF6* is located on chrom. 1q23.2, encoding Ly108. This locus was also among the newly identified hits in the GWAS by Zhao et al., in which rs1265883 in intron 1 of *SLAMF6* was associated with an increased risk of GD (235).

The most significant new hit in the GD GWAS by Chu *et al.* was the 6q27 locus (234). This locus contains the *RNASET2*, *FGFR1OP*, and *CCR6* genes**.** The top SNP rs9355610 was associated with RNASET2 and FGFR1OP expression levels. Previous GWAS have also associated this locus with other autoimmune diseases, including rheumatoid arthritis, Crohn's disease, and vitiligo (226, 254, 255). Although these studies suggest a role for

these genes in immune regulation, little is known about the exact molecular mechanisms behind the observed associations, which needs to be investigated in future studies.

Finally, the 14q32.2 locus emerged as a possible GD candidate gene in the GWAS by Zhao *et al.* (235). The top SNP was located in an intergenic region, where the authors identified 2 new non-coding RNAs which they designated C14orf64 and "GD Candidate Gene at 14q32.2" (*GDCG14q32.2*). These were shown to be highly expressed in immune related tissues, including thymus and CD4+ and CD8+ T-cells. Finally, it is interesting to note that the 14q32.2 locus has previously been identified as a DM1 susceptibility locus (218).

Other loci

The *ABO* and 4p14 loci were also detected as susceptibility loci for hyperthyroidism, but do not have an established role in (auto)immunity (234, 235). The *ABO* gene encodes a glycosyltransferase that catalyzes the transfer of carbohydrates to the H antigen, forming the antigenic structure of the ABO blood groups. In recent years, the *ABO* gene has been associated with a wide range of diseases, such as myocardial infarction (256), ischemic stroke (257), venous thromboembolism (258), and oesophageal and pancreatic cancer (259). However, there is no clear documented role for ABO in thyroid physiology or autoimmunity. The mechanisms by which genetic variation in *ABO* alters the risk of GD therefore remain to be explored.

The 4p14 locus was identified as a new GD candidate locus in the GWAS by Chu et al (234). The top SNP rs683215 is located between the *CHRNA9* and *RHOH* genes, but is not in LD with variants in these genes. However, the authors identified a new gene 5 kb downstream of rs683215, which they designated "GD Candidate Gene at 4p14" (*GDCG4p14*), which was shown to be highly expressed in CD4+ and CD8+ T-cell subsets. Finally, rs683215 was shown to be correlated with *GDCG4p14* and *CHRNA9* expression levels. However, the exact roles of these genes in the pathogenesis of GD need to be established in future studies.

C. GWAS on TPO-antibodies

Whereas the previously discussed GWAS included cases with GD or hypothyroidism, we recently took a different approach to identify new AITD susceptibility loci, namely by performing a GWAS on TPOAbs including 27,200 subjects from 16 populations (Figure 2) (260). As TPOAb-positivity is not only associated with an increased risk of hypothyroidism (Hashimoto's thyroiditis), but also with an increased risk of hyperthyroidism (GD), GWAS significant hits were additionally tested in relation to hypo- and hyperthyroidism. We refer the reader to Chapter 9, where the results and potential implications of these findings have been extensively discussed.

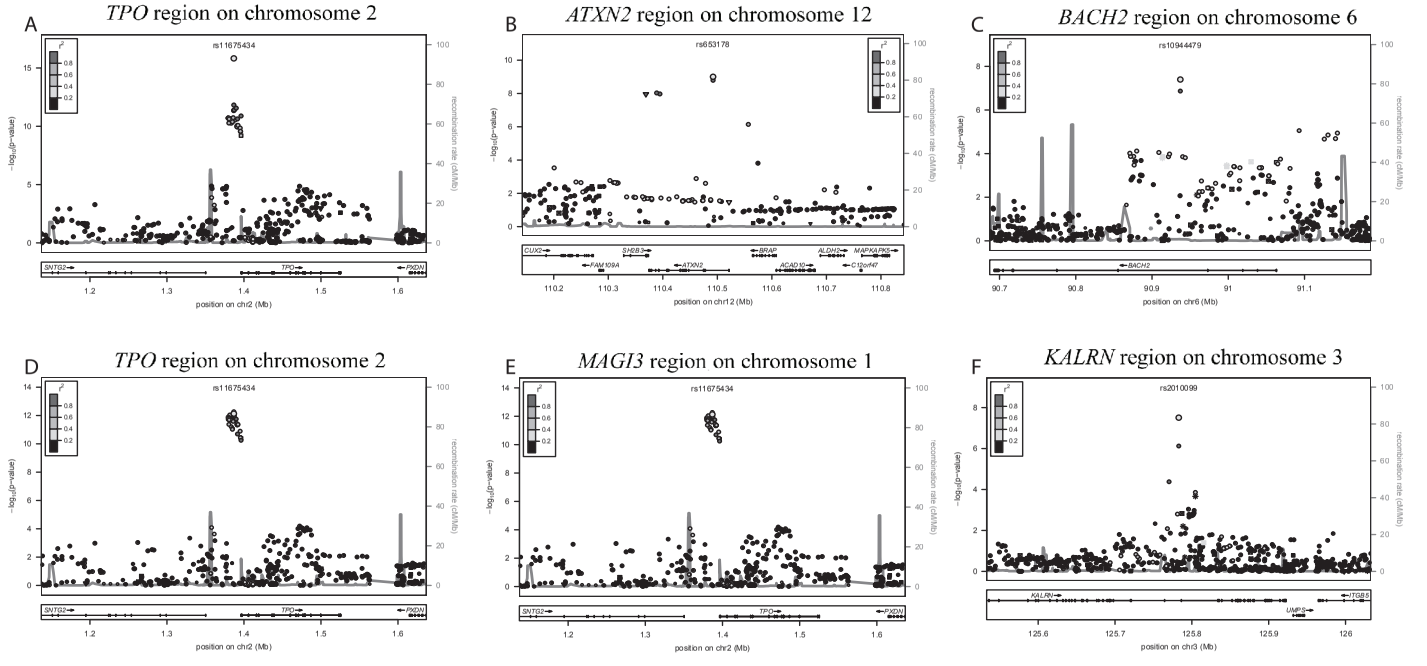


Figure 1. Genome wide association studies meta-analyses: Loci associated with TPOAb-positivity (a-c) and TPOAb levels (d-f) on a genome-wide level of significance. Regional association plots of the genome-wide significant loci associated with TPOAb-positivity (a-c) and TPOAb levels (d-f). The y-axis on the left indicates the $-\log_{10}$ *P* value for the association with TPOAb –positivity (a-c) or TPOAb levels (d-f). Single nucleotide polymorphisms (SNPs) are plotted on the x-axis according to their chromosomal position against the association with the phenotype on the y-axis. The most significant stage 1 SNP is indicated in purple. The combined stage 1 and 2 result of this SNP is indicated in yellow. The SNPs surrounding the most significant SNP are color-coded to reflect their LD with this SNP. Symbols reflect functional genomic annotation, as indicated in the legend. The blue y-axes on the right of each plot indicate the estimated recombination rates (based on HapMap Phase II); the bottom of each panel shows the respective annotated genes at the locus and their transcriptional direction. Mb, megabases
A color figure is available at: http://www.plosgenetics.org/article/info%3Adoi%2F10.1371%2Fjournal.pgen.1004123

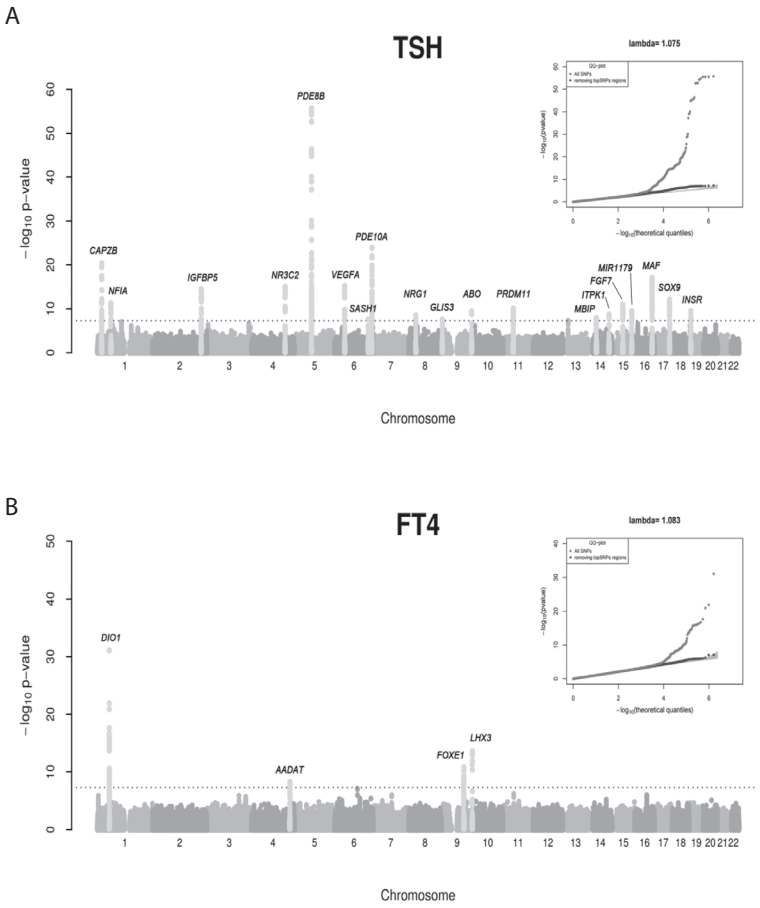


Figure 3 Manhattan plots from GWAS meta-analysis results of serum TSH (panel A) and FT4 (panel B) levels (112). SNPs are plotted on the x axis according to their position (build 36) on each chromosome against association with TSH (A) and FT4 (B) on the y axis (shown as –log10 P value) in. The loci highlighted in green are those that reached genome-wide significance. In each panel, quantile-quantile plots obtained with all SNPs (red dots) and after removal of SNPs within associated regions (blue dots) are also shown. The gray area corresponds to the 90% confidence region from a null distribution of P values (generated from 100 simulations). A color figure is available at: http://www.plosgenetics.org/article/info%3Adoi%2F10.1371%2Fjournal.pgen.1003266#s5

D. GWAS on hypothalamus-pituitary-thyroid axis setpoint

In the last 6 years, 5 GWAS on serum TSH and/or FT4 levels have been published (112, 135-137, 261), the largest being the GWAS by Porcu *et al.* (Figure 3) and Gudmundsson *et al* (112, 135). These GWAS have led to an enormous increase in the number of identified susceptibility loci for serum TSH and FT4 levels, as illustrated in Figure 4. Porcu *et al.* performed a GWAS on normal-range TSH and FT4 levels in 26,400 and 17,500 individu-

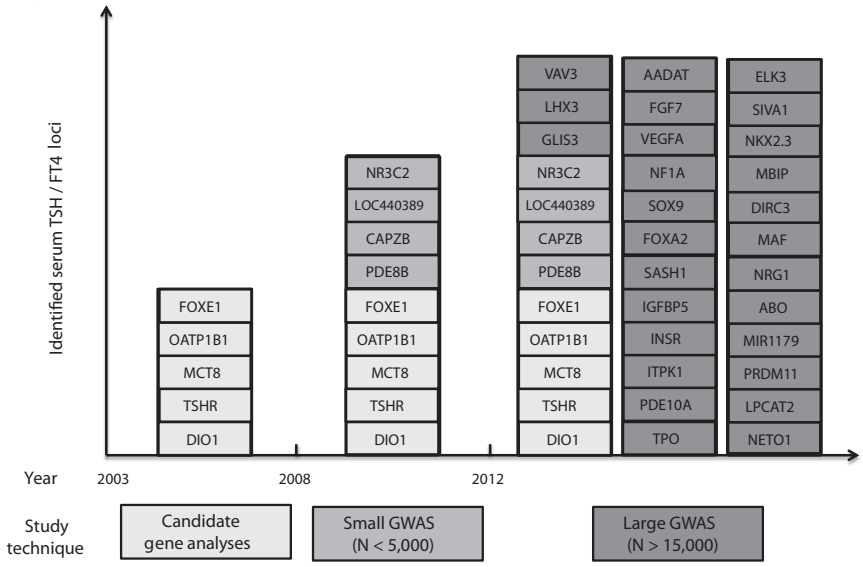


Figure 4. Identified serum TSH and/or FT4 associated single nucleotide polymorphisms (SNPs) over time, using different study techniques. Candidate gene analyses hits were included when associations were replicated in at least one independent population (N >500) or in case of *in-vitro* evidence for functionality.

als, respectively, from 18 populations, resulting in the identification of 26 genome-wide significant hits (112). A genetic risk score was calculated based on these new hits, indicating that carriage of multiple risk alleles was associated with a higher risk of an increased TSH level. Gudmundsson *et al.* performed a GWAS on TSH in 27,700 Icelanders and identified 22 GWAS significant loci, 3 of which were also associated with thyroid cancer (135). Part of the identified loci in these GWAS included genes that were already known to affect thyroid parameters, such as *DIO1*, *FOXE1*, *GLIS3*, *LHX3*, *TPO* and *VAV3*, but also included a large number of new candidate genes, which are discussed below.

Hits in the TSH signaling cascade

Part of the recently discovered candidate genes for serum thyroid parameters include genes with a role in the TSH signaling cascade. After binding of TSH to TSHR, the cAMP signaling cascade is activated, and the family of phosphodiesterases is responsible for cAMP degradation, thereby inactivating this pathway. Genetic variation in intron 1 of the *PDE8B* gene is the most consistently reported significant hit in the various GWAS on serum TSH levels (112, 135, 136, 261). PDE8B is highly expressed in the thyroid and has the highest affinity for cAMP of any known phosphodiesterase (262). Their associations with serum FT4 levels remain controversial (104, 112, 135, 263-265), but it is speculated that the identified variants increase PDE8B activity, resulting in lower cAMP levels in response to TSH. Consequently, a higher TSH level is required to maintain normal levels

of TH. More recent studies have shown that genetic variation in *PDE8B* is associated with subclinical hypothyroidism in pregnancy and recurrent miscarriage (266, 267). In addition, Jorde *et al.* found in a large population study in Norway that genetic variation in *PDE8B* was not only associated with higher serum TSH levels, but also with a smaller height and an increased risk of myocardial infarction (263). However, the association with myocardial infarction was not corrected for thyroid function, in order to see if the association was mediated by TH.

PDE10A is another PDE which is also highly expressed in the thyroid and has been associated with TSH levels in 2 GWAS (112, 135). It has been shown to degrade both cAMP and cGMP (268). Furthermore, genetic variation in *PDE10A* was associated with increased serum TSH levels in a large Alpine population (269).

In turn, the activities of not only PDE10A but also of CAPZB are modulated by cAMP (270, 271). Genetic variation in *CAPZB* has been associated with serum TSH levels in various GWAS (112, 135, 261, 272). *CAPZB* is highly expressed in the thyroid and encodes the two β subunit isoforms of the capping protein, also known as the barbed-end actin-binding protein. The TSH-induced extension of microvilli and filopedia protruding from the thyrocyte surface in the follicular lumen is an important step in TH production. In this way, Tg is endocytosed, endocytotic vesicles fuse with lysosomes, and proteolysis of Tg leads to the release of the iodothyronines. Teumer *et al.* postulated that the *CAPZB* variants result in an altered capping capacity, thereby affecting TH synthesis and leading to altered TSH levels (271). Depending on the directions of the effects, one could also expect compensatory hypo- or hyperplasia of the thyroid. In this respect it is noteworthy that *CAPZB* was indeed one of the genome-wide significant hits in the thyroid volume and goiter GWAS by Teumer *et al* (271).

Another candidate gene in the TSH signaling cascade was identified in the GWAS by Porcu *et al.* (112). An intergenic SNP on chr 14q31 (rs11624776) was associated with serum TSH levels. This locus contained *ITPK1*, encoding the enzyme inositol 1,3,4-trisphosphate 5/6-kinase, which catalyzes the rate-limiting step in the formation of phosphorylated forms of inositol. It has been known for several years that TSHR does not only couple to G_s, leading to cAMP activation, but also to G_q, activating the inositol phosphatase pathway (273). These data therefore suggest that ITPK1 plays a role in the TSH signaling cascade.

Hits in the GH/IGF1 signaling pathway

It has already been known for long that the GH/IGF1 and TH signaling pathways interact (274, 275). TH influences GH/IGF1 production and signaling at various levels, but several lines of evidence also suggest that GH/IGF1 influences thyroid growth, TH production, production of TH-binding proteins and peripheral deiodination (274, 275). It is therefore interesting that a number of hits in the serum TSH and FT4 GWAS were located in or near

members of the GH/IGF1 signaling pathway. Hits included the *INSR*, encoding the insulin receptor, and *IGFBP5*, a member of the IGF-1 binding protein family (112, 135). Enhanced thyroidal IGFBP5 production is correlated with inhibition of thyroid function, and has been shown to be significantly down-regulated in GD patients with ophthalmopathy compared to GD patients without ophthalmopathy (276, 277). Further hits associated with serum TSH levels included SASH1 and FOXA2 (112, 135), which are downstream targets of the GH/IGF1 signaling pathway, while Lantz *et al.* have shown that FOXA2 also regulates insulin secretion (278-280).

Hits encoding transcription factors expressed in the thyroid

Two of the newly identified loci in GWAS of serum thyroid parameters included transcription factors expressed in the thyroid. In the GWAS by Porcu *et al.*, a SNP on chr 17q23 located 5 kb downstream of *SOX9* (rs9915657) was associated with serum TSH levels (112). Besides being a transcription factor involved in chondrocyte differentiation and male sex determination, SOX9 is highly expressed in the thyroid. In 2002, Zhou et al. showed that SOX9 interacts with TRAP230, a component of the T3 receptor-associated protein (TRAP) complex, suggesting an interaction between the TH signaling and SOX9 pathways (281).

Furthermore, genetic variation in *NF1A* has also been associated with serum TSH levels (112, 135). *NF1A* encodes a member of the NF1 (nuclear factor 1) family of transcription factors, which play a pivotal role in various developmental processes (282). However, several lines of evidence support an important role of these transcription factors in the control of TH synthesis. Fernandez *et al.* showed that NF1 binds simultaneously with FOXE1 to the *NIS* upstream enhancer region, both of which can also activate the *NIS* promoter (283). Nakazato *et al.* have found that NF1 proteins, including NF1A, control constitutive repression of *TTF1* expression (284). Finally, NF1 proteins have been shown to interact with TTF-2 to control the expression of TPO (285).

Hits encoding growth factors expressed in the thyroid

VEGFA is a growth factor with a well-established role in angiogenesis, and since long has been associated with benign and malignant tumors of the thyroid (286). However, in the GWAS by Gudmundsson *et al.* and Porcu *et al*., genetic variation in *VEGFA* was also found to be associated with serum TSH levels (112, 135). Angiogenesis is particularly essential for the thyroid as the microvasculature continuously supplies iodine, a key element in TH synthesis, and iodine deficiency stimulates VEGFA secretion from thyrocytes (287). In addition, it has been shown that in the developing mouse thyroid, epithelial VEGFA production is necessary for endothelial cells recruitment and expansion, controlling epithelial reorganization in follicles and C-cell differentiation (288).

Fibroblast growth factor 7 (*FGF7*) has also been identified as a candidate gene for serum TSH levels. FGFs play an important role in the development of the thyroid, as well as in the progression of thyroid cancer (289, 290). Furthermore, *FGF7* was one of the significant hits in the goiter GWAS by Teumer *et al.* (271). Future studies should clarify the exact molecular mechanism behind the observed associations between genetic variation in *FGF7*, serum thyroid parameters, and goiter.

Finally, there are a number of GWAS significant hits associated with serum thyroid parameters which do not have a documented role in thyroid signaling pathways, including *AADAT*, *NETO1/FBXO15*, *LPCAT2/CAPNS2*, *PRDM11*, *MIR1179*, *NRG1*, *MAF*, *DIRC3*, *NR3C2, MBIP*, *NKX2.3*, *SIVA1* and *ELK3* (112, 135). We refer to the respective GWAS for further details on these genes (112, 135). Future studies should obviously clarify the biological mechanisms behind the observed associations, possibly elucidating new pathways in thyroid (patho)physiology.

VII. DISCUSSION AND FUTURE PERSPECTIVES

Over the past few years, the introduction of GWAS has led to the identification of a large number of new candidate genes for thyroid (dys)function. As a proof of concept it is reassuring to note that the hits also included well-known genes that had already been identified in candidate and linkage analyses. As effect sizes are small, the individual variants have no direct clinical relevance in predicting thyroid disease, but the observed associations could elucidate new pathways in the pathogenesis of thyroid dysfunction. Many of the identified new variants are non-coding, located intergenic or in loci that have no known role in TH signaling or autoimmunity. Unfortunately, for most of these variants no attempts have been made to further understand the exact biological mechanism behind the observed associations, which is a crucial step in unravelling the pathogenesis of thyroid diseases.

Identification of new candidate genes and associated pathways can be of clinical importance for a number of reasons. First of all, new pathways might form a focus for the design of new drugs that could be used in the treatment of thyroid diseases. Genetic variation may furthermore have a role in the choice and prediction of drug dosing and response. This has been nicely shown for a number of drugs in the cardiovascular field, including the cytochrome P450 (CYP)2C9 and vitamin K epoxide reductase (VKORC1) for warfarin treatment (291). Despite the few reports on the effects of D1 and D2 on treatment response, much remains to be learned about the role of genetic variation in TH pathway genes in the treatment of thyroid disorders (113, 172, 173).

The identification of new candidate gene variants can also be of potential use in the field of thyroid diagnostics. Porcu *et al.* have shown that subgroups with a substantially increased risk of hypothyroidism can be identified by combining multiple risk alleles (112). In our GWAS on TPOAbs, we have shown that with the use of only 5 SNPs, a large subgroup with an increased risk of both TPOAb-positivity as well as increased TSH levels can be identified (260). Despite this, these currently available genetic markers for thyroid disorders lack sensitivity and specificity to be clinically useful. Further studies are therefore required to explain the remainder of the variation.

Studies have shown that patients on LT4 replacement therapy have a decreased well-being, despite having serum TH parameters within the normal range (175, 292). This suggests that these "normal" serum TH parameters do not match the patient's physiological set-point. Therefore, the ultimate application of genetics in the treatment of thyroid diseases would be the use of genetic markers to reliably estimate an individual's set-point, towards which then a personalized treatment can be directed. We are still very far from personalized treatment, although many risk loci have been identified in GWAS over the last few years. This is illustrated by the fact that, when combining all identified risk loci in the GWAS by Porcu *et al.*, only 5.6% and 2.3% of the total variation in serum TSH and FT4 levels, respectively, could be explained (112).

Various approaches can be taken in the continuing search of this missing genetic heritability. One obvious way is performing GWAS including larger number of samples, thereby increasing power. The benefits of increasing sample size in GWAS has been comprehensively reviewed by Lindquist *et al*., who estimated that only one-fifth of all GWAS detectable SNPs underlying chronic diseases have been detected by GWAS so far (293). They furthermore conclude that increasing sample size has a much larger impact than increasing coverage on the potential of future GWAS to detect additional SNP-disease associations and heritability. This also seems to hold true for the thyroid field, where the benefits of increasing GWAS sample size have been illustrated by the fact that the more recent GWAS including more than 15,000 samples have been much more successful in identifying risk loci than the first GWAS including 2,000 – 4,000 samples (Fig. 4) (112, 135-137, 261). It has been known for long that there are substantial differences in the prevalence of thyroid diseases between men and women, and the GWAS by Porcu *et al.* on serum TSH and FT4 levels detected a number of loci with gender-specific effects (112). It would therefore be interesting to also include the X-chromosome in these analyses, which has not been studied in most of the published thyroid GWAS. Furthermore, finemapping involves screening all known risk variants from any available data sources, including HapMap, sequencing data etc, around the GWAS identified variant. In this way, one can determine if the identified effect is actually driven by another (more rare) marker which is in LD with the identified SNP. As previously mentioned, finemapping

is not only important to further unravel the molecular mechanism underlying the observed associations, but also to determine the true effects of a locus on thyroid function.

In recent years it has become increasingly clear that various autoimmune disease have a shared genetic basis (294). This is illustrated by the fact that a large part of the discussed GWAS hits in section VI had also been associated with other autoimmune diseases. Newly identified susceptibility genes for other autoimmune diseases should therefore also be considered as potential candidate genes for thyroid (dys)function.

However, besides candidate gene analyses, GWAS and finemapping, a number of novel methods and approaches have emerged that will further improve our understanding of the genetic basis of thyroid (dys)function in the coming years. Copy number variations (CNVs) are genetic variations of a larger part of the genome, including duplications, deletions and inversions. For long, it has been known that CNVs play an important role in the genetic basis of intellectual deficiencies, congenital anomalies, and autism spectrum disorders. However, very little is known about the role of CNVs in human thyroid (dys) function. Huber *et al.* studied the effects of CNVs in *PTPN22* and *CD40* on GD, but these were too rare to be informative (295). Therefore, the potential role of CNVs in thyroid (dys)function still needs to be clarified by large-scale studies.

The fact that various loci associated with thyroid (dys)function are located within the same pathways, suggests that also gene-gene interactions could occur. However, also gene-environment interactions should be taken into consideration in explaining the remaining part of the susceptibility and variability of thyroid (dys)function. Despite the various challenges involved in these kind of studies (296), including the requirement of even larger sample sizes, studies on gene-environment interactions would be especially interesting for thyroid disease, considering the multiple environmental factors that play a role in its pathogenesis, such as iodine status, smoking, and viral infection (208, 215, 297, 298).

Furthermore, GWAS only assesses 0.1% of the nucleotides of the genome, and therefore much can be expected from exome and whole-genome sequencing, providing a complete catalog of all variants within the studied genomic region, rather than relying on markers or LD. Sequencing only the part of the genome that is protein coding (i.e., the exome), is more cost-effective and targets the part that is most likely to directly affect protein structure/function, simplifying its biological interpretation. This technique has already proved itself in the thyroid field, elucidating mutations involved in the pathogenesis of familial goiter and thyroid cancer, and more recently the detection of mutations in *THRA* as a novel cause of RTH (24, 88, 90, 299, 300). Besides higher laboratory and computational costs, more challenges have to be faced when sequencing the whole genome, mainly because of the fact that this technique identifies thousands of new variants in each individual, requiring an effective way to filter out the non-causing mutations by sequencing non-affected related family members (301). Despite this,

much is expected from this approach, given its success in identifying the genetic causes of many other human disorders in the past few years (302). Above discussed techniques will likely lead to the identification of variants over the entire spectrum depicted in Figure 1, ranging from rare variants with large effects causing monogenic thyroid diseases to common variants with small effects causing polygenic thyroid diseases and variations in thyroid function tests.

Finally, besides investigating genetic variants, new technologies have emerged that investigate gene expression and its regulation. Epigenetics study the control of gene expression, including DNA methylation, micro RNAs and histone modification, whereas transcriptomics study the actual RNA levels. These techniques have also entered the thyroid field. For example, Ambrosio *et al.* have shown that LSD-1 and FoxO3 play an important role in the epigenetic control of *DIO2* and *DIO3* in myogenesis (303). As discussed, after *DIO2* had been suggested as a susceptibility locus for osteoarthritis, Bos *et al.* showed an increased amount of D2 protein in osteoarthritic versus normal cartilage (182). The addition of these dynamic expression data to the available data on genetic variation of the static DNA backbone is a crucial next step in unraveling the molecular mechanisms underlying thyroid function and dysfunction.

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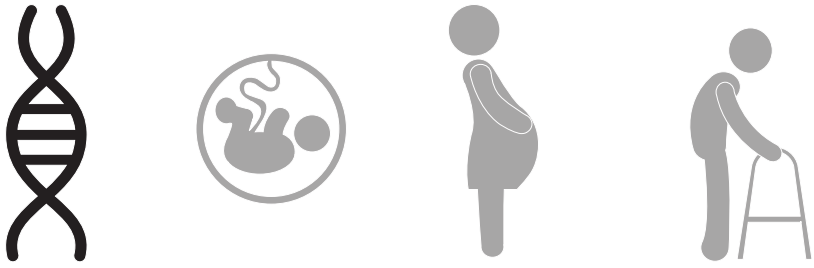
Appendix 1

(Supplemental data Chapter 8)



SUPPLEMENTAL TEXT S1

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COHORT DESCRIPTION

The following cohorts are part of the Meta-Thyroid consortium:

Baltimore longitudinal study on Aging (BLSA): BLSA is a population-based study aimed to evaluate contributors of healthy aging in the older population residing predominantly in the Baltimore-Washington DC area [1]. Starting in 1958, participants are examined every one to four years depending on their age. Currently there are approximately 1100 active participants enrolled in the study. The BLSA has continuing approval from the Institutional Review Board (IRB) of Medstar Research Institute.

Cardiovascular Health Study (CHS): CHS is a population-based cohort study of risk factors for coronary heart disease and stroke in adults aged 65 years or older conducted across four field centers in the United States [2]. The original cohort of 5,201 persons consisting of 4,925 Caucasians was recruited in 1989-1990 from a random sample of people on Medicare eligibility lists. An additional 687 African-Americans were enrolled subsequently. African American participants were excluded from this analysis of individuals of European ancestry. CHS participants completed standardized clinical examinations and questionnaires at study baseline and at nine annual follow-up visits. DNA was extracted from blood samples drawn on all participants who consented to genetic testing at the 1989-90 examination.

Framingham Heart Study (FHS): The original cohort of FHS was recruited in 1953 in the town of Framingham in Massachusetts. In 1971, an Offspring cohort of 5,124 (2,483 men with average age of 37 and 2,641 women with average age of 36) was recruited and has been examined every four years. The subjects in the Offspring cohort include adult children of Original cohort and spouses of offspring. All participants provided a written informed consent and all study protocols were approved by Boston University [3,4].

Genetics, Arthrosis, and Progression study (GARP): The GARP study has been described in detail previously [5]. It aimed at identifying determinants of osteoarthritis and the progression of this disease. The study is based on sibships of white Dutch ancestry with clinical- and radiographically-confirmed osteoarthritis at two or more joint sites of the hand, spine (cervical or lumbar), knee or hip. In the current analyses we included 359 subjects from whom we had genome wide scan data and thyroid levels available.

Helsinki Birth Cohort Study (HBCS): The HBCS is composed of 8,760 individuals born between the years 1934-44 in one of the two main maternity hospitals in Helsinki, Finland. Between 2001 and 2003, a randomly selected sample of 928 males and 1,075 females participated in a clinical follow-up study with a focus on cardiovascular, metabolic and reproductive health, cognitive function and depressive symptoms. Detailed information on the selection of the HBCS participants and on the study design can be found elsewhere [6]. Research plan of the HBCS was approved by the Institutional

Review Board of the National Public Health Insitute and all participants have signed an informed consent.

Invecchiare in Chianti study (InCHIANTI): The InCHIANTI study is a population-based epidemiological study aimed at evaluating the factors that influence mobility in the older population living in the Chianti region in Tuscany, Italy. The details of the study have been previously reported [7,8]. Briefly, 1616 residents were selected from the population registry of Greve in Chianti (a rural area: 11,709 residents with 19.3% of the population > 65 years of age), and Bagno a Ripoli (Antella village near Florence; 4,704 inhabitants, with 20.3% of the population > 65 years of age). The participation rate was 90% (n=1453), and the subjects ranged between 21-102 years of age.

LBC1921: The LBC1921 cohort consists of 550 relatively healthy individuals, 316 females and 234 males, assessed on cognitive and medical traits at around 79 years of age. They were born in 1921, most took part in the Scottish Mental Survey of 1932, and almost all lived independently in the Lothian region (Edinburgh City and surrounding area) in Scotland. When tested, the sample had a mean age of 79.1 years (SD = 0.6). A full description of participant recruitment and testing can be found elsewhere [9]. Ethics permission for the study was obtained from the Multi-Centre Research Ethics Committee for Scotland (MREC/01/0/56) and from Lothian Research Ethics Committee (LREC/1998/4/183). The research was carried out in compliance with the Helsinki Declaration. All subjects gave written, informed consent.

LBC1936: The LBC1936 consists of 1,091 relatively healthy individuals assessed on cognitive and medical traits at around 70 years of age. They were born in 1936, most took part in the Scottish Mental Survey of 1947, and almost all lived independently in the Lothian region of Scotland. The sample of 548 men and 543 women had a mean age 69.6 years (SD = 0.8). A full description of participant recruitment and testing can be found elsewhere [10]. Ethics permission for the study was obtained from the Multi-Centre Research Ethics Committee for Scotland (MREC/01/0/56) and from Lothian Research Ethics Committee (LREC/2003/2/29). The research was carried out in compliance with the Helsinki Declaration. All subjects gave written, informed consent.

LifeLines: The LifeLines Cohort Study is a multi-disciplinary prospective population-based cohort study examining in a unique three-generation design the health and health-related behaviours of 165,000 persons living in the North East region of The Netherlands [11]. It employs a broad range of investigative procedures in assessing the biomedical, socio-demographic, behavioural, physical and psychological factors which contribute to the health and disease of the general population, with a special focus on

multimorbidity. All survey participants are between 18 and 90 years old at the time of enrollment. Recruitment has been going on since the end of 2006, and until January 2011 over 40,000 participants have been included.

Leiden Longevity Study (LLS): For the Leiden Longevity Study, long-lived siblings of European descent were recruited together with their offspring and the partners of the offspring. Families were recruited if at least two long-lived siblings were alive and fulfilled the age criterion of 89 years or older for males and 91 years or older for females, representing less than 0.5% of the Dutch population in 2001 [12]. In total 944 long-lived siblings were included with a mean age of 94 years (range 89-104), 1671 offspring (61 years, 39-81) and 744 partners (60 years, 36-79). DNA from the Leiden Longevity Study was extracted from samples at baseline using conventional methods [13].

MICROS: The *MICROS* study is part of the genomic health care program 'GenNova' and was carried out in three villages of the Val Venosta, South Tyrol (Italy), in 2001-2003. It comprised members of the populations of Stelvio, Vallelunga and Martello. A detailed description of the *MICROS* study is available elsewhere [14]. Briefly, study participants were volunteers from three isolated villages located in the Italian Alps, in a German-speaking region bordering with Austria and Switzerland. Owing to geographical, historical and political reasons, the entire region experienced a prolonged period of isolation from surrounding populations. The study participants are connected among each other in a unique genealogy for the three villages. Information on the participant's health status was collected through a standardized questionnaire.

Nijmegen Biomedical Study (NBS): Details of the NBS have been described before.[15] In brief, the Nijmegen Biomedical Study is a population- based cross-sectional study conducted by the Radboud University Nijmegen Medical Centre. Approval to conduct the study was obtained from the Institutional Review Board. Nijmegen is a town in the eastern part of The Netherlands with 156,000 inhabitants, approximately 87% of Caucasian descent. Age and sex stratified randomly selected adult (age 18 years and older) inhabitants of Nijmegen (N=22,452) received an invitation to fill out a postal questionnaire on lifestyle and medical history. A total of 6,434 participants donated blood for measurement of thyroid function and DNA-isolation. Informed consent was obtained from each participant, and the Institutional Review Board of the Radboud University Nijmegen Medical Centre approved the study.

Old Order Amish (OOA): The Old Order Amish (OOA) study participants reported here were from ongoing studies of cardiovascular disease and longevity [16,17]. Nearly all of the enrolled individuals are descendants of a small number of Amish who settled in

Lancaster County, Pennsylvania, in the mid-eighteenth century. A total of 1,136 individuals from these two studies had serum TSH measured by Immulite 2000 (Siemens), using a standardized third generation assay, and were previously genotyped with the 500K Affymetrix Mapping Array set. This study was approved by the Institutional Review Boards of the University of Maryland and the National Cancer Institute.

PROSPER/PHASE: All data come from the PROspective Study of Pravastatin in the Elderly at Risk (PROSPER). A detailed description of the study has been published elsewhere [18,19]. PROSPER was a prospective multicenter randomized placebo-controlled trial to assess whether treatment with pravastatin diminishes the risk of major vascular events in elderly. Between December 1997 and May 1999, we screened and enrolled subjects in Scotland (Glasgow), Ireland (Cork), and the Netherlands (Leiden). Men and women aged 70-82 years were recruited if they had pre-existing vascular disease or increased risk of such disease because of smoking, hypertension, or diabetes. A total number of 5,804 subjects were randomly assigned to pravastatin or placebo. A large number of prospective tests were performed including Biobank tests and cognitive function measurements.

Rotterdam Study (RS): The RS is a prospective population-based cohort study on determinants of chronic diseases in the elderly, which has been described previously [20,21]. The study comprised 7983 men and women aged 55 years and over, living in a district of Rotterdam, The Netherlands. Informed consent was obtained from each participant, and the Medical Ethics Committee of the Erasmus Medical Center Rotterdam approved the study.

SardiNIA: The SardiNIA study consists of 6,148 volunteers, males and females, ages 14–102 yr, recruited and phenotyped from a cluster of four towns in the Ogliastra province of Sardinia [22,23] The local ethical committee approved the study protocol and all participants provided a written informed consent.

Twins UK: The Twins UK cohort consists of 2,217 female twins of northern European/UK ancestry (1,831 dizygotic and 386 monozygotic), aged 18–82 yr, from St Thomas' UK Adult Twin Registry (TwinsUK), a volunteer sample recruited in the United Kingdom without selection for particular traits (www.twinsuk.ac.uk/) [24].

ValBorbera (INGI): The Val Borbera (INGI) population is a collection of 1,664 genotyped samples collected in the Val Borbera Valley, a geographically isolated valley located within the Appennine Mountains in NorthWest Italy [25]. The valley is inhabited by about 3000 descendants from the original population, living in 7 villages along the valley and

in the mountains. The valley was inhabited by about 10,000 people in the 19th century when endogamy was >80%. Participants were healthy people between 18 and 102 years of age that had at least one grandfather living in the valley.

SUPPLEMENTAL MATERIALS AND METHODS

Genotyping and imputation

Nine different genotyping platforms were used by the cohorts included in this meta-analysis: Illumina HumanHap 610K (HBCS, LBC1921, LBC1936), Illumina HumanHap 550K (BLSA, InCHIANTI, RS), Illumina HumanHap 660K (LLS, PROSPER), Illumina HumanHap 370K (CHS, NBS, ValBorbera), Illumina Cyto-SNP12 v2- 300K (LifeLines), Illumina HumanHap 300K (MICROS), the Affymetrix 500K in combination with the 10K supplemental array (SardiNIA), the Affymetrix 500K in combination with the 50K supplemental array (FHS), the Illumina HumanHap 300K, 550K and 610K arrays (TwinsUK). Each study performed genotyping quality control checks based on duplicate sample genotyping, SNP call rate, Hardy-Weinberg equilibrium, Mendelian inconsistencies, sex mismatch, and principle components methods were used to evaluate the presence of population stratification. Each study imputed 2.5 million HapMap SNPs for each participant using currently available imputation methods. BLSA, FHS, HBCS, InCHIANTI, LBC1921, LBC1936, MICROS, PROSPER, RS, SardiNIA, and ValBorbera used the MACH algorithm (http://www.sph.umich.edu/csg/abecasis/MaCH/); LifeLines, LLS and TwinsUK used IMPUTE (http://www.stats.ox.ac.uk/~marchini/software/gwas /impute), and CHS used BimBam. Further details are summarized in Table S1.

Thyroid function measurements

Methods used by each study cohort to measure TSH and FT4 levels are reported in Table S1.

Statistical analyses

All cohorts excluded subjects with thyroid medication, thyroid surgery, or with out-of-range TSH values (TSH > 4.0 mIU/L or TSH < 0.4 mIU/L). Linear regression analyses (additive model) were performed after applying inverse normal transformation to both TSH and FT4. Age, age-squared, and gender were used as covariates, as well as principal components axes or additional variables, when needed. Further details are summarized in Table S1.

Association of TSH SNPs in pregnant women

Association of TSH lead SNPs was tested in pregnant women of the Exeter Family Study of Childhood Health (EFSOCH). EFSOCH is a consecutive birth cohort consisting of children

born between 2000 and 2004 in central Exeter, UK, and their parents [26]. Both parents attended a study visit at 28 weeks of gestation, at which DNA was collected and a fasting blood sample was taken for biochemical assays. The local research ethics committees approved the study, and all adult participants gave informed written consent. Serum TSH and FT4 levels were determined in 974 pregnant women using an electrochemiluminescent immunoassay, run on the Modular E170 Analyzer (Roche, Burgess Hill, UK). The manufacturer's population reference ranges (for non-pregnant samples) were: TSH, 0.35–4.5 mIU/L; and FT4, 11–24 pmol/L. For the analyses of the pregnant women, we used reference ranges specific for the assay and 28th week of gestation based on our own set of TPOAb-negative, healthy, pregnant women (n=901): TSH, 0.49–4.21 mIU/L and FT4, 9.13–15.17 pmol/L [27]. Serum TPOAb levels were determined using the competitive immunoassay (Roche) in 970 pregnant women. A titer above 34 IU/mL was considered positive.

DNA samples were genotyped at KBiosciences (Hoddesdon, UK; www.kbioscience.co.uk), using their own system of fluorescence-based competitive allele-specific PCR (KASPar). Call rates of the 9 SNPs analyzed were >95% and there was no evidence of deviation from Hardy-Weinberg equilibrium ($P > 0.05$). Concordance between duplicate samples (10% of total) was >99% for all SNPs.

Association analyses were carried out using Stata SE v.10 (StataCorp, Texas, USA). A total of 862 pregnant women with TSH levels and genotype were available for analysis (mean age 30.4 years, s.d. 5.3 years). Women taking medication for thyroid disorders were excluded ($n = 14$). We used linear regression to analyse the association between TSH level (inverse normal transformation) and each individual SNP (coded as 0, 1 or 2 TSH-increasing alleles), with age and age-squared as covariables. We then constructed a genetic risk score (GRS) for all women with at least 7 available SNPs:

GRS = weighted score x N available SNPs / sum of weights of available SNPs, where weighted score = $w1$ x SNP1 + $w2$ x SNP2 +…wi x SNPi and wi is the beta coefficient from the association between TSH levels and SNPi. We performed linear regression of TSH level against the GRS (additive model), with age and age-squared as covariables. We performed all analyses twice: first including, and then excluding the women who tested positive for TPO antibodies (7.5% of the sample). We also verified that the results were not materially altered on adjustment for fetal genotype at all 9 SNPs. Finally, we used logistic regression to assess the association between subclinical hypothyroidism in pregnancy and the GRS, again adjusting for age and age-squared. We defined cases as TSH > 4.21 mIU/L (the upper limit of the reference range) and controls as TSH <=4.21 mIU/L [27].

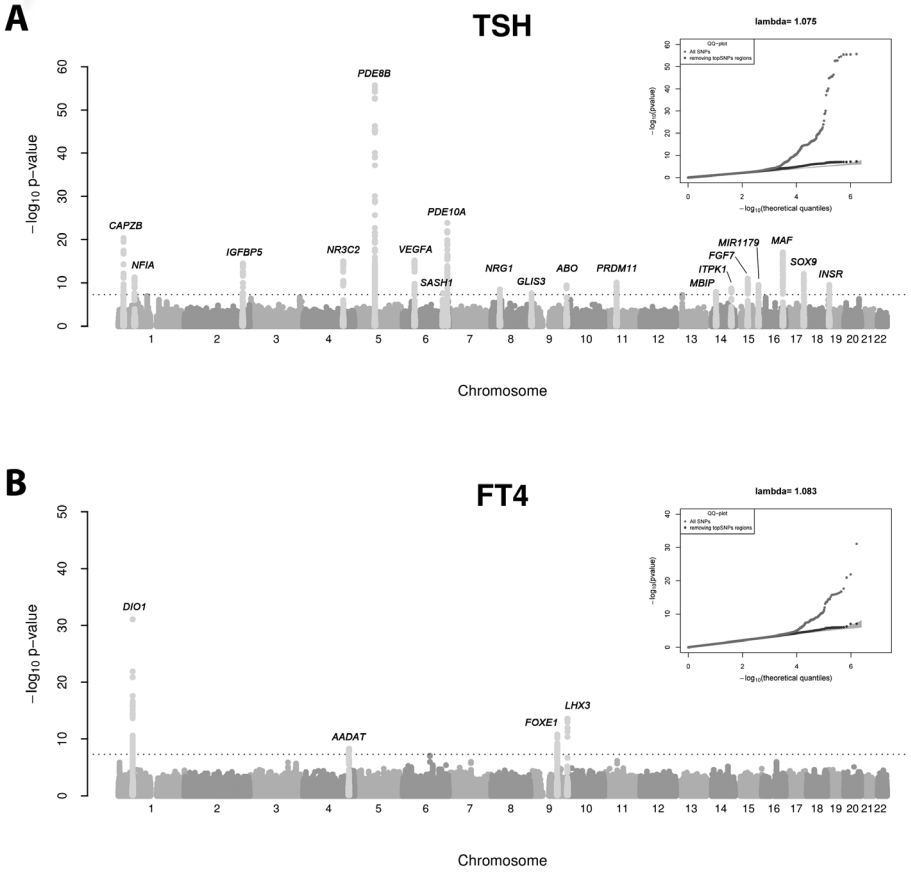

Figure S1 Manhattan plots from GWAS meta-analysis results of serum TSH (panel A) and FT4 (panel B) levels. SNPs are plotted on the x axis according to their position (build 36) on each chromosome against association with TSH (A) and FT4 (B) on the y axis (shown as –log10 P value) in. The loci highlighted in green are those that reached genome-wide significance. In each panel, quantile-quantile plots obtained with all SNPs (red dots) and after removal of SNPs within associated regions (blue dots) are also shown. The gray area corresponds to the 90% confidence region from a null distribution of P values (generated from 100 simulations). A color figure is available at: http://www.plosgenetics.org/article/info%3Adoi%2F10.1371%2Fjournal.pgen.1003266#s5

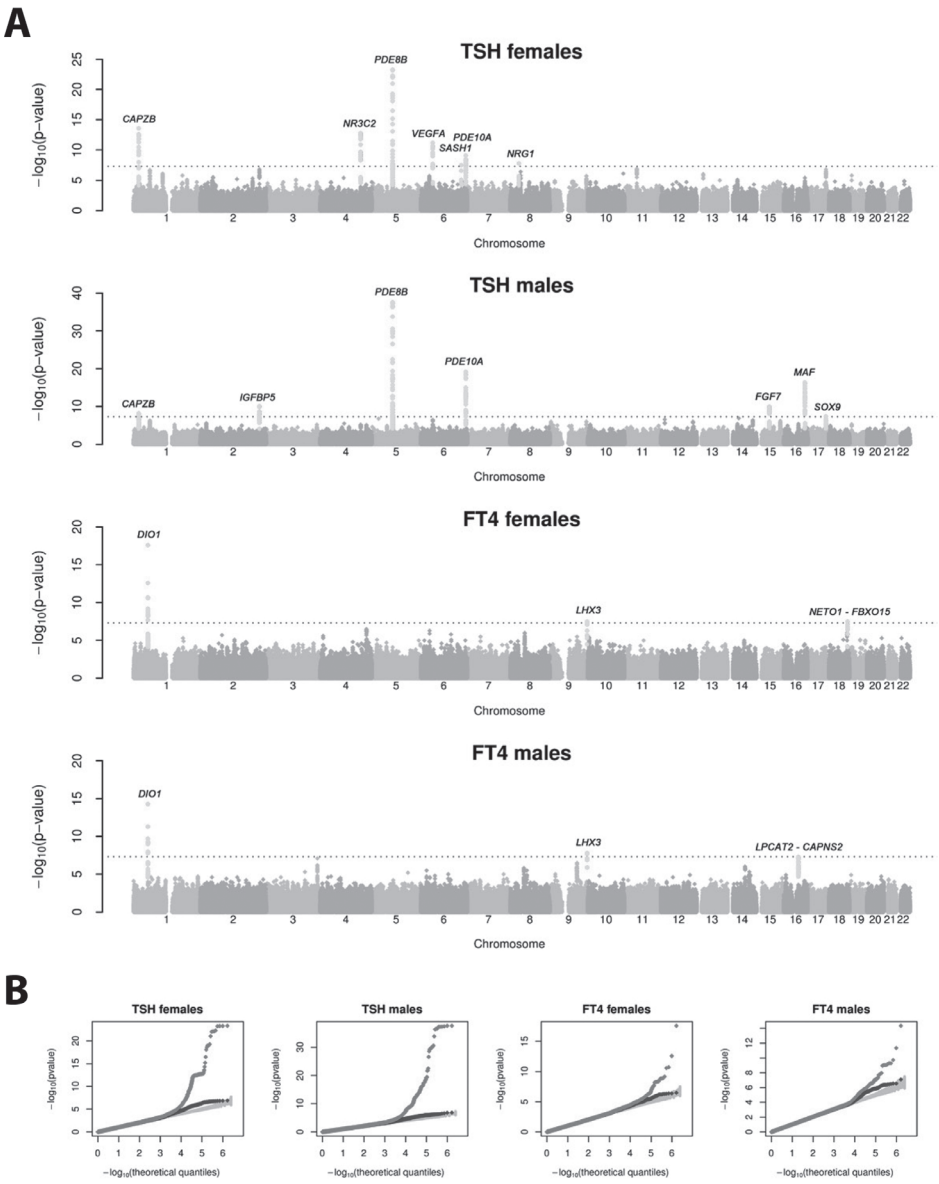

Figure S2

Panel A. Manhattan plots from meta-analysis results of serum TSH and FT4 levels for men and women separately are shown as indicated. SNPs are plotted on the x axis according to their position (build 36) on each chromosome; association with TSH and FT4 is indicated on the y axis (as –log10 P value). Signals reaching genome-wide statistical significance in the gender specific analysis are shown in green. Panel B. Quantile-quantile plots are shown for all SNPs (red dots) and after removal of SNPs within associated regions (blue dots). A color figure is available at: http://www.plosgenetics.org/article/info%3Adoi%2F10.1371%2Fjournal.pgen.1003266#s5

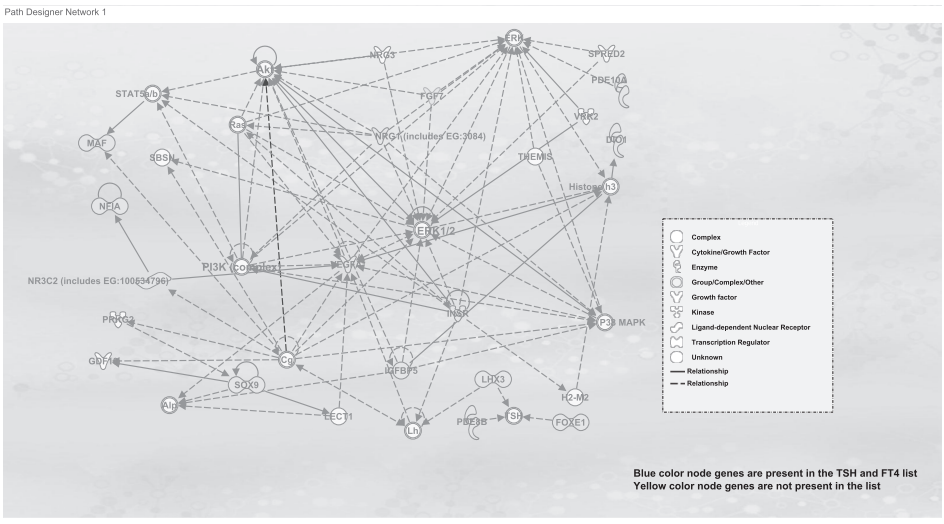

Figure S3 Ingenuity Pathway Analysis of the TSH and FT4 top hits

Tables S1 and **S2** are available at: http://www.plosgenetics.org/article/info%3Adoi%2F10.1371%2Fjournal.pgen.1003266#s5

Table S3. Heterogeneity analysis of South European vs North American cohorts.

			South Europeans (S.E.)						North Americans (N.A.)						S.E vs N.A.	
Gene	SNP	A1/A2	FreqA1	Effect	StdErr	*P*	Het *P*	N	FreqA1	Effect	StdErr	*P*	Het *P*	N	Het *P*	N
TSH levels																
PDE8B	rs6885099	A/G	0.5484	-0.1511	0.0182	1.12 X 10-16	0.125	7488	0.6125	-0.1419	0.0200	1.17 X 10-12	0.799	5407	0.7353	12895
PDE10A	rs753760	C/G	0.6949	0.1058	0.0198	8.81 X 10-08	0.343	7488	0.6701	0.0737	0.0223	0.0009389	0.208	5407	0.2847	12895
CAPZB	rs10799824	A/G	0.1707	-0.0869	0.0243	0.0003451	0.457	7488	0.1709	-0.0818	0.0276	0.003078	0.004	5407	0.8904	12895
MAF/LOC440389	rs3813582	T/C	0.6434	0.1125	0.0191	4.17 X 10-09	0.296	7488	0.6452	0.0747	0.022	0.0006724	0.203	5407	0.1973	12895
VEGFA	rs9472138	T/C	0.2714	-0.0807	0.0205	8.20 X 10-05	0.077	7488	0.2915	-0.0903	0.0217	3.14 X 10-05	0.097	5407	0.7492	12895
VEGFA	rs11755845	T/C	0.3185	-0.0746	0.0199	0.0001809	0.464	7488	0.2474	-0.1134	0.0237	1.65 X 10-06	0.947	5407	0.2129	12895
NR3C2	rs10032216	T/C	0.7660	0.1069	0.022	1.20 X 10-06	0.577	7488	0.7923	0.0679	0.0246	0.00577	0.533	5407	0.2402	12895
IGFBP5	rs13015993	A/G	0.7501	0.0656	0.0207	0.001543	0.928	7488	0.7379	0.1042	0.0227	4.37 X 10-06	0.470	5407	0.2117	12895
NR3C2	rs10032216	T/C	0.7660	0.1069	0.022	1.20 X 10-06	0.577	7488	0.7923	0.0679	0.0246	0.00577	0.533	5407	0.2402	12895
SOX9	rs9915657	T/C	0.6129	-0.0658	0.0188	0.0004785	0.224	7488	0.5473	-0.0827	0.0202	4.22 X 10-05	0.964	5407	0.5427	12895
NFIA	rs334699	A/G	0.0543	-0.1218	0.0423	0.003965	0.005	7488	0.0471	-0.0588	0.048	0.2202	0.589	5407	0.3278	12895
FGF7	rs10519227	A/T	0.2765	-0.0612	0.0207	0.003175	0.937	7488	0.2224	-0.077	0.0249	0.001966	0.313	5407	0.6278	12895
PRDM11	rs17723470	T/C	0.2052	-0.0551	0.025	0.0274	0.709	7488	0.2923	-0.0691	0.0221	0.001782	0.646	5407	0.6764	12895
MIR1179	rs17776563	A/G	0.3340	-0.1087	0.0198	3.76 X 10-08	0.901	7488	0.3488	-0.049	0.0218	0.02484	0.225	5407	0.04392	12895
INSR	rs4804416	T/G	0.5395	-0.0432	0.0187	0.02081	0.173	7488	0.5894	-0.0605	0.0202	0.002797	0.142	5407	0.5322	12895
ABO	rs657152	A/C	0.3166	0.1382	0.0196	1.919 X 10-12	0.001	7488	0.4100	0.0416	0.0205	0.04215	0.092	5407	0.00071	12895
ITPK1	rs11624776	A/C	0.6104	-0.0937	0.0200	$2.72 X 10^{-06}$	0.924	7488	0.6419	-0.0447	0.0264	0.08989	0.378	5407	0.1418	12895
NRG1	rs7825175	A/G	0.2009	-0.0735	0.0238	0.001987	0.8	7488	0.2338	-0.033	0.0252	0.1918	0.754	5407	0.2454	12895
MBIP	rs1537424	T/C	0.6301	-0.0444	0.0185	0.01646	0.195	7488	0.5899	-0.0376	0.0213	0.0777	0.645	5407	0.8107	12895
SASH1	rs9497965	T/C	0.4062	0.0716	0.019	0.0001637	0.874	7488	0.4493	0.0077	0.0211	0.7155	0.514	5407	0.02531	12895
GLIS3	rs1571583	A/G	0.2820	0.0328	0.0200	0.1012	0.846	7488	0.2742	0.0911	0.0223	$4.36 X 10^{-05}$	0.360	5407	0.05308	12895
FT4 levels																
DIO1	rs2235544	A/C	0.4698	0.0.1257	0.0.0214	$4.24 X 10^{-09}$	0.0.198	6084	0.5046	0.0.1421	0.0.0313	$5.52 X 10^{-06}$	0.0.354	2077	0.0.666	8161
LHX3	rs7860634	A/G	0.4733	0.0984	0.0215	$4.5 X 10^{-06}$	0.229	6084	0.5557	0.0874	0.0315	0.005526	0.901	2077	0.774	8161
FOXE1	rs7045138	T/C	0.5413	0.0467	0.0337	0.1665	0.656	1997	0.5057	0.1415	0.0372	0.0001443	0.540	2077	0.059	4074
AADAT	rs11726248	A/G	0.0913	0.0.0971	0.0385	0.01159	0.927	6084	0.1100	0.0963	0.0494	0.05119	0.244	2077	0.990	8161
LPCAT2/CAPNS2	rs6499766	A/T	0.4300	0.088	0.0218	$5.33 X 10^{-05}$	0.861	6084	0.5014	-0.0198	0.0319	0.5354	0.447	2077	0.005	8161
NETO1/FBXO15	rs7240777	A/G	0.5567	-0.041	0.0217	0.05939	0.512	6084	0.5617	-0.0086	0.0329	0.7926	0.841	2077	0.412	8161

Table S4. Association results for TSH and FT4 overlapping loci and their involvement in the negative feedback loop

TSH associated markers			**TSH**			**FT4**		
Gene	Marker Name	A1/A2	Effect (StdErr)	*P*	N	Effect (StdErr)	*P*	N
PDE8B	**rs6885099**	**A/G**	**-0.141 (0.009)**	**1.95 X 10^{-56}**	**26042**	**0.025 (0.012)**	**0.034**	**17505**
PDE10A	rs753760	C/G	0.100 (0.010)	1.21 X 10^{-24}	25988	-0.020 (0.013)	0.114	17451
CAPZB	rs10799824	A/G	-0.113 (0.012)	3.60 X 10^{-21}	26031	0.020 (0.016)	0.194	17494
MAF/LOC440389	**rs3813582**	**T/C**	**0.082 (0.010)**	**8.45 X 10^{-18}**	**25948**	**-0.031 (0.013)**	**0.013**	**17411**
VEGFA	**rs9472138**	**T/C**	**-0.079 (0.010)**	**6.72 X 10^{-16}**	**25767**	**0.037 (0.013)**	**4.13 X 10^{-03}**	**17229**
VEGFA	rs11755845	T/C	-0.065 (0.010)	1.68 X 10^{-10}	25710	0.019(0.013)	0.153	17172
NR3C2	rs10032216	T/C	0.087 (0.011)	9.28 X 10^{-16}	26053	0.014 (0.014)	0.332	17516
IGFBP5	**rs13015993**	**A/G**	**0.078 (0.010)**	**3.24 X 10^{-15}**	**26016**	**-0.026 (0.013)**	**0.047**	**17479**
SOX9	rs9915657	T/C	-0.064 (0.009)	7.53 X 10^{-13}	25692	0.005 (0.012)	0.642	17154
NFIA	**rs334699**	**A/G**	**-0.141 (0.021)**	**5.40 X 10^{-12}**	**25757**	**0.053 (0.027)**	**0.048**	**17219**
FGF7	rs10519227	A/T	-0.072 (0.011)	1.02 X 10^{-11}	25988	0.013 (0.014)	0.352	17451
PRDM11	rs17723470	T/C	-0.065 (0.010)	8,83 X 10^{-11}	26054	0.005 (0.013)	0.709	17517
MIR1179	**rs17776563**	**A/G**	**-0.060 (0.010)**	**2.89 X 10^{-10}**	**25758**	**0.027 (0.013)**	**0.032**	**17221**
INSR	rs4804416	T/G	-0.057 (0.009)	3.16 X 10^{-10}	25632	0.021 (0.012)	0.076	17094
ABO	rs657152	A/C	0.058 (0.009)	4.11 X 10^{-10}	25765	-0.013 (0.012)	0.271	17227
ITPK1	rs11624776	A/C	-0.064 (0.011)	1.79 X 10^{-9}	23482	0.011 (0.014)	0.450	14945
NRG1	rs7825175	A/G	-0.066 (0.011)	2.94 X 10^{-9}	25996	0.020 (0.015)	0.171	17459
ITPK1	rs11624776	A/C	-0.064 (0.011)	1.79 X 10^{-9}	23482	0.011 (0.014)	0.450	14945
SASH1	rs9497965	T/C	0.051 (0.009)	2.25 X 10^{-0}	25980	-0.013 (0.012)	0.277	17443
GLIS3	**rs1571583**	**A/G**	**0.057 (0.010)**	**2.55 X 10^{-8}**	**25766**	**-0.028 (0.013)**	**0.040**	**17228**
FT4 associated markers			**FT4**			**TSH**		
Gene	Marker Name	A1/A2	Effect (StdErr)	*P*	N	Effect (StdErr)	*P*	N
DIO1	rs2235544	A/C	0.138 (0.012)	7.87 X 10^{-32}	17226	-0.013 (0.009)	0.136	25764
LHX3	**rs7860634**	**A/G**	**0.102 (0.013)**	**2.30 X 10^{-14}**	**14529**	**0.031 (0.011)**	**5.25 X 10^{-3}**	**20933**
AADAT	rs11726248	A/G	0.111 (0.019)	5.20 X 10^{-9}	17515	0.014 (0.015)	0.327	26052
FOXE1	rs7045138	T/C	0.098 (0.015)	1.50 X 10^{-11}	10997	0.008 (0.010)	0.451	19535
LPCAT2/CAPNS2	rs6499766	A/T	0.056 (0.012)	1.18 X 10^{-6}	17489	-0.009 (0.009)	0.3374	26026
NETO1/FBXO15	rs7240777	A/G	-0.049 (0.012)	3.13 X 10^{-5}	17146	0.005 (0.009)	0.559	25684

Table S5. Genotype risk score for TSH alleles in pregnant women.

Modela	N	Betab	SE	P-value
GRS[c]	858	0.109	0.015	3×10^{-12}
GRS in TPO-Ab negative women	794	0.117	0.015	3×10^{-14}
GRS adjusted for fetal genotypes	561	0.106	0.023	8×10^{-6}

[a] Linear regression of TSH level in pregnancy (inverse-normal transformation) against genotype risk score, with age and age-squared as covariates, excluding women on thyroid function medication and those of non-European descent.
[b] beta per TSH-raising allele.
[c] GRS= Genotype risk score was calculated as described in the Supplementary Methods in women with up to 2/9 SNPs missing.

Table S6. Association between TSH genetic risk score in pregnant women and subclinical hypothyroidism in pregnancy.

Modela	N	Effectc	StdErr	OR	*P*
GRS[b]	858	0.162	0.078	1.176	0.039
GRS in TPO-Ab negative women	794	0.257	0.093	1.292	0.006

[a] Logistic regression of outcome against genotype risk score, with age and age-squared as covariates, excluding women taking thyroid function medication and those non-European descent.
[b] GRS= Genotype risk score was calculated as described in the Supplementary Methods in women with up to 2/9 SNPs missing
[c] beta per TSH-raising allele.

Table S7. Association of SNPs reported by Gudmundsson and colleagues in our data-set.

MarkerName	MarkerTested	chr	Nearest Gene	TSH				FT4			
				Effect	StdErr	*P*	N	Effect	StdErr	*P*	N
rs10799824	**rs10799824**	**1**	***CAPZB***	**-0.113**	**0.012**	**3.60 X 10-21**	**26031**	**0.020**	**0.016**	**0.194**	**17494**
rs334725	**rs334725**	**1**	***NFIA***	**0.135**	**0.021**	**4.85 X 10^{-11}**	**25767**	**-0.053**	**0.027**	**0.050**	**17229**
rs17020124	rs17020124	1	*VAV3*	0.081	0.017	9.95 X 10^{-07}	26055	-0.006	0.022	0.798	17518
rs11694732	rs11694732	2	*TPO*	0.027	0.009	0.00257	25968	-0.027	0.012	0.022	17431
rs737308	**rs737310 (r2=1)**	**2**	***IGFBP5***	**-0.078**	**0.010**	**1.14 X 10^{-14}**	**26013**	**0.027**	**0.013**	**0.038**	**17476**
rs966423	rs966423	2	*DIRC3*	0.016	0.009	0.0726	25758	-0.006	0.012	0.586	17220
rs10030849	**rs11935941 (r2=0.99)**	**4**	***NR3C2***	**0.084**	**0.011**	**3.77 X 10-15**	**26052**	**0.010**	**0.014**	**0.492**	**17515**
rs2046045	**rs2046045**	**5**	***PDE8B***	**-0.142**	**0.009**	**2.14 X 10-55**	**25767**	**0.017**	**0.012**	**0.151**	**17229**
rs729761	**rs729761**	**6**	***VEGFA***	**-0.088**	**0.011**	**2.83 X 10^{-15}**	**23073**	**0.042**	**0.015**	**0.0038**	**14535**
rs6923866	**rs11755845 (r2=0.95)**	**6**	***VEGFA***	**-0.065**	**0.010**	**1.68 X 10-10**	**25710**	**0.019**	**0.013**	**0.153**	**17172**
rs3008043	**rs3008043**	**6**	***PDE10A***	**-0.092**	**0.010**	**5.23 X 10-20**	**21923**	**0.019**	**0.014**	**0.152**	**13386**
rs2439302	**rs2439302**	**8**	***NRG1***	**0.037**	**0.009**	**3.54 X 10-05**	**25932**	**-0.002**	**0.012**	**0.878**	**17395**
rs965513	**rs965513**	**9**	***FOXE1***	**-0.022**	**0.009**	**0.0196**	**25764**	**-0.068**	**0.012**	**3.45 X 10^{-08}**	**17226**
rs7913135	rs7913750 (r2=1)	10	*NKX2-3*	-0.029	0.009	0.00122	26043	-0.026	0.012	0.028	17506
rs7128207	**rs7128207**	**11**	***PRDM11***	**0.050**	**0.009**	**3.05 X 10^{-08}**	**25756**	**-0.005**	**0.012**	**0.665**	**17218**
rs61938844	NO PROXY	12	*ELK3*	ND	ND	ND	ND	ND	ND	ND	ND
rs944289	rs944289	14	*MBIP**	-0.043	0.009	2.22 X 10^{-06}	25746	0.0211	0.012	0.077	17208
rs116909374	NO PROXY	14	*MBIP*	ND	ND	ND	ND	ND	ND	ND	ND
rs34269820	**rs957362 (r2=0.89)**	**14**	***ITPK1***	**-0.052**	**0.011**	**9.75 X 10-07**	**25766**	**0.0114**	**0.014**	**0.407**	**17228**
rs73362602	not in HapMap2	14	*SIVA1*	ND	ND	ND	ND	ND	ND	ND	ND
rs73398284	**rs17477923 (r2=0.95)**	**15**	***FGF7***	**0.068**	**0.010**	**1.40 X 10-11**	**26033**	**-0.022**	**0.013**	**0.099**	**17496**
rs7190187	**rs7188445 (r2=0.99)**	**16**	***MAF/LOC440389***	**-0.079**	**0.010**	**9.66 X 10^{-17}**	**25903**	**0.036**	**0.013**	**4.91 X 10^{-03}**	**17366**
rs10420008	**rs10420008**	**19**	***INSR***	**-0.074**	**0.012**	**7.23 X 10-10**	**21333**	**0.008**	**0.016**	**0.630**	**12795**
rs6082762	rs2424440 (r2=1)	20	*FOXA2*	0.031	0.011	4.08 X 10^{-03}	26054	0.016	0.014	0.255	17517

ND, not determined

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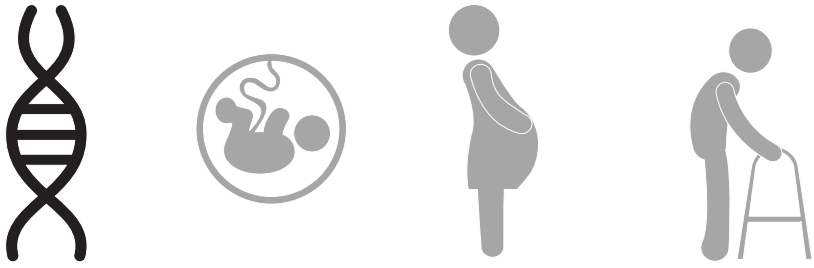
Appendix 2

(Supplemental data Chapter 9)



SUPPLEMENTARY TEXT S1

COHORT DESCRIPTIONS

Stage 1 cohorts

Busselton Health Study (BHS): The BHS includes a series of cross-sectional health surveys carried out since 1966 of residents of Busselton, a rural town with a predominantly Caucasian population, located in the southwest of Western Australia [74]. In 1994-5, there was a follow-up study of people who had participated in previous studies. Participants completed a health questionnaire, underwent physical examination, and gave a venous blood sample in the morning after an overnight fast.

Cardiovascular Health Study (CHS): The CHS is a population-based cohort study of risk factors for coronary heart disease and stroke in adults aged 65 years or older conducted across four field centers in the United States [75]. The original cohort of 5,201 persons consisting of 4,925 Caucasians was recruited in 1989-1990 from a random sample of people on Medicare eligibility lists. CHS participants completed standardized clinical examinations and questionnaires at study baseline and at nine annual follow-up visits.

Helsinki Birth Cohort Study (HBCS): The HBCS is composed of 8,760 individuals born between the years 1934-44 in one of the two main maternity hospitals in Helsinki, Finland. Between 2001 and 2003, a randomly selected sample of 928 males and 1,075 females participated in a clinical follow-up study with a focus on cardiovascular, metabolic and reproductive health, cognitive function and depressive symptoms. Detailed information on the selection of the HBCS participants and on the study design can be found elsewhere [76,77]. Details of the thyroid studies have been described for women and are similar for men in the cohort [78].

KORA: The KORA discovery study is a population-based cohort study including 1287 probands aged 32 to 79 from the Cooperative Health Research in the Region of Augsburg Study (KORA F4, Southern Germany), which has been described in detail previously [79]. All individuals were of European ancestry.

Nijmegen Biomedical Study (NBS): The NBS is a population-based survey on lifestyle and medical history in 9350 men and women living in Nijmegen, The Netherlands. Rationale and design have been described previously [80].

Rotterdam Study (RS): The Rotterdam Study is a prospective population-based cohort study on determinants of chronic diseases in the elderly, which has been described previously [81,82]. The study comprised 7983 men and women aged 55 years and over, living in a district of Rotterdam, The Netherlands.

SardiNIA: The SardiNIA study consists of 6,148 volunteers, males and females, ages 14–102 years, recruited and phenotyped from a cluster of four towns in the Ogliastra province of Sardinia [73]. The local ethical committee approved the study protocol and all participants provided a written informed consent. Genotyping was performed in 4,694 individuals using either the Affymetrix 10K, Affymetrix 500K or Affymetrix 6.0 arrays, and missing genotypes imputed using a within-families approach, as previously described [83].

Study of Health in Pomerania (SHIP): The SHIP is a cross-sectional survey in West Pomerania, the north-east area of Germany [84,85]. A sample from the population aged 20 to 79 years was drawn from population registries. First, the three cities of the region (with 17,076 to 65,977 inhabitants) and the 12 towns (with 1,516 to 3,044 inhabitants) were selected, and then 17 out of 97 smaller towns (with less than 1,500 inhabitants), were drawn at random. Second, from each of the selected communities, subjects were drawn at random, proportional to the population size of each community and stratified by age and gender. Only individuals with German citizenship and main residency in the study area were included. Finally, 7,008 subjects were sampled, with 292 persons of each gender in each of the twelve five-year age strata. In order to minimize drop-outs by migration or death, subjects were selected in two waves. The net sample (without migrated or deceased persons) comprised 6,267 eligible subjects. The SHIP population finally comprised 4,308 participants (corresponding to a final response of 68.7%).

SHIP–Trend: The SHIP-Trend is a longitudinal population-based cohort study assessing the prevalence and incidence of common, population relevant diseases and their risk factors [85]. Baseline examinations have started in 2008 and were finished in 2012. The study region is essentially the same as the study region of the initial SHIP cohort. The sample was drawn randomly from population registries.

TwinsUK: The TwinsUK cohort consists of 2455 female twins of Western European/UK ancestry, aged 18–82 years, from St Thomas' UK Adult Twin Registry, a volunteer sample recruited in the United Kingdom without selection for particular traits, which has previously been shown to be representative of singleton populations and the UK population in general [86].

Val Borbera: The INGI-Val Borbera population is a collection of 1,664 genotyped samples collected in the Val Borbera Valley, a geographically isolated valley located within the Appennine Mountains in Northwest Italy [87]. The valley is inhabited by about 3,000 descendants from the original population, living in 7 villages along the valley and in the mountains. Participants were healthy people 18-102 years of age that had at least one grandfather living in the valley.

Stage 2 cohorts

Asklepios: The Asklepios Study is a longitudinal population study focusing on better understanding of –and the interplay between- cardiovascular function and human aging with an eventual goal of developing better risk prevention models [88]. The 2524 participants are a population-representative cohort of 35-55 year old men and women, free from cardiovascular disease at study initiation (2002), randomly sampled from the twinned Belgian communities of Erpe-Mere and Nieuwerkerken.

CARLA: The CARLA study is an ongoing cohort study of a representative sample of the inhabitants of the city of Halle, eastern Germany, comprising 1,779 men and women aged 45–83 years at baseline [89]. The baseline examination took place between December 2002 and January 2006. A multi-step recruitment strategy aimed to achieve a high response rate. The final response rate after subtracting exclusions (individuals who were deceased prior to the invitation, had moved away, or were unable to participate due to illness) was 64.1%.

Exeter Family of Childhood Health (EFSOCH): The EFSOCH is a consecutive birth cohort consisting of children born between 2000 and 2004 in central Exeter, UK, and their parents [90]. Both parents attended a study visit at 28 weeks of gestation, at which DNA was collected and a fasting blood sample was taken for biochemical assays. In addition, a sample of mothers attended a follow-up visit at a median of 5 years post-pregnancy, at which a further fasting blood sample was taken. 1289 fathers and post-pregnancy mothers were included in the stage 2 analyses. The effects of the 5 GWAS significant SNPs on clinical thyroid disease in pregnancy was studied in 859 pregnant mothers.

Health2006 Study: The Health2006 Study is a cross-sectional population-based cohort study on lifestyle factors in relation to risk of chronic disease. The participants in the Health2006 Study were drawn as a random sample from the background population aged 18 to 69 years living in 11 municipalities in the South-western part of the greater Copenhagen area. A sample of 7770 persons eligible for invitation with Danish citizenship and born in Denmark was obtained from the Danish Central Personal Register, Min-

istry of Internal Affairs. A total of 3471 persons entered the study and participated in the health examinations at the RCPH which took place between June 2006 and June 2008. In addition, the study was registered at www.clinical.trials.com (Unique ID: KA20060011).

SardiNIA2: The SardiNIA stage 2 cohort consist of 1,392 individuals from the SardiNIA cohort unrelated (kinship coefficient=0) to the individuals in Stage 1 [73,91].

Graves' disease and thyroid cancer cohorts

Graves' disease and controls cohorts: The United Kingdom (UK) Graves' disease cases consisted of 2478 patients, with a mean age of 32 years and consisted of 82% females [92]. The control population consisted of 2682 geographically matched subjects drawn from the British 1958 Birth Cohort [93] (http://www.b58cgene.sgul.ac.uk/index.php). All subjects from the 1958 Birth Cohort were between the ages of 44-46 years when DNA was obtained and consisted of 50% females. All subjects were of white European ancestry with written informed consent and Ethics Committee/Institutional Review Board approval.

Nijmegen thyroid cancer and controls cohorts: Participants consisted of 154 individuals with non-medullary thyroid cancer (73% females, mean age at diagnosis 39.3 (SD 12.7)) and 2019 cancer-free individuals (51% females, mean age 61.5 (SD 10.3)). Affected individuals were recruited from the Department of Endocrinology, Radboud University Nijmegen Medical Centre (RUNMC), Nijmegen, The Netherlands from November 2009 to June 2010. All affected individuals were of self-reported European descent. The unaffected individuals were recruited from the Nijmegen Biomedical Study (NBS) [94]. The study was approved by the Ethical Committee and the Institutional Review Board of the RUNMC, Nijmegen, The Netherlands and all study subjects gave written informed consent.

Ohio thyroid cancer and controls cohorts: The study was approved by the Institutional Review Board of the Ohio State University. All subjects gave written informed consent before participation. Cases (n= 181) were histologically confirmed papillary thyroid carcinoma (PTC) patients (including traditional PTC and follicular variant PTC). These patients were admitted to the Ohio State University (OSU) Comprehensive Cancer Center. All cases were Caucasian; 39 men, 142 women, with a mean age at diagnosis of 39 (median age 38 years, range 12 to 88). Controls (n= 192) were individuals without clinically diagnosed thyroid cancer from the central Ohio area who were randomly picked from a pool of controls for genetics projects. All controls were Caucasian, 53 men, 139 women, with a mean age of 49 (median age 50 years, range 18 to 82).

SUPPLEMENTARY MATERIALS AND METHODS

Genotyping methods

Stage 2 cohorts

Asklepios: Genomic DNA was extracted from samples of peripheral venous blood according to standard procedures. DNA was available in all 2524 subjects. Genotypes for all 20 stage 1 SNPs were determined by Kbioscience (Unit 7, Maple Park, Hoddesdon, Herts, England UK) using the KASP on demand genotyping reagent system.

CARLA: Genomic DNA was extracted from samples of peripheral venous blood according to standard procedures. 1491 subjects were genotyped for rs11675434, rs653178, rs3094228, rs301799, and rs1230666. Genotypes were determined using the pre-developed TaqMan® SNP Genotyping Assays (Applied Biosystems, Darmstadt, Germany).

EFSOCH: Genomic DNA was extracted from samples of peripheral venous blood according to standard procedures. DNA was available in 1842 fathers and mothers with TPOAb data. Subjects were genotyped for all 20 stage 1 SNPs, except for rs3094228, rs1894407, and rs9277555. DNA samples were genotyped at KBiosciences (Hoddesdon, UK; www.kbioscience.co.uk), using their own system of fluorescence-based competitive allele-specific PCR (KASPar).

Of note, serum TSH levels were determined in 964 men and 974 pregnant women, and again in 572 women post-pregnancy. Serum FT4 levels were determined in 973 men, 974 pregnant women and 567 women post-pregnancy. Serum TSH and FT4 were analyzed using an electrochemiluminescent immunoassay, run on the Modular E170 Analyzer (Roche, Burgess Hill, UK). The manufacturer's population reference ranges (for non-pregnant samples) were: TSH, 0.35–4.5 mIU/L; and FT4, 11–24 pmol/L. For the analyses of the pregnant women, we used reference ranges specific for the assay and 28th week of gestation based on our own set of TPOAb-negative, healthy, pregnant women (n=901): TSH, 0.49–4.21 mIU/L and FT4, 9.13–15.17 pmol/L [95].

Health2006 Study: Genomic DNA was extracted from samples of peripheral venous blood according to standard procedures. DNA was available in 3287 subjects with TPOAb data. Genotypes for all 20 stage 1 SNPs were determined using KBiosciences allele-specific PCR (KASPar) (KBiosciences, Hoddesdon, UK).

SardiNIA2: Genomic DNA was extracted from samples of peripheral venous blood according to standard procedures. DNA was available in all subjects with TPOAb data.

Genotypes for rs1230666, rs3094228, rs1894407, rs9277555, rs10944479, and rs653178 were determined by ImmunoChip array, which was recently genotyped in the full Sardinia cohort but not included in the current GWAS data set. Genotyping was performed according to manufacturer's protocol, and quality control criteria have been described previously [73].

Graves' disease and thyroid cancer cohorts

Graves' disease and controls cohorts: Both rs10944479 and rs1230666 genotyping assays were purchased from Applied Biosystems, UK as pre-validated assays on demand. All genotyping was performed using Taqman genotyping technology on an ABI7900HT (Applied Biosystems, Warrington, UK) and all genotyping plots were independently verified by two investigators to prevent sample miscalling.

Nijmegen thyroid cancer and controls cohorts: Genotype data of most (N=1832) of the Dutch controls were already available at the start of the study and were obtained by Illumina HumanHap300 or HumanCNV370-Duo BeadChips [96]. Genotyping of the thyroid cancer cases (N=154) and the remaining controls (N=187) was performed by either TaqMan single nucleotide polymorphism (SNP) assays (rs11675434, rs653178, rs10944479 and rs1230666) on the 7300 ABI Real-Time polymerase chain reaction system (Applied Biosystems, CA, USA) or conventional PCR and Sanger sequencing (rs2010099).

Ohio thyroid cancer and controls cohorts: For both cases and controls, genomic DNA from blood samples was extracted by a standard phenol-chloroform procedure. To genotype the five SNPs, SNaPshot assay (ABI) was used as described [97]. Primer extension was carried out using the SNaPshot Multiplex Kit (ABI) according to the manufacturer's instructions. The allele analysis was performed using an ABI 3730 DNA Analyzer.

Thyroid ultrasound measurements and diagnosis of goiter

Thyroid ultrasound measurements in SHIP/SHIP-Trend and KORA were performed using the Ultrasound VST-Gateway 5 MHz linear array transducer (Diasonics) and the SONOLINE G50 5 MHz linear array transducer (Siemens Medical), respectively. Thyroid volume was calculated as length * width * depth * 0.479 (mL) for each lobe. Goiter was defined as a thyroid volume > 18 mL in women and > 25 mL in men, as has been described in detail previously [98].

Heritability analyses of TPOAb-positivity and TPOAb levels

Heritability analyses for TPOAb-positivity and TPOAb levels were performed in SardiNIA, TwinsUK and Val Borbera using SOLAR (Sequential Oligogenic Linkage Analysis Routines;

http://bioweb2.pasteur.fr/docs/solar/). For the TPOAb levels a rank-based transformation method, using an inverse normal transformation as performed by SOLAR, was used to deal with kurtosis and skewness. For both traits, age and sex were included in the analyses. A basic model was used to estimate the additive polygenic component σ_a^2 and environmental component σ_e^2 of each trait variance due to mean effects of single alleles. Polygenic model as performed by solar quantified heritability as $h^2 = \sigma_a^2/\sigma_a^2+\sigma_e^2$ and provided an estimate of the degree to which the offspring phenotypes are explained by parental phenotypes.

Stage 1 meta-analyses

Before meta-analysis, SNPs with a minor allele frequency (MAF) < 1% or a low imputation quality (< 0.3 for MACH and < 0.4 for IMPUTE/BIMBAM) were excluded. In addition, SNPs were excluded from the GWAS on TPOAb-positives and –negatives if (2 * N * MAF * imputation quality) < 30, where N was the total number of subjects included in the analysis of the respective SNP. The results of each GWAS were combined using a population size weighted z-score based meta-analysis, as TPOAb levels were determined with a range of different TPOAb assays (Table S1).

Selection of stage 2 SNPs

Stage 1 SNPs with a *P*-value ≤ 10^{-5} were separated into independent loci by clumping based on LD (r2>0.2) using PLINK. SNPs with MAF < 5 % or high heterogeneity ($I^2 \geq 50$ %) were excluded. In this way, based on $I^2 = 80.8$ and heterogeneity *P*-value = $1.5x10^{-6}$, we excluded rs133994 from the TPOAb level analyses. Based on the MAF criteria, we excluded rs547165 (MAF = 0.01) in the TPOAb level analyses, and rs9563708 (MAF = 0.03) in the TPOAb-positivity analyses. To make the most efficient use of available resources, not all promising stage 1 SNPs had to be followed up in all stage 2 replication cohorts (see Supplementary Material S1section on genotyping methods). In this way, taking sample size and financial constraints of the stage 2 replication cohorts into account, we calculated that we were powered to establish associations at GWAS significant levels for stage 1 SNPs with $P < 5 \times 10^{-6}$.

Variance explained

To evaluate the variance explained for TPOAb-positivity and serum TPOAb levels by the GWAS significant hits, we subtracted, in each GWAS study, the variance explained by the basic regression model (only including the covariates age and sex) from that explained by the full model, in which also the 5 GWAS significant SNPs were included. A weighted average of study-specific variances was calculated by combining the variance explained with weights proportional to the study sample size.

Association analyses clinical thyroid disease

In clinical practice, the TPOAb status (positive or negative) rather than the TPOAb level is important in decision making. As the identified 3 GWAS significant SNPs for TPOAb levels also showed associations with TPOAb-positivity (*TPO*-rs11675434*:* OR, 1.21 [95% CI, 1.15-1.28)], $P = 1.5 \times 10^{-16}$; *MAGI3*-rs1230666*:* OR, 1.23 [95% CI, 1.14-1.33], $P = 1.5 \times 10^{-6}$; *KALRN*-rs2010099*:* OR, 1.24 [95% CI, 1.12-1.37], $P = 7.4 \times 10^{-5}$), we studied the (combined) effects of all 5 SNPs on clinical thyroid disease as specified below.

Genetic risk score and TPOAb-positivity

A genetic risk score based on the 5 GWAS significant SNPs was calculated for every individual as the weighted sum of TPOAb-positivity risk alleles, with weights proportional to the effect estimated in the stage 1 + 2 meta-analysis. As a z-score based meta-analysis does not provide betas, we calculated betas using a fixed effects (inverse variance based) meta-analysis for TPOAb-positivity.

In each stage 2 study, we calculated genetic risk score quartiles from the global distribution of the scores. The number of TPOAb-positives and –negatives were compared between the genetic risk score quartiles, using logistic regression analyses, adjusting for age and sex. The results of each study were combined using a population size weighted z-score based meta-analysis.

Associations with hypo- and hyperthyroidism

The associations between genetic risk score quartiles and the risk of increased TSH levels, overt hypothyroidism, decreased TSH levels and overt hyperthyroidism were studied as well, using the same method as for the genetic risk score vs TPOAb-positivity analyses. The associations with the individual SNPs were studied as well, using logistic regression analyses, adjusting for age and sex. The results of each study were combined using a population size weighted z-score based meta-analysis. Bonferroni threshold was used to define significance of associations.

As thyroid hormone metabolism significantly changes during pregnancy [24], we additionally studied the individual and combined effects of the GWAS significant SNPs on the risk of thyroid dysfunction during pregnancy. These effects were studied in 859 pregnant women from the EFSOCH cohort, including 59 TPOAb-positives, 38 women with increased TSH and 13 women with suppressed TSH levels. As there were only 2 overt hypothyroid and 7 overt hyperthyroid women, we were unable to study the effects on overt hypo- and hyperthyroidism during pregnancy.

Associations with goiter

Thyroid ultrasound data were available in 3614 SHIP, 887 SHIP-Trend, and 1290 KORA subjects. Subjects using thyroid medication and pregnant women were excluded, ex-

cept in SHIP-Trend in which pregnancy data were unavailable. The associations between the individual SNPs, genetic risk scores and goiter were studied using logistic regression analyses, adjusting for age, sex and body surface area (BSA). BSA was calculated as $0.007184 \times (\text{weight[kg]})^{0.425} \times (\text{height[cm]})^{0.725}$. The results of each study were combined using a population size weighted z-score based meta-analysis.

Associations with thyroid disease in independent populations

Since Graves' disease is the major cause of hyperthyroidism, and considering the fact that Hashimoto's thyroiditis and Graves' disease co-segregate in families [17,20,21], we selected those SNPs that showed promising associations with hyperthyroidism in our meta-analyses (i.e., $P \leq 0.05$). These SNPs were tested in an independent population of 2478 patients with Graves' disease and 2682 controls using logistic regression analyses, adjusting for age and sex.

As both thyroid autoimmunity and abnormal TSH levels have been implicated in the development of thyroid cancer [99,100], we tested the risk of thyroid cancer for the GWAS significant SNPs in the Nijmegen and Ohio cohorts, including in total 333 cases and 2209 controls. Associations were studied using logistic regression analyses, adjusting for age and sex. The results of these studies were combined using a population size weighted z-score based meta-analysis.

Effects of previously identified thyroid related SNPs in the stage 1 TPOAb-positivity and TPOAb level meta-analyses

Table S4 shows the stage 1 TPOAb-positivity and TPOAb level meta-analyses results for GWAS significant SNPs reported in 15 previous GWAS on thyroid related phenotypes [36,37,41,91,101-107]. We reported a proxy ($r^2>0.8$) and the relative r^2 in case the marker was not available. The most significant associations were found for SNPs in or near *CTLA4*, *PTPN22*, *SH2B3*, and *MAF*. The rs11694732 variant, which is in LD with our top hit *TPO*-rs11675434 ($r^2=0.97$ in HapMap2), was also associated with both TPOAb-positivity and TPOAb levels. However, given the higher *P*-value this association is most likely driven by *TPO*-rs11675434.

Bioinformatics tools search for functional relevance

For the GWAS significant SNPs, bioinformatics tools were searched for functional relevance including Gwava (www.sanger.ac.uk/sanger/StatGen_Gwava), Haploreg [108], and eQTL resources (http://eqtl.uchicago.edu). However, this did not lead to additional insights (data not shown).

Pathway analyses

Pathway analyses were performed using the Ingenuity Pathway Analysis software tool Network (IPA; Ingenuity Systems, Ca, USA) in order to get more insight into possible pathways and networks involved. Focus genes for network inquiry were selected using the top 20 stage 1 GWAS SNPs (Tables S2 and S3). Molecules and/or relationships considered were the ones available in the IPA Knowledge Base for mammals (human, mouse or rat). Confidence filters were set to consider only relationships where the confidence is Experimentally Observed or High (Predicted). Networks were generated with a maximum size of 35 genes and allowing up to 25 networks per analysis. The networks are constructed using the IPA algorithm which generates a score as well as a *P*-value. IPA computes a score for each network according to the fit of that network to the set of focus genes. The *P*-value is calculated using the right-tailed Fisher Exact test. The 20 promising loci were mapped in the Ingenuity Knowledge base and all were considered for network construction on available interactions. In this way, Ingenuity generated 4 networks (Table S7) which included 196 nodes. There were no overlapping networks. The top 2 networks incorporated genes that code for cell death, survival and movement as well as molecular transport and carbohydrate metabolism. We assessed how genes in the networks overlap with canonical pathways in the Ingenuity database (Table S8.). The top three networks included the OX40 Signaling Pathway, Antigen Presentation Pathway and Autoimmune Thyroid Disease Signaling pathways.

The same 20 loci were used to examine functional connectivity with the Gene Relationships Among Implicated Loci Package (GRAIL; www.broad.mit.edu/mpg/grail/) [109]. In short, GRAIL is a tool that searches for relationships between genes in different disease associated loci for a given set of genes or SNP's. GRAIL mines PubMed archives looking for similarities in the published scientific text among the associated genes. Genes for text mining of the functional data source were identified using HapMap2 Release 22 CEU samples. The search included indexed abstracts from PubMed last curated on December 2006. The results of GRAIL are summarized in Figure S6. The most significant relation was found between *BACH2* and *MAF*, which were also both prominent in the cell death, survival and movement IPA pathway (Table S7). In humans, *MAF* has been shown to play a role in IL10 and IL4 expression [110] and CD4+ T-lymphocytes transcription [111].

Figure S1 TPOab level distribution in the stage 1 and 2 cohorts

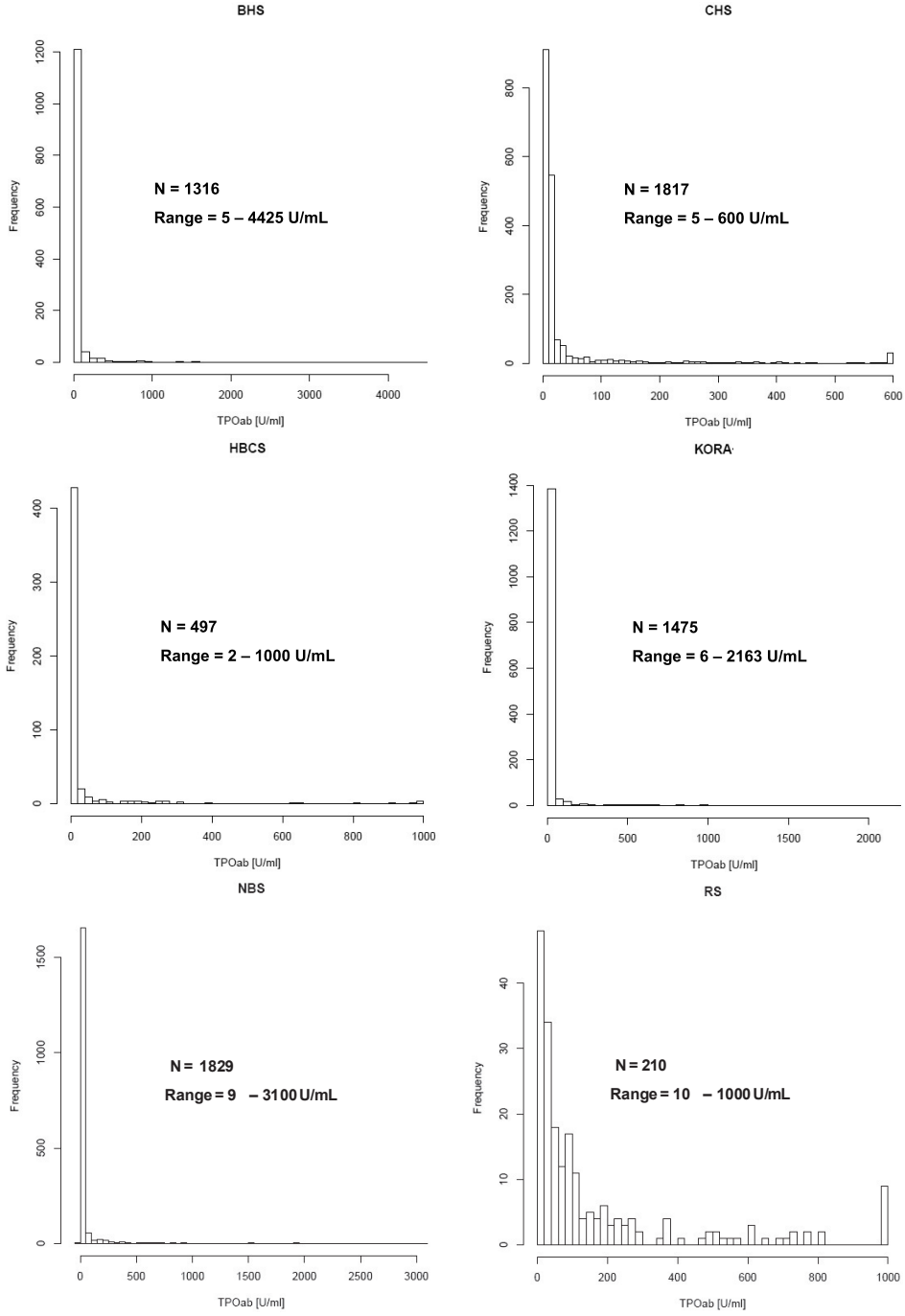


Figure S1 (Continued)

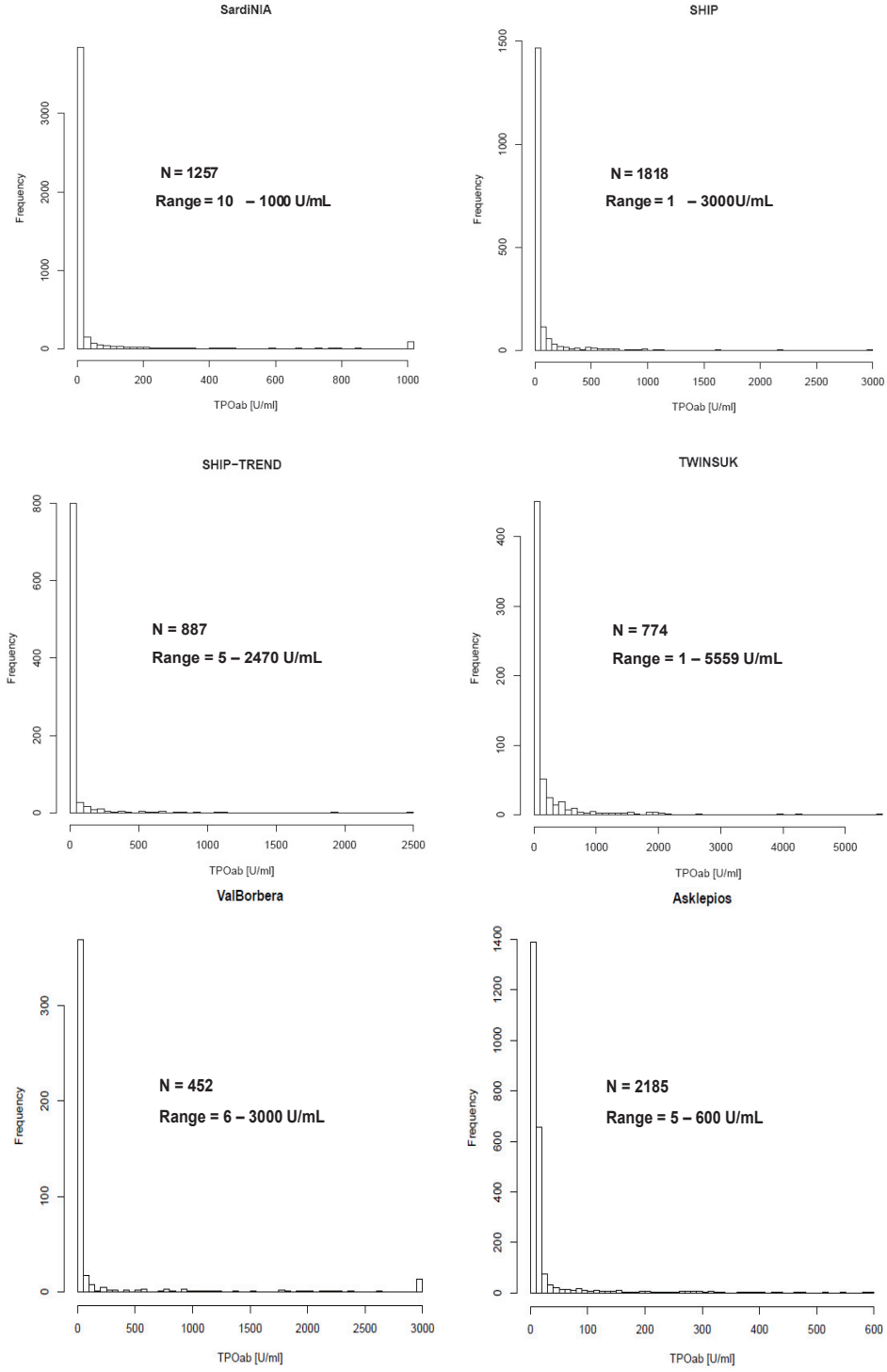


Figure S1 (Continued)

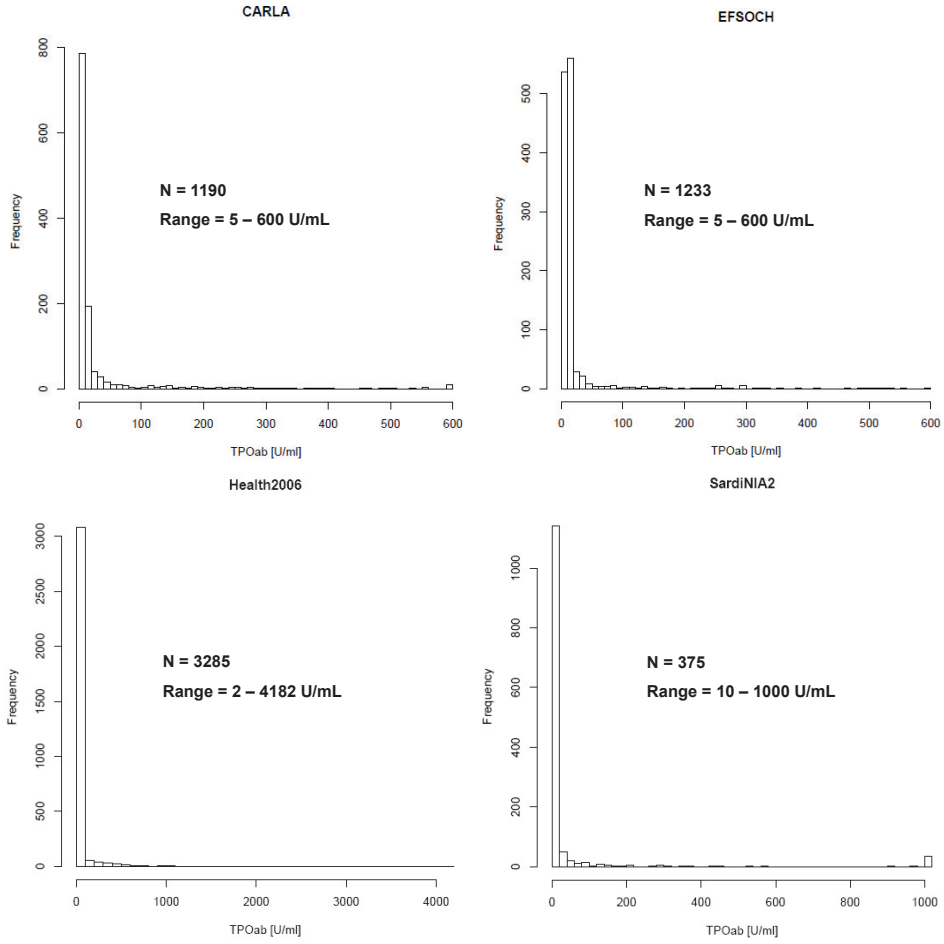


Figure S1 TPOAb level distributions in stage 1 and 2 cohorts.

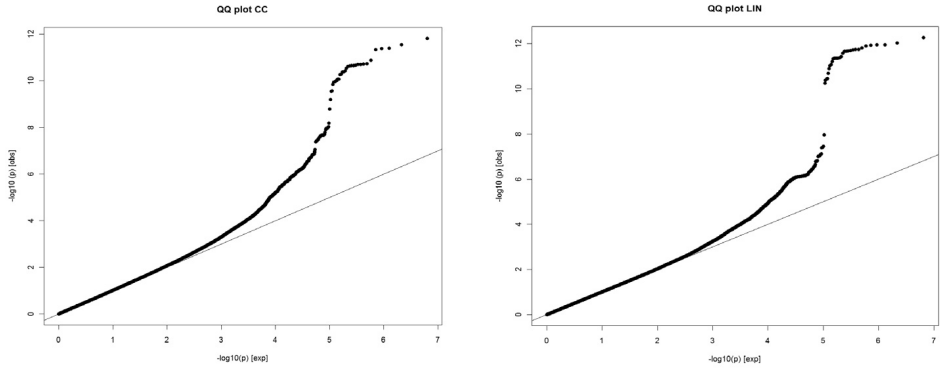


Figure S2. Quantile-quantile (QQ) plots for the TPOAb-positivity and TPOAb level stage 1 meta-analyses

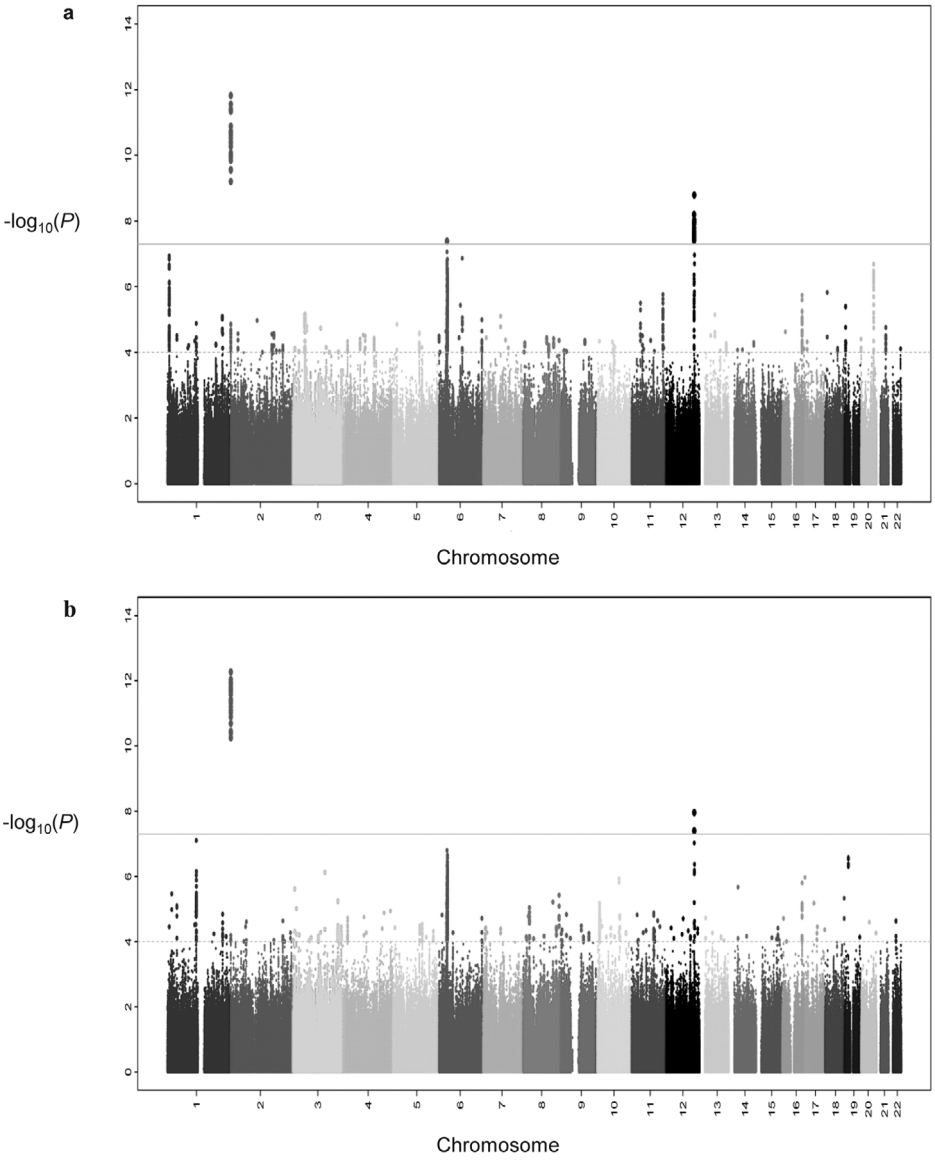


Figure S3. Manhattan plots for stage 1 meta-analyses for TPOAb-positivity (a) and TPOAb levels (b). SNPs are plotted on the x-axis according to their chromosomal position against TPOAb-positivity (a) or TPOAb levels (b) (shown as – $\log_{10}$ *P* value) on the y-axis. The horizontal grey line indicates the threshold for genome-wide statistical significance ($P < 5 \times 10^{-8}$). Genome-wide significant associations were observed near *TPO* (Chr 2p25; $P = 1.5 \times 10^{-12}$), at *ATXN2* (Chr 12q24.1; $P = 1.6 \times 10^{-9}$) and near *HCP5* (Chr 6p21.3; $P = 4.1 \times 10^{-8}$) for TPOAb-positivity, and near *TPO* (Chr 2p25; $P = 5.4 \times 10^{-13}$) and at *ATXN2* (Chr 12q24.1; $P = 1.1 \times 10^{-8}$) for TPOAb levels. A color figure is available at: http://www.plosgenetics.org/article/info%3Adoi%2F10.1371%2Fjournal.pgen.1004123#s5

Figure S4. Regional association plots of stage 1 lead loci for TPOAb-positivity.

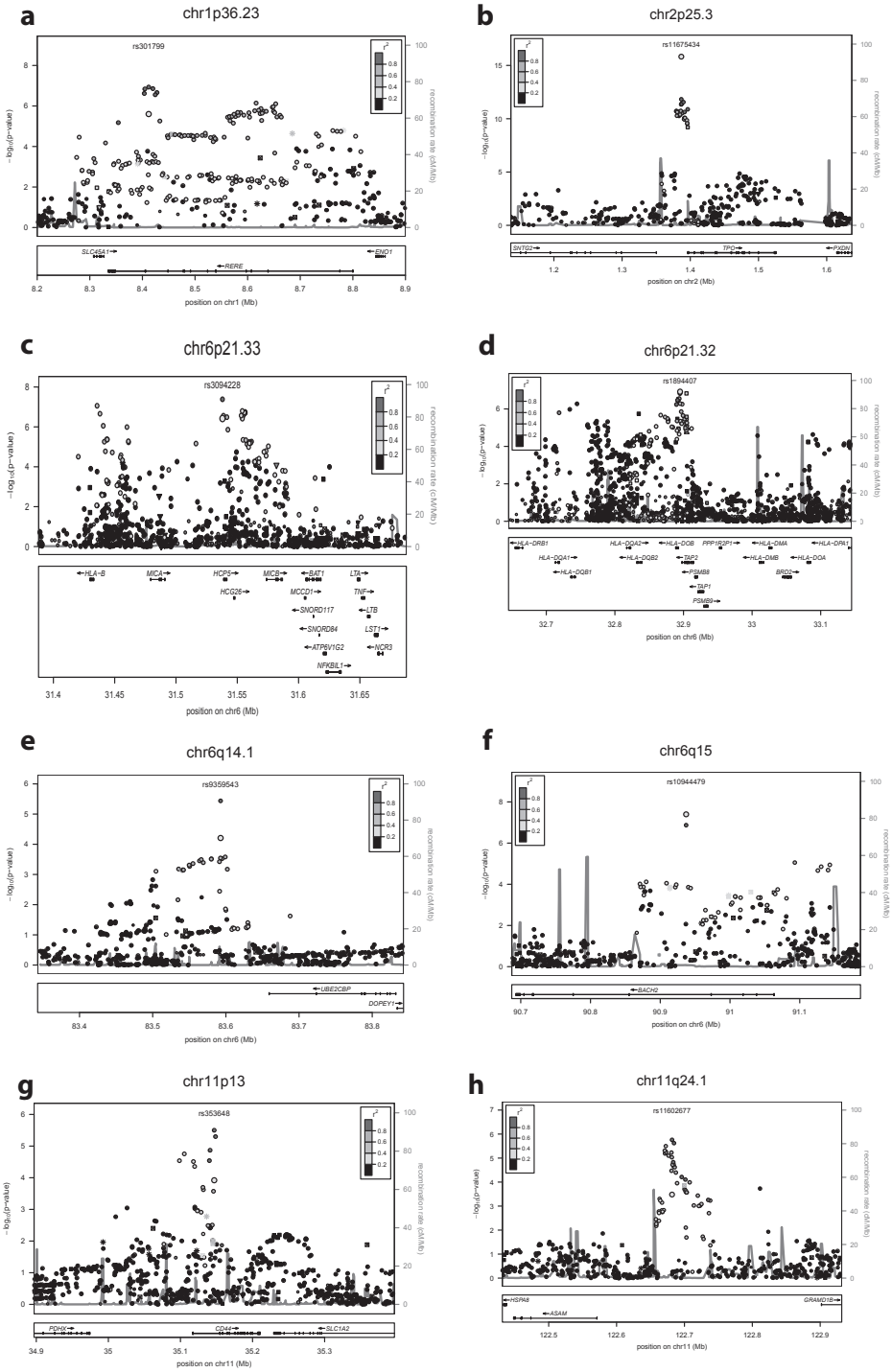
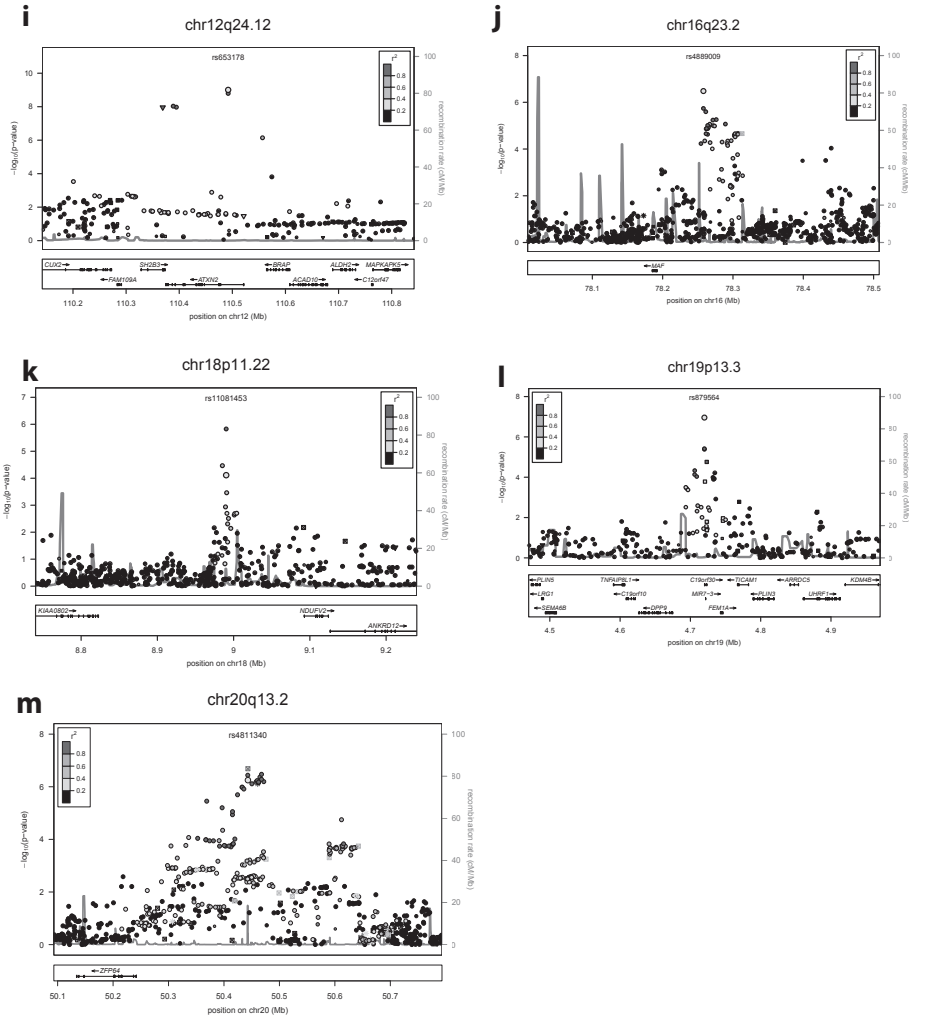


Figure S4 (continued)**.**



The y-axis on the left indicates the – $\log_{10}$ *P* value for the association with TPOAb –positivity. SNPs are plotted on the x-axis according to their chromosomal position. The most significant stage 1 SNP is indicated in purple. The combined stage 1 and 2 result of this SNP is indicated in yellow. The SNPs surrounding the most significant SNP are color-coded to reflect their LD with this SNP. Symbols reflect functional genomic annotation, as indicated in the legend. The blue y-axes on the right of each plot indicate the estimated recombination rates (based on HapMap Phase II); the bottom of each panel shows the respective annotated genes at the locus and their transcriptional direction. Mb, megabases
A color figure is available at: http://www.plosgenetics.org/article/info%3Adoi%2F10.1371%2Fjournal.pgen.1004123#s5

Figure S5. Regional association plots of stage 1 lead loci for TPOAb levels.

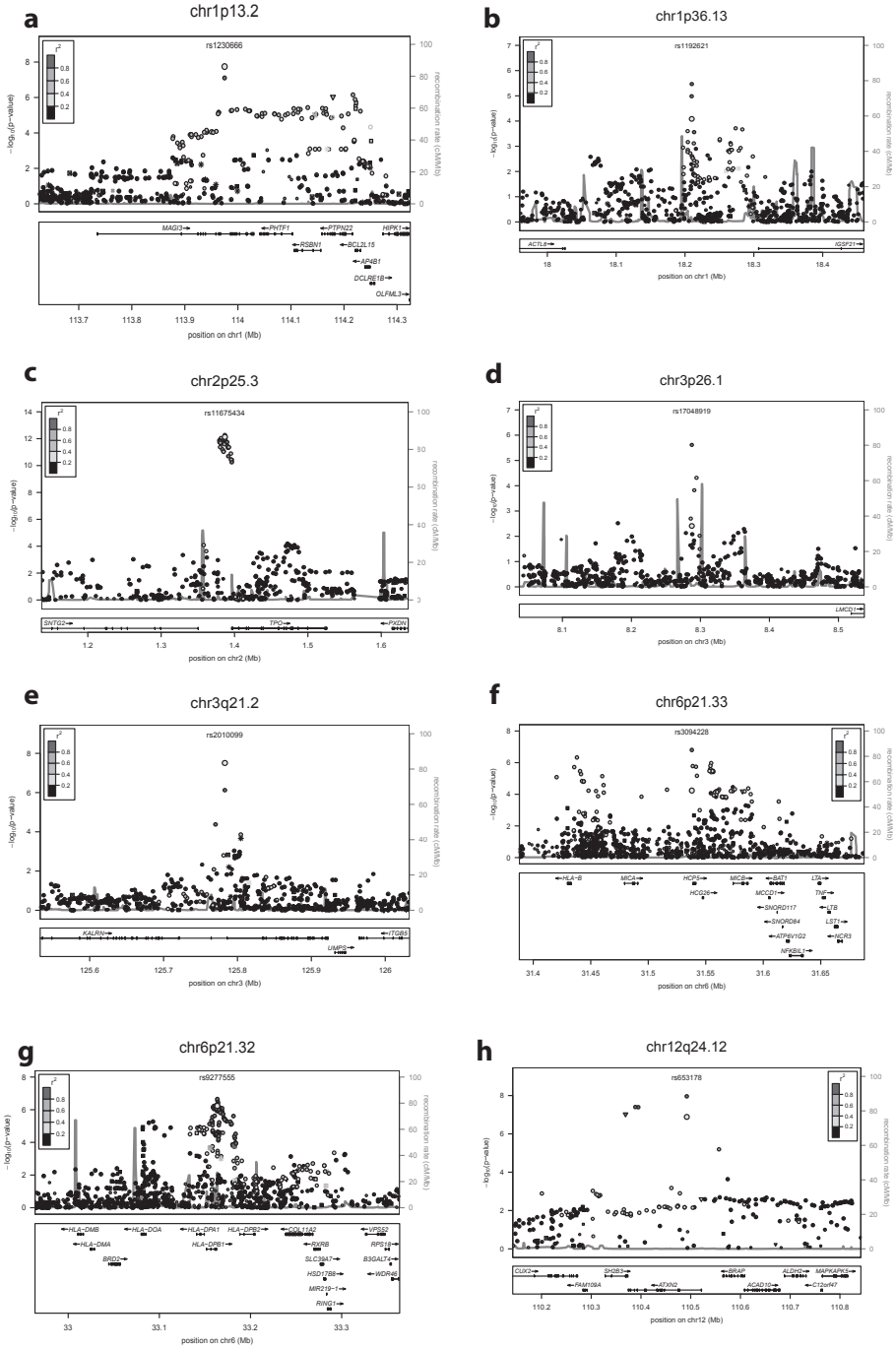
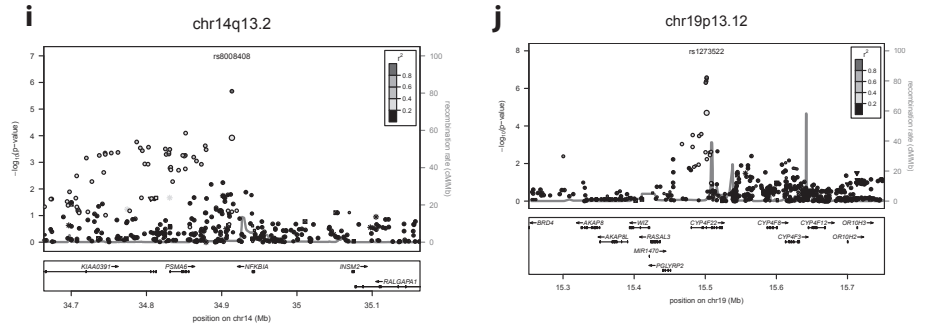


Figure S5 (Continued).



The y-axis on the left indicates the – $\log_{10}$ *P* value for the association with TPOAb levels. SNPs are plotted on the x-axis according to their chromosomal position. The most significant stage 1 SNP is indicated in purple. The combined stage 1 and 2 result of this SNP is indicated in yellow. The SNPs surrounding the most significant SNP are color-coded to reflect their LD with this SNP. Symbols reflect functional genomic annotation, as indicated in the legend. The blue y-axes on the right of each plot indicate the estimated recombination rates (based on HapMap Phase II); the bottom of each panel shows the respective annotated genes at the locus and their transcriptional direction. Mb, megabases
A color figure is available at: http://www.plosgenetics.org/article/info%3Adoi%2F10.1371%2Fjournal.pgen.1004123#s5

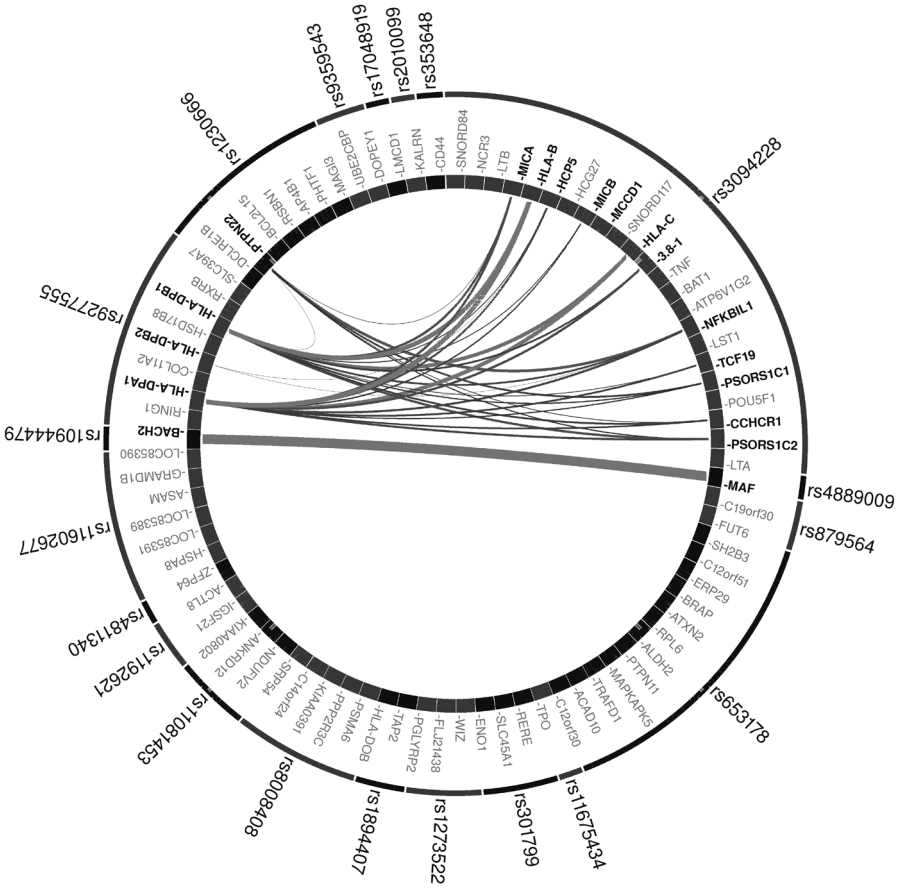


Figure S6. GRAIL results for the stage 1 TPOAb-positivity and TPOAb level lead SNPs
GRAIL circle plot of locus connectivity where each locus is plotted in a circle, where significant connections ($P < 0.05$) based on PubMed abstracts are drawn spanning the circle. Analyses were based on the 20 stage 1 TPOAb-positivity and TPOAb level lead SNPs.

Table S1. Study sample genotyping, quality control and association analyses for stage 1 populations.

	Genotyping		Sample QC		SNP QC			Imputation						Case-control analyses		Continuous analyses	
Study	N	Platform	Call rate	Exclusion criteria	Call rate	HWE	MAF	N of SNPs for imputation	Reference panel	Software	N of SNPs in analyses	Covariates used in all analyses	Population stratification correction or assessment[a]	Software	λ	Software	λ
BHS	1366	Illumina Human 610 quad	≥ 0.95	Relatedness and duplicates	≥ 0.90	$P>10^{-6}$	≥ 0.01	549,294	HapMap II, CEU, Build 36	MACH	2,543,887	Age, sex, asthma status	Yes (no stratification)	GenABEL	1.01	GenABEL	1.01
CHS	3271	Illumina 370CNV BeadChip	≥ 0.95	Non-European descent	≥ 0.97	$P>10^{-5}$	-	306,655	HapMap II, CEU, Build 36	BIMBAM v0.99	2,573,510	Age, sex, clinic site	Yes (Exclusion non-European descent)	R	1.03	R	1.01
HBCS	1728	Illumina 610k	≥ 0.95	Relatedness and duplicates	≥ 0.95	$P>10^{-5}$	≥ 0.01	509,948	HapMap II, CEU, Build 36	MACH	2,543,887	Age, sex	Yes (no stratification)	Plink and ProbABEL	1.00	Plink and ProbABEL	1.00
KORA	1814	Affymetrix 6.0 (1000K)	≥ 0.95	Relatedness and duplicates	≥ 0.93	$P>10^{-6}$	≥ 0.01	909,622	HapMap II, CEU, Build 36	IMPUTE	2,743,205	Age, sex	Yes (PCA)	SNPTEST	1.03	SNPTEST	1.01
NBS	1980	Illumina 370 CNV BeadChip	≥ 0.95	Non-Caucasian ancestry	≥ 0.95	$P>10^{-6}$	≥ 0.01	311,918	HapMap II, CEU, Build 36	IMPUTE	2,542,995	Age, sex	Yes (Exclusion non-Caucasian ancestry)	SNPTEST	1.01	SNPTEST	1.00
RS	5974	Illumina HumanHap 550K v.3	≥ 0.98	Relatedness and duplicates	≥ 0.98	$P>10^{-6}$	≥ 0.01	512,349	HapMap II, CEU, Build 36	MACH	2,543,887	Age, sex	Yes (no stratification)	Mach2dat (GRIMP)	1.02	Mach2qtl (GRIMP)	1.01
SardiNIA	4694	Affymetrix 10K, 500K, 6.0	>0.95	NA	>0.90 (10/500K) >0.95(6.0K)	$P>10^{-6}$	>0.05 (10K,500K) >0.01(6.0)	731,209	HapMap II, CEU, Build 36	MACH	2,353,985	Age, sex	No (high isolation)	Mach2dat	1.08	MERLIN (—fastassoc)	1.13
SHIP	4081	Affymetrix SNP Array 6.0	≥ 0.92	Duplicates	-	-	-	869,224	HapMap II, CEU, Build 36	IMPUTE	2,748,910	Age, sex	Yes (no stratification)	SNPTEST	1.05	SNPTEST	1.01
SHIP-Trend	986	Illumina Human Omni 2.5	≥ 0.94	Duplicates	≥ 0.90	$P>10^{-4}$	-	1,782,967	HapMap II, CEU, Build 36	IMPUTE	3,437,411	Age, sex	Yes (no stratification)	SNPTEST	1.06	SNPTEST	0.88
TwinsUK	2455	Illumina Hap300, Hap550, Hap610	≥ 0.95	Relatedness and duplicates	≥ 0.90	$P>10^{-6}$	≥ 0.01	295,702	HapMap II, CEU, Build 36	IMPUTE	2,544,232	Age	Yes (Exclusion non-European descent)	GenABEL	1.00	GenABEL	1.00
ValBorbera	1664	Illumina 370K - HumanCN370-Quadv3	> 0.95	Relatedness and duplicates	≥ 0.90	$P>10^{-4}$	≥ 0.01	324,326	HapMap II, CEU, Build 36	MACH	2,412,004	Age, sex	No (high isolation)	GenABEL	1.01	GenABEL	1.01

Table S2. Associations of stage 1 lead SNPs with TPOAb-positivity in stage 1 and 2

			Alleles		*Stage 1* Up to 1769 cases + 16,528 controls		*Stage 2* Up to 922 cases + 8068 controls		*Stage 1 + 2* Up to 2691 cases + 24,596 controls		
SNP	Chr	Position (Build 36)	Risk	Other	OR (95% CI)	*P*	OR (95% CI)	*P*	OR (95% CI)	*P*	Het *P*
rs11675434	2	1386822	T	C	1.20 (1.13-1.27)	$1.5x10^{-12}$	1.28 (1.14-1.42)	$1.9x10^{-5}$	1.21 (1.15-1.28)	$1.5x10^{-16}$	0.08
rs653178	12	110492139	C	T	1.14 (1.08-1.20)	$1.6x10^{-9}$	1.13 (1.02-1.25)	0.04	1.14 (1.08-1.19)	$9.9x10^{-10}$	0.29
rs3094228	6	31537906	C	T	1.33 (1.20-1.47)	$4.1x10^{-8}$	1.08 (0.94-1.23)	0.31	1.23 (1.13-1.33)	$3.8x10^{-7}$	0.08
rs301799	1	8411889	C	T	1.13 (1.07-1.20)	$1.2x10^{-7}$	1.02 (0.90-1.16)	0.72	1.11 (1.06-1.17)	$2.5x10^{-6}$	0.39
rs10944479	6	90937114	A	G	1.31 (1.16-1.47)	$1.4x10^{-7}$	1.16 (1.01-1.34)	0.04	1.25 (1.14-1.37)	$4.0x10^{-8}$	0.35
rs1894407	6	32895014	C	A	1.19 (1.12-1.27)	$1.5x10^{-7}$	1.10 (0.96-1.25)	0.13	1.17 (1.11-1.24)	$1.2x10^{-7}$	0.41
rs4811340	20	50443164	G	C	1.14 (1.08-1.21)	$2.1x10^{-7}$	1.08 (0.95-1.24)	0.28	1.13 (1.07-1.20)	$5.6x10^{-7}$	0.09
rs11081453	18	8990246	T	C	1.26 (1.14-1.40)	$1.5x10^{-6}$	1.01 (0.89-1.15)	0.98	1.16 (1.07-1.26)	$7.7x10^{-5}$	0.10
rs11602677	11	122681726	A	G	1.19 (1.11-1.27)	$1.7x10^{-6}$	0.93 (0.81-1.07)	0.34	1.13 (1.07-1.20)	$3.3x10^{-4}$	0.08
rs4889009	16	78257948	G	C	1.14 (1.08-1.22)	$1.8x10^{-6}$	1.14 (1.00-1.29)	0.05	1.14 (1.08-1.21)	$3.3x10^{-7}$	0.82
rs353648	11	35146865	T	G	1.28 (1.15-1.41)	$3.2x10^{-6}$	1.03 (0.86-1.24)	0.83	1.19 (1.09-1.30)	$1.2x10^{-4}$	0.24
rs9359543	6	83592789	T	C	1.32 (1.17-1.50)	$3.7x10^{-6}$	1.04 (0.88-1.22)	0.71	1.21 (1.10-1.34)	$6.2x10^{-5}$	0.05
rs879564	19	4719917	A	G	1.14 (1.07-1.21)	$3.9x10^{-6}$	1.20 (1.05-1.38)	0.01	1.15 (1.09-1.22)	$1.1x10^{-7}$	0.89

Chr., chromosome
Het *P*, heterogeneity *P*-value (significance threshold $P = 0.004$).
All analyses adjusted for age and gender

Table S3. Associations of stage 1 lead SNPs with serum TPOAb levels in stage 1 and 2

			Alleles		*Stage 1* Up to 12,353 subjects		*Stage 2* Up to 8159 subjects		*Stage 1 + 2* Up to 20,512 subjects		
SNP	Chr	Position (Build 36)	Risk	Other	β (SE)	*P*	β (SE)	*P*	β (SE)	*P*	Het *P*
rs11675434	2	1386822	T	C	0.0182 (0.0048)	$5.4x10^{-13}$	0.0429 (0.0229)	0.01	0.0202 (0.0046)	$7.4x10^{-13}$	0.06
rs653178	12	110492139	C	T	0.0144 (0.0047)	$1.1x10^{-8}$	0.0607 (0.0224)	0.18	0.0147 (0.0045)	$1.3x10^{-7}$	0.14
rs3094228	6	31537906	C	T	0.0179 (0.0062)	$1.6x10^{-7}$	0.1730 (0.0342)	0.84	0.0240 (0.0059)	$5.8x10^{-5}$	0.31
rs1230666	1	113974933	A	G	0.0241 (0.0067)	$7.9x10^{-8}$	0.0495 (0.0321)	0.02	0.0269 (0.0064)	$1.8x10^{-8}$	0.23
rs9277555	6	33163583	G	A	0.0198 (0.0052)	$2.2x10^{-7}$	0.0322 (0.0299)	0.20	0.0199 (0.0050)	$5.8x10^{-7}$	0.36
rs1273522	19	15501972	G	A	0.0211 (0.0053)	$2.7x10^{-7}$	-0.0047 (0.0231)	0.76	0.0195 (0.0050)	$2.0x10^{-5}$	0.01
rs2010099	3	125782947	C	T	0.0197 (0.0080)	$7.5x10^{-7}$	0.0565 (0.0360)	0.01	0.0240 (0.0076)	$3.1x10^{-8}$	0.23
rs8008408	14	34913567	A	G	0.0285 (0.0081)	$2.1x10^{-6}$	0.0301 (0.0353)	0.81	0.0261 (0.0077)	$1.2x10^{-4}$	0.19
rs17048919	3	8287555	G	C	0.0401 (0.0077)	$2.4x10^{-6}$	0.0004 (0.0313)	0.27	0.0344 (0.0073)	$3.9x10^{-3}$	0.03
rs1192621	1	18209837	A	T	0.0229 (0.0052)	$3.4x10^{-6}$	0.0026 (0.0247)	0.73	0.0279 (0.0050)	$8.2x10^{-5}$	0.11

Chr., chromosome

Het *P*, heterogeneity *P*-value (significance threshold $P = 0.005$).

Effects are expressed in SD of natural logarithm transformed serum TPOAb level, adjusted for age and gender.

Table S4 is available at: http://www.plosgenetics.org/article/info%3Adoi%2F10.1371%2Fjournal.pgen.1004123#s5

Table S5. Genetic risk score and the risk of increased TSH levels

GRS Quartile	% Increased TSH levels (N cases/total)	OR (95% CI)[a]	*P* value
1 (reference)	4.2 % (212 / 5051)	-	-
2	5.7 % (290 / 5050)	1.37 (1.13-1.66)	1.5×10^{-3}
3	5.8 % (299 / 5185)	1.45 (1.20-1.75)	6.8×10^{-4}
4	6.2 % (309 / 5013)	1.51 (1.26-1.82)	2.9×10^{-6}

GRS, genetic risk score (based on rs11675434, rs653178, rs10944479, rs1230666, rs2010099).
[a] Adjusted for age and gender

Table S6. Newly identified TPOAb associated loci and the risk of thyroid cancer

		Alleles		Nijmegen cohort (154 cases/ 2019 controls)		Ohio cohort (179 cases / 190 controls)		Combined (333 cases / 2209 controls)	
Nearby Gene	SNP	Risk	Other	OR (95% CI)	*P*	OR (95% CI)	*P*	OR (95% CI)	*P*
TPO	rs11675434	T	C	1.01 (0.68-1.50)	0.97	1.04 (0.75-1.43)	0.83	1.03 (0.80-1.32)	0.85
ATXN2	rs653178	C	T	1.36 (0.87-2.12)	0.17	1.30 (0.95-1.77)	0.10	1.32 (1.02-1.70)	0.03
BACH2	rs10944479	A	G	1.69 (1.14-2.53)	0.01	0.86 (0.56-1.33)	0.50	1.24 (0.92-1.66)	0.15
MAGI3	rs1230666	A	G	0.99 (0.63-1.55)	0.95	0.92 (0.57-1.52)	0.75	0.89 (0.64-1.25)	0.51
KALRN	rs2010099	C	T	1.22 (0.77-1.94)	0.40	1.51 (0.97-2.34)	0.07	1.37 (0.99-1.88)	0.06

Adjusted for age and gender

Table S7. Top IPA associated networks for the stage 1 TPOAb-positivity and TPOAb level lead SNPs

Associated Network Functions	Score	Focus Molecules	Molecules in Network
Cell Death and Survival, Renal Necrosis/ Cell Death, Cellular Movement	24	10	ARHGEF1, **BACH2**, CD3, **CD44**, CYB5R3, DOK3, DR4/5, EZH2, FUCA1, GZMK, **HCP5**, Igm, ITGB1, Jnk, **KALRN**, **MAF**, **MAGI3**, NFkB (complex), **NFKBIA**, PELI3, PRMT2, RAC1, **RERE**, RIOK3, RTKN, SCFD1, SUMO4,Taok2,TNIP3, **TPO**, VOPP1, ZFAND6, **ZFP64**, Zfp125, ZMYND11
Carbohydrate Metabolism, Molecular Transport, Small Molecule Biochemistry	5	3	**ATXN2**, CD36, FABP4, FBP1, **FEM1A**, FFAR4, GCG, HADHA, IGF2, Ins1, INSR, IRS2, LCP1, LRP5, MIF, **NDUFV2**, NKX2-2, NOS3, PDE3B, PDK4, PDX1, PIK3R2, PLA2G1B, PPARGC1A, PRKCI, PTPN1, PTPRN, RPS6KB1, SIRT1, SIRT6, SLC2A1, SLC2A4, TFAM, UCP2, UCP3
Cellular Growth and Proliferation, Respiratory System Development and Function, Immunological Disease	3	1	**HLA-DPB1**, MAGEA3/MAGEA6
Immunological Disease, Infectious Disease, Cell Morphology	2	1	CD63, CD82, EBI3, **HLA-DOB**, Hla-Drb, IL27, mir-223

Physical function analysis for the 20 stage 1 lead SNPs using IPA (Ingenuity Pathway Analysis). Four networks were generated which are ordered by a score denoting significance.

Table S8. Top IPA associated canonical pathways for the stage 1 TPOAb-positivity and TPOAb level lead SNPs

Canonical Pathways	*P*-value
OX40 Signaling Pathway	7.6×10^{-5}
Antigen Presentation Pathway	5.9×10^{-4}
Autoimmune Thyroid Disease Signaling	1.0×10^{-3}
Cytotoxic T Lymphocyte-mediated Apoptosis of Target Cells	2.8×10^{-3}
Allograft Rejection Signaling	2.9×10^{-3}

Top Canonical Pathways for the 20 stage 1 lead SNPs using IPA (Ingenuity Pathway Analysis).

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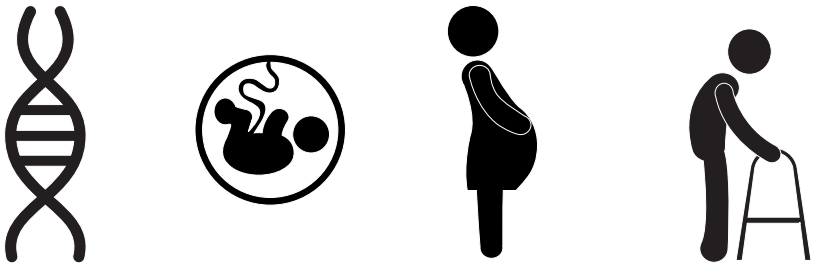
Summary



SUMMARY

Although it has been appreciated for long that thyroid hormone is crucial for the development and function of virtually all tissues, much remains to be learned about the determinants and effects of thyroid function throughout life. In the introduction (**Chapter 1**), a brief overview of thyroid hormone synthesis, metabolism, and action is given, and we provide a general background for the studies described in this thesis. These studies can be divided in three parts.

The first part of this thesis (Chapters 2-6) involves studies on determinants and effects of thyroid function during pregnancy. Reference ranges for TSH and FT4 are different in the pregnant state compared to the non-pregnant state. For this reason, the guidelines of the Endocrine Society and American Thyroid Association recommend to calculate trimester specific reference ranges per centre. If these calculated ranges are not available in the laboratory, TSH reference ranges of 0.1–2.5 mU/L for the first trimester and of 0.2–3.0 mU/L for the second trimester are recommended. In **Chapter 2**, we calculated pregnancy-specific population-based TSH and FT4 reference ranges in the Generation R Study. The importance of calculating population-based reference ranges was illustrated by the fact that 8.6 and 4.9 % of the TPOAb-negative women with normal range TSH levels had a TSH level above 2.5 mU/L and 3.0 mU/L in the first and second trimesters, respectively. The effects of ethnicity on serum thyroid parameter reference ranges were studied in **Chapter 3**. Ethnic differences were shown in serum TSH, T4, and TPO-antibody positivity and we found significant diagnostic discrepancies depending on whether population or ethnicity-specific reference ranges were used to diagnose thyroid disease.

Little is known about the effects of maternal iodine status on maternal and child thyroid function in iodine-sufficient areas. In **Chapter 4**, we studied these associations in the iodine-sufficient Generation R Study, and showed that mothers with higher early-pregnancy urinary iodine levels have an increased risk of hyperthyroid newborns. These data should prompt further studies on the identification and closer monitoring of these mothers with a higher iodine status during pregnancy.

Various studies have shown that, in the non-pregnant state, even minor variation in thyroid function within the normal range is associated with detrimental health outcomes. However, little is known about the effects of normal-range thyroid function on the risk of pregnancy complications. The associations between early-pregnancy maternal thyroid status and child birth weight were analyzed in **Chapter 5**. We showed that high-normal FT4 levels are associated with lower birth weight and an increased risk of small size for gestational age at birth (SGA) newborns. In **Chapter 6**, we investigated the effects of early-pregnancy maternal thyroid status on the risk of hypertensive disorders of pregnancy, including pregnancy-induced hypertension and preeclampsia. We show

that not only hyperthyroidism but also high-normal FT4 levels during early-pregnancy are associated with an increased risk of hypertensive disorders. These data demonstrate that even mild variation in thyroid function within the normal range can have important maternal and fetal consequences. The results of the third part of this thesis are put into perspective in the first part of the discussion (**Chapter 13**). In this chapter, we discuss the definition of normal serum thyroid parameter reference ranges during pregnancy, different factors that contribute to these reference ranges, as well as the association of subclinical thyroid disease and the risk of maternal and child complications in relation to these ranges. Based on these results, we conclude that it is essential that institutions calculate their own pregnancy-specific populated-based reference ranges.

Consequently, clinicians should also use these population-based ranges for the diagnosis and treatment of thyroid diseases, instead of using the fixed serum TSH cut-off levels of 2.5 and 3.0 mU/L.

The second part (Chapters 7-9) of this thesis involves multi-centre studies in which we searched for new genetic determinants of thyroid function and thyroid autoimmunity. In **Chapter 7**, we studied the effects of common genetic variation in 68 thyroid hormone pathway genes on serum TSH and FT4 levels in the Rotterdam Study, and promising results were replicated in 3 independent populations. Significant associations were found for *PDE8B* polymorphisms with serum TSH levels, and *DIO1* and *FOXE1* polymorphisms with serum FT4 levels. As the identified variants in this study and other previous candidate gene analyses only explained a minor part of the total variation in thyroid function, a consortium was started in order to perform large-scale genome-wide association studies (GWAS). **Chapter 8** describes a GWAS on serum TSH and FT4 levels in up to 26,420 and 17,520 euthyroid individuals, respectively. This resulted in 26 significant loci, including several novel loci for TSH (*PDE10A*, *VEGFA*, *IGFBP5*, *NFIA*, *SOX9*, *PRDM11*, *FGF7*, *INSR*, *ABO*, *MIR1179*, *NRG1*, *MBIP*, *ITPK1*, *SASH1*, *GLIS3*) and FT4 (*LHX3*, *FOXE1*, *AADAT*, *NETO1/FBXO15*, *LPCAT2/CAPNS2*). The TSH-associated loci contributed not only to variation within the normal range, but also to TSH values outside the reference range, suggesting that they may be involved in thyroid dysfunction. In **Chapter 9**, we performed a GWAS in 18,297 individuals for TPO-antibody positivity and in 12,353 individuals for TPOAb serum levels, with replication in 8,990 individuals. Significant associations were detected at *TPO*, *ATXN2*, *BACH2*, *MAGI3*, and *KALRN*. By combining multiple risk alleles, we were able to identify a subgroup with a substantially increased risk of TPO-antibody positivity. Additionally, the *MAGI3* and *BACH2* variants were associated with an increased risk of hyperthyroidism, which was replicated in an independent cohort of patients with Graves' disease. The *MAGI3* variant was also associated with an increased risk of hypothyroidism. These results are put into perspective in the first part of the discussion (**Chapter 14**), in which we provide an overview of the current literature on genetic determinants

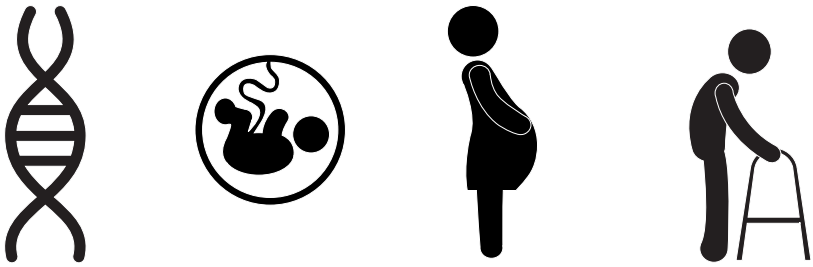
of thyroid function. This review discusses both monogenic and polygenic causes of thyroid dysfunction and altered thyroid function, including new candidate genes identified by GWAS, and what insights these genes provide about the genetic basis of thyroid (dys) function. New techniques are discussed which will help to further unravel the genetic basis of thyroid (dys)function in the near future.

In the third part of this thesis (Chapters 10-12) we investigated the effects of genetic variation in the thyroid hormone receptor alpha (*THRA*) locus on human bone and brain. **Chapter 10** demonstrates that common genetic variation in this locus is not associated with bone mineral density, bone geometry and fracture risk. In **Chapter 11**, we investigated the effects of genetic variation in this locus on MRI-derived brain volumes. The circadian clock gene *REV-ERBα* overlaps with *THRA*, and this study demonstrated that a haplotype block in *REV-ERBα* was associated with more white matter lesions in women.

Although both hypo- and hyperthyroidism have been associated with an increased risk of depression, little is known about the effects of normal-range thyroid function on the risk of depression. In **Chapter 12**, we therefore investigated these effects in elderly from a population-based cohort study, who were depression-free at baseline and were followed-up for 8 years for the occurrence of depressive syndromes. Elderly with low-normal TSH levels had an increased risk of developing a depressive syndrome, compared to individuals with high-normal TSH levels. This study therefore identified low-normal TSH as an important risk factor for depression in the elderly.




Samenvatting



De essentiële rol van schildklierhormoon in de ontwikkeling en functie van verscheidene weefsels is al lang geleden onderkend. Desondanks is er nog veel onbekend over de determinanten en effecten van schildklierfunctie. In de introductie van dit proefschrift (**Hoofdstuk 1**) werd een overzicht gegeven van schildklierhormoonproduktie, -metabolisme en –actie. Tevens werden de navolgende hoofdstukken geintroduceerd, die in drie delen zijn opgedeeld.

Het eerste deel van dit proefschrift (hoofdstukken 2-6) omvat studies die betrekking hebben op de determinanten en effecten van schildklierfunctie tijdens de zwangerschap. Referentiewaarden voor TSH (schildklier stimulerend hormoon) en FT4 (schildklierhormoon) verschillen tussen zwangeren en niet-zwangeren. Om deze reden adviseren de Endocrine Society en de American Thyroid Association het gebruik van trimesterspecifieke referentiewaarden. Indien deze referentiewaarden niet in het betreffende laboratorium of kliniek beschikbaar zijn, wordt het gebruik van TSH referentiewaarden van 0.1-2.5 mU/L (eerste trimester) en 0.2-3.0 mU/L (tweede en derde trimester) aanbevolen.

In **hoofdstuk 2** zijn zwangerschapsspecifieke TSH en FT4 referentiewaarden berekend in een groot zwangerencohort (de Generation R studie). 8.6% en 4.9% van de TPO-antilichaam negatieve zwangeren met normale TSH waarden had een TSH boven de 2.5 muU/L (eerste trimester) of 3.0 mU/L (tweede trimester). Deze resultaten illustreren het belang van het berekenen van zwangerschapsspecifieke populatie-gebaseerde referentiewaarden voor TSH en FT4. In **hoofdstuk 3** werden de effecten van etniciteit op TSH en FT4 referentiewaarden in de zwangerschap bestudeerd. TSH, FT4 en TPO-antilichaam serumwaarden verschilden tussen de ethische groepen. Daarnaast toonden wij aan dat het gebruik van etniciteitsspecifieke referentiewaarden een significante invloed had op de diagnostiek van schildklierafwijkingen tijdens de zwangerschap.

Jodium is essentieel voor de synthese van schildklierhormoon. Desondanks is er in jodium sufficiënte gebieden maar weinig bekend over de invloed van jodium status op de schildklierfunctie van de zwangere en het kind. In **hoofdstuk 4** werden deze associaties in de Generation R studie bestudeerd, waarbij zwangeren met hogere jodium concentraties in de urine een hoger risico hadden op een hyperthyreoot kind. Het is daarom van belang dat toekomstige studies zich richten op de identificatie en monitoring van deze vrouwen met een hogere jodium status tijdens de zwangerschap.

Studies in niet-zwangere populaties hebben aangetoond dat zelfs kleine variaties in schildklierfunctie binnen de normale referentiewaarden geassocieerd zijn met een hoger risico op verscheidene ziektebeelden. Er is echter weinig bekend over de effecten van variatie in schildklierfunctie binnen de normale range op het risico op zwangerschapscomplicaties. De associaties tussen schildklierfunctie van de zwangere en het geboortegewicht van het kind werden daarom bestudeerd in **hoofdstuk 5**. Zwangeren

met hoog-normale FT4 concentraties hadden een hoger risico op een kind met een lager geboortegewicht. In **hoofdstuk 6** analyseerden wij de relaties tussen schildklierfunctie van de zwangere en het risico op hypertensieve aandoeningen in de zwangerschap (zwangerschapshypertensie en pre-eclampsie). Niet alleen hyperthyreoïdie maar ook hoog-normale FT4 concentraties waren geassocieerd met een hoger risico op deze hypertensieve complicaties. Deze resultaten tonen aan dat zelfs kleine variaties in schildklierfunctie van de zwangere belangrijke effecten kunnen hebben op zowel moeder als kind.

De resultaten van het eerste deel van dit proefschrift werden in perspectief gezet in **hoofdstuk 13**. In dit hoofdstuk werden de definitie en determinanten van TSH en FT4 referentiewaarden tijdens de zwangerschap bediscussieerd, alsmede de effecten van subklinische schildklieraandoeningen op het risico op complicaties bij moeder en kind. Daarbij concludeerden wij dat het essentieel is dat instituten hun eigen zwangerschapsspecifieke populatiegebaseerde TSH en FT4 referentiewaarden berekenen. Deze referentiewaarden dienen gebruikt te worden voor zowel de diagnose als behandeling van schildklierziekten in de zwangerschap, in plaats van de TSH afkapwaarden van 2.5 en 3.0 mU/L.

Het tweede deel van dit proefschrift (hoofdstukken 7-9) omvat multicenter studies waarin werd gezocht naar nieuwe genetische determinanten van schildklierfunctie en –autoimmuniteit. In **hoofdstuk 7** werden de effecten van frequent voorkomende genetische varianten (polymorfismen) in 68 schildklier gerelateerde genen op TSH en FT4 concentraties in de Rotterdam Studie bestudeerd, waarbij veelbelovende associaties werden gerepliceerd in drie onafhankelijke populaties. Significante associaties werden gevonden voor *PDE8B* polymorfismen met TSH concentraties en *DIO1* en *FOXE1* polymorfismen met FT4 concentraties. Aangezien deze varianten maar een klein deel van de totale variatie in schildklierfunctie konden verklaren, werd een consortium opgezet om grootschalige zogenaamde genoom-wijde associatie studies (GWAS) uit te kunnen voeren. **Hoofdstuk 8** beschreef een GWAS voor serum normale range TSH en FT4 concentraties in 26420 en 17520 participanten. Dit resulteerde in 26 significant geassocieerde genetische loci, waaronder nieuwe loci voor TSH (*PDE10A*, *VEGFA*, *IGFBP5*, *NFIA*, *SOX9*, *PRDM11*, *FGF7*, *INSR*, *ABO*, *MIR1179*, *NRG1*, *MBIP*, *ITPK1*, *SASH1*, *GLIS3*) en FT4 (*LHX3*, *FOXE1*, *AADAT*, *NETO1/FBXO15*, *LPCAT2/CAPNS2*). De TSH geassocieerde loci waren niet alleen geassocieerd met TSH concentraties binnen de referentierange, maar ook met TSH concentraties buiten de referentierange, wat suggereert dat deze loci tevens een rol spelen in schildklierziekten. In **hoofdstuk 9** werd een GWAS uitgevoerd in 18297 participanten voor TPO-antilichaam positiviteit en in 12353 participanten voor TPO-antilichaam serum concentraties. Significante associaties werden gevonden voor *TPO*, *ATXN2*, *BACH2*, *MAGI3*, en *KALRN*. Door het combineren van meerdere risicoallelen in een

risicoscore kon een subgroep worden geidentificeerd met een substantieel verhoogd risico op TPO-antilichaam positiviteit. Daarnaast waren de *MAGI3* en *BACH2* polymorfismen geassocieerd met een verhoogd risico op hyperthyreoïdie, wat gerepliceerd werd in een onafhankelijk cohort van patiënten met de ziekte van Graves. Het *MAGI3* polymorfisme was tevens geassocieerd met een verhoogd risico op hypothyreoïdie.

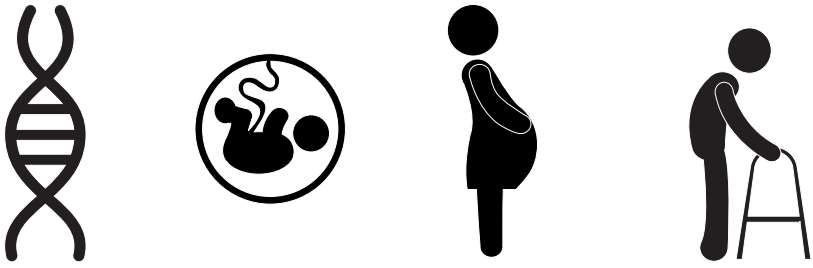
De resultaten van het tweede deel van dit proefschrift werden bediscussieerd in **hoofdstuk 14**, waarin een overzicht werd gegeven van de beschikbare literatuur rond genetische determinanten van schildklierfunctie. Deze review behandelde zowel mono- als polygenetische oorzaken van schildklierdysfunctie en afwijkende schildklier serum parameters, inclusief nieuw geidentificeerde kandidaatgenen in GWAS. Tevens werden nieuwe technieken besproken die in de nabije toekomst zullen bijdragen aan de ontrafeling van de genetische basis van schildklier(dys)functie.

In het derde deel van dit proefschrift (hoofdstukken 10-12) werden de effecten van genetische variatie in de schildklierhormoon receptor alfa (*THRA*) locus op humaan bot en hersenen bestudeerd. **Hoofdstuk 10** toonde aan dat polymorfismen in deze locus niet geassocieerd waren met botdichtheid, botgeometrie en risico op fracturen. In **hoofdstuk 11** werden de effecten van deze polymorfismen bestudeerd op hersenvolumina, verkregen via MRI scans. Het circadiane klokgen *REV-ERBα* overlapt met *THRA*, waarbij een haplotype blok in *REV-ERBα* geassocieerd was met meer witte stof laesies in vrouwen.

De laatste studie in dit proefschrift (**hoofdstuk 12**) bestudeerde de relatie tussen schildklierfunctie en de kans op depressie. Zowel hypo- als hyperthyreoïdie zijn geassocieerd met een verhoogd risico op het ontwikkelen van een depressie. Er is echter maar weinig bekend over de effecten van een schildklierfunctie binnen de normale range op het risico op een depressie. In hoofdstuk 12 bestudeerden wij deze effecten in een populatie ouderen die bij het begin van de studie geen depressie hadden en vervolgens 8 jaar gevolgd werden voor het ontwikkelen van depressieve syndromen. Ouderen met een laag-normaal TSH hadden een verhoogd risico op het ontwikkelen van een depressief syndroom, in vergelijking tot ouderen met een hoog-normaal TSH. Zodoende identificeerde deze studie een laag-normaal TSH als een belangrijke risicofactor voor depressie in ouderen. Dankwoord




Dankwoord
Acknowledgements



Onderzoek doe je niet alleen. De afgelopen jaren zijn een fantastische ervaring geweest waarin ik mij naast mijn opleiding tot internist heb kunnen ontwikkelen tot onderzoeker, mede dankzij de vele mensen met wie ik heb mogen samenwerken. Daarom wil ik in deze sectie mensen binnen en buiten het onderzoek bedanken zonder wie de voorgaande hoofdstukken niet mogelijk waren geweest.

Allereerst natuurlijk Prof.dr.ir. T.J. Visser. Beste Theo, als ik de proefschriften van al jouw promovendi erop na sla is het vocabulaire nogal eentonig: enkel superlatieven. Ondanks dat ik graag tegen de draad in ga kan ik daarin enkel mee gaan. Je enthousiasme en laagdrempeligheid vormen de basis voor een altijd goede sfeer op het lab. Ik sta nog steeds met verbazing te kijken als je na een praatje (dat je al talloze keren hebt gehoord) steeds weer met interessante vragen en ideeën komt. Ik hoop dan ook nog lang met je te mogen brainstormen over nieuwe resultaten en daarnaast te genieten van de foute tenten waarin we met het lab in de vroege uurtjes op congressen belanden!

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Prof.dr. Hooijkaas, Dr. Jaddoe, Dr. (Willy) Visser en Dr. De Muinck Keizer-Schrama. Allen dank voor de altijd levendige discussies die ieder manuscript sterker hebben gemaakt!

I have the privilege to collaborate with various cohorts in our thyroid GWAS consortium and CHARGE thyroid function working group. I would especially like to thank those people who have been a continuous support in our projects: Eleonora Porcu, Serena Sanna and Silvia Naitza (SardiNIA), Anne Cappola (CHS), Giorgio Pistis and Daniela Toniolo (ValBorbera), Scott Wilson (Twins UK), and Alexander Teumer and Henry Völzke (SHIP). Alexander, it is a great pleasure to work with you on the next thyroid GWAS, which will definitely lead to new insights into the genetics of thyroid dysfunction! Anna Köttgen (ARIC), our collaboration has only just started and is already a success!

Tevens wil ik Fernando Rivadeneira, Joyce van Meurs, Michael Verbiest, Lisette Stolk, Marjolein Peters en Karol Estrada van het genetisch lab bedanken voor hun steun bij genetische analyses en gezellige uitstapjes op de CHARGE meetings.

Gelukkig heb ik ook in de kliniek leuke collega's: Dank voor alle gezellige borrels, skireizen en de dagelijkse discutabele filmpjes op de whatsapp… Nog groen in het SFG mocht ik tijdens een skireis ook eens de patiëntenrol innemen tgv een schouderluxatie, en kan jullie zeggen dat het met de "menselijke kant" van deze aanstaand geneesheren en –dames ook meer dan goed zit! Tevens ben ik mijn opleiders Prof.dr. van Saase en A.P. Rietveld zeer erkentelijk voor hun flexibiliteit rond mijn geplande onderzoeksperiode in het buitenland.

En dan de belangrijkste mensen in mijn leven: familie en vrienden.

Allereerst de vaste vriendengroep: Rene, Pieter-Jan, Patrick, Jos, Erwin, Jan-Willem en Wouter. Al sinds '98 kennen wij elkaar en hebben dan ook een onuitputtelijk scala aan memorabele/kansloze momenten van alle vakanties en weekenden die we samen hebben doorgebracht. Het is schitterend te zien hoe ondanks ieders drukke bestaan het altijd weer als "vanouds" is als we met de groep samen zijn! En ook zeker mag ik tegenwoordig de partners niet vergeten: dank voor het in acceptabele banen leiden van deze soms ongeleide projectielen! Bob, van iedereen ken ik jou het langst. Ik geniet nog steeds van ons relaxte avondjes met een pizza fresco met extra kaas en knoflooksaus en een filmpje...

Toen ik Evita leerde kennen kreeg ik er tevens plots een grote schoonfamilie bij, waar ik mij vanaf het begin af aan altijd thuis bij heb gevoeld. Frank, Chu, John, Olga, Uschi, Alex, Richard en Saimira: De laatste jaren hebben wij samen al vele feestdagen, etentjes (en tegenwoordig zelfs hardloopwedstrijden…) doorgemaakt, wat altijd zowel een sociaal maar ook zeker een culinair genot is! Maureen en Willem: het is een eer dat jullie ceremoniemeesters willen zijn bij onze bruiloft. Wij kunnen ons geen betere wensen!

Ans, Peter en Sabine wil ik bedanken voor de gezellige en vertrouwde momenten samen: wij zien uit naar jullie komst op 22 augustus!

Voor mijn familie in Italië: Cara Giorgina, Giovanna, Roberto, Beatrice, Francesco e naturalmente Giuditta: i miei primi ricordi da bambino vanno alle lunghe ed indimenticabili vacanze estive presso di voi. La consuetudine delle lunghe sedute a tavola, le discussioni piene di calore e di passione le sento ormai come una Cosa Mia e non mi lasceranno mai per tutta la vita! Vi ringrazio per avermi accolto con la cordialita' tipica che solo voi sapete dare. Nonostante in questi ultimi anni ci siamo visti meno spesso, Padova rimane sempre "Un Ritorno a Casa".

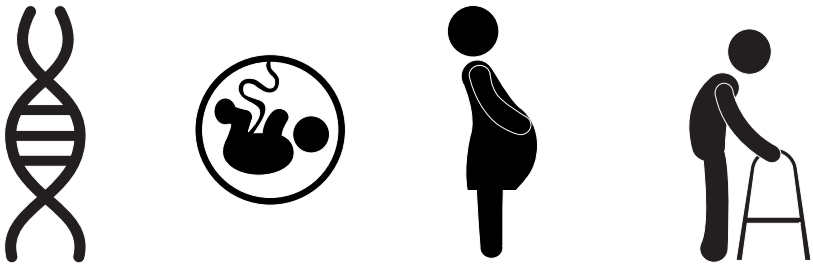
Hilly, Ad en Sharita: ik prijs mij zeer gelukkig met zo'n schoonouders en –zus. Al lange tijd vragen jullie je af waarom ik op vrije dagen altijd weer aan "dat onderzoek" zit. Dank voor alle gezellige momenten samen, en de steun die jullie ons bieden in de drukke dagelijkse rompslomp van twee arts-assistenten!

Ma en Pa, dit proefschrift is aan jullie opgedragen. Daar hoefde ik niet lang over na te denken daar jullie de fundamenten zijn van al dit werk. Dank voor alle onvoorwaardelijke liefde, steun en vertrouwen die jullie mij altijd hebben gegeven. Ook na al jaren in Rotterdam te wonen voelt thuiskomen nog altijd als een warm nest en dat zegt meer dan genoeg. De vastberadenheid en nieuwsgierigheid die jullie mij hebben meegegeven is de afgelopen jaren meer dan ooit van pas gekomen!

En dan Evita, de liefde van mijn leven. Ik vroeg je al gekscherend hoe je hier bedankt zou willen worden. Maar na al het wetenschappelijk verantwoord schrijven val ik hier dan toch echt stil daar woorden de betekenis van je dagelijkse liefde en steun nooit zouden kunnen dekken. Ik houd het dan ook kort en krachtig, zoals een sterke conclusie van een wetenschappelijk schrijven hoort te zijn: Ik hou van je.




PhD PORTFOLIO



Research skills	**Year**	**Workload**
Classical methods for Data-analysis, Rotterdam	2008	2 weeks
Basic fellows track, ATA, Palm Beach, USA	2009	3 days
SPSS course, Rotterdam	2009	1 day
Genome browsers, Rotterdam	2009	2 days
SNPs and human disease, Rotterdam	2009-2010	8 days
Basic and translational endocrinology, Rotterdam	2009-2010	10 days
ZonMw workshop on grant proposals	2011	1 day
Endocrine Trainee Day, Endocrine Society, Houston, USA	2012	1 day
Clinical fellows track, ATA, San Juan, Puerto Rico	2013	3 days

Clinical courses		
Rotterdamse internistendag, Rotterdam	2009	1 day
Dutch Internal Medicine days, Maastricht	2012	3 days
MedicALS, Houten	2013	2 days
FCCS provider course, Houten	2014	2 days
DESG course on diabetes, Hoevelaken	2014	2 days

Presentations (international meetings)	**Year**	**Type**
The thyroid hormone receptor alpha locus and bone: a role for the circadian clock gene Rev-Erba, ETA, Lisbon, Portugal	2008	Oral
Preliminary evidence that a functional polymorphism in D1 is associated with enhanced potentiation of the antidepressant effect of sertraline by T3, ATA, Chicago, USA	2008	Poster
A large-scale association analysis of 68 thyroid hormone pathway genes with TSH and FT4 levels, ATA, Palm Beach, USA	2009	Oral
Maternal first trimester thyroid hormone levels within the normal range affect birth weight, ESE, Rotterdam (Young Investigator Award)	2011	Oral
Relations between maternal first-trimester and newborn thyroid hormone serum levels, ETA, Krakow, Poland (Young Investigator Award)	2011	Oral
Identification of new loci associated with serum TSH or FT4 levels: results from a genome wide scan in 23,600 subjects, ATA, Indian Wells, USA	2012	Oral
Women with high early pregnancy urinary iodine levels have an increased risk of hyperthyroid newborns, ENDO, Houston, USA	2012	Oral
Urinary iodine excretion in 1100 pregnant women from Rotterdam, the Netherlands, ICCIDD, Leiden	2013	Oral

A genome wide meta-analysis in 27,300 subjects identifies novel loci associated with thyroid peroxidase antibodies and clinical thyroid disease, CHARGE, Rotterdam	2013	Poster
*A genome wide meta-analysis in 27,300 subjects identifies novel loci associated with thyroid peroxidase antibodies and clinical thyroid disease,*ETA, Leiden	2013	Oral
Effects of maternal thyroid state on development of the offspring, ICCID, Leiden	2013	Oral
Genetic variants associated with thyroid autoimmunity and clinical thyroid disease: new insights from a genome wide meta-analysis in 27,000 subjects, ETA, Leiden	2013	Oral

Presentations (national meetings)

The thyroid hormone receptor alpha locus and bone: a role for the circadian clock gene Rev-Erba, Science days Internal Medicine, Antwerp, Belgium	2008	Poster
Low-normal TSH levels are associated with depression, Science days Internal Medicine, Antwerp, Belgium	2010	Oral
Relations between maternal first-trimester and newborn thyroid hormone serum levels, Dutch Endocrine Meeting, Noordwijkerhout	2012	Oral
Women with high early pregnancy urinary iodine levels have an increased risk of hyperthyroid newborns, Science days Internal Medicine, Antwerp, Belgium	2013	Poster
Women with high early pregnancy urinary iodine levels have an increased risk of hyperthyroid newborns, Dutch Endocrine Meeting, Noordwijkerhout	2013	Oral
A large-scale population-based analysis of common genetic variation in the thyroid hormone receptor alpha locus and bone, Dutch Thyroid Society, Amsterdam	2013	Oral
Genetic studies on serum thyroid parameters, thyroid dysfunction and autoimmunity, Dutch Thyroid Society *(award lecture MSD Thyroid Award)*	2013	Oral
Early-pregnancy hyperthyroidism and high-normal FT4 levels are risk factors for hypertensive disorders during pregnancy, Dutch Endocrine Meeting, Noordwijkerhout	2014	Oral

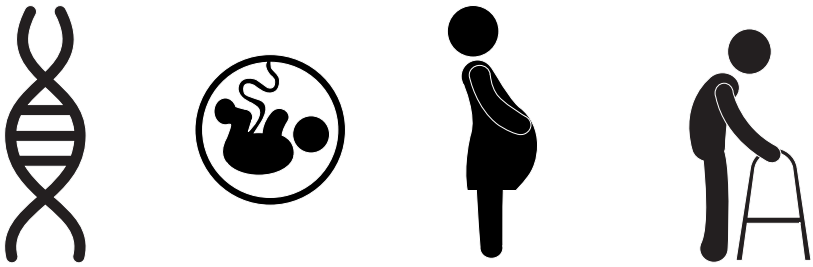
(Inter)national conferences	Year	Workload
Annual meeting of the European Thyroid Association, Lisbon, Portugal	2008	4 days
Science days Internal Medicine, Antwerp, Belgium	2008	2 days
Annual meeting of the American Thyroid Association, Chicago, USA	2008	4 days
Annual meeting of the American Thyroid Association, Palm Beach, USA	2009	4 days
CHARGE meeting, Houston, USA	2009	3 days
Science days Internal Medicine, Antwerp, Belgium	2010	2 days
CHARGE meeting, Boston, USA	2011	3 days
Annual meeting of the European Society of Endocrinology, Rotterdam	2011	3 days
Annual meeting of the European Thyroid Association, Krakow, Poland	2011	4 days
Dutch Endocrine Meeting, Noordwijkerhout	2012	2 days
Annual meeting of the American Thyroid Association, Indian Wells, USA	2012	4 days
Annual meeting of the Endocrine society, Houston, USA	2012	4 days
Dutch Endocrine Meeting, Noordwijkerhout	2013	2 days
CHARGE meeting, Rotterdam	2013	2 days
Annual meeting of the European Thyroid Association, Leiden	2013	4 days
ICCIDD meeting, Leiden	2013	1 day
Annual meeting of the American Thyroid Association, San Juan, Puerto Rico	2013	1 day
Dutch Endocrine Meeting, Noordwijkerhout	2014	2 days

Teaching activities		
Lectures on thyroid (dys)function second year medical students	2009	3 days
Lectures on adrenal (dys)function second year medical students	2009	2 days
Lectures on thyroid (dys)function second year medical students	2010	3 days
Lectures on adrenal (dys)function second year medical students	2010	2 days
Lectures on thyroid (dys)function second year medical students	2011	3 days
Lectures on adrenal (dys)function second year medical students	2011	2 days

Other		
Coding of fractures in the Rotterdam Study	2010	40 hours
Exit interview with Rotterdam Study participants	2010-2011	90 hours
Referee activities for various international journals	2009-2014	
PI CHARGE Thyroid Function working group	2009-2014	




List of publications



PUBLICATIONS BASED ON THE STUDIES DESCRIBED IN THIS THESIS

[#*] *Joint first or last authors*

1. **M Medici**, WM van der Deure, M Verbiest, SH Vermeulen, PS Hansen, LA Kiemeney, ARMM Hermus, MM Breteler, A Hofman, L Hegedüs, KO Kyvik, M den Heijer, AG Uitterlinden, TJ Visser, RP Peeters. A large-scale association analysis of 68 thyroid hormone pathway genes with serum TSH and FT4 levels. *European Journal of Endocrinology 2011;164(5):781-188.*

2. **M Medici**, YB de Rijke, RP Peeters, W Visser, SM de Muinck Keizer-Schrama, VWV Jaddoe, A Hofman, H Hooijkaas, EA Steegers, H Tiemeier, JJ Bongers-Schokking, TJ Visser. Maternal early pregnancy and newborn thyroid hormone parameters: The Generation R Study. *Journal of Clinical Endocrinology and Metabolism 2012;97(2):646-652*.

3. **M Medici**, F Rivadeneira, WM van der Deure, A Hofman, JBJ van Meurs, The GEFOS Consortium, AG Uitterlinden, TJ Visser, RP Peeters. A large-scale population-based analysis of common genetic variation in the thyroid hormone receptor alpha locusand bone. *Thyroid 2012;22(2):223-224.*

4. **M Medici**, MA Ikram, F van der Lijn, T den Heijer, MW Vernooij, A Hofman, WJ Niessen, TJ Visser, MM Breteler, RP Peeters. The thyroid hormone receptor alpha locus and white matter lesions: a role for the clock gene *REV-ERBα*. *Thyroid 2012;22(11):1181- 1186.*

5. **M Medici**, S Timmermans, W Visser, SM de Muinck Keizer-Schrama, VWV Jaddoe, A Hofman, H Hooijkaas, YB de Rijke, H Tiemeier, JJ Bongers-Schokking, TJ Visser, RP Peeters, EA Steegers. Maternal thyroid hormone parameters during early pregnancy and birth weight: the Generation R Study. *Journal of Clinical Endocrinology and Metabolism 2013;98(1):59-66.*

6. E Porcu[*], **M Medici***, G Pistis*, CB Volpato, SG Wilson, AR Cappola, SD Bos, J Deelen, M den Heijer, RM Freathy, J Lahti, C Liu, LM Lopez, IM Nolte, JR O'Connell, T Tanaka, S Trompet, A Arnold, S Bandinelli, M Beekman, S Böhringer, SJ Brown, BM Buckley, C Camaschella, AJM de Craen, G Davies, MCH de Visser, I Ford, T Forsen, TM Frayling, L Fugazzola, M Gögele, AT Hattersley, AR Hermus, A Hofman, JJ Houwing-Duistermaat, RA Jensen, E Kajantie, M Kloppenburg, EM Lim, C Masciullo, S Mariotti, C Minelli, BD Mitchell, R Nagaraja, RT Netea-Maier, A Palotie, L Persani, MG Piras, BM Psaty, K Räikkönen, JB Richards, F Rivadeneira, C Sala, MM Sabra, N Sattar, BM Shields, N Soranzo, JM Starr, DJ Stott, FCGJ

Sweep, G Usala, MM van der Klauw, D van Heemst, A van Mullem, SH Vermeulen, JP Walsh, RGJ Westendorp, E Widen, G Zhai, F Cucca, IJ Deary, JG Eriksson, L Ferrucci, CS Fox, WJ Jukema, LA Kiemeney, PP Pramstaller, D Schlessinger, AR Shuldiner, EP Slagboom, AG Uitterlinden, B Vaidya, TJ Visser, BHR Wolffenbuttel, I Meulenbelt, JI Rotter, TD Spector, AA Hicks, D Toniolo, S Sanna#, RP Peeters#, S Naitza#. A meta-analysis of thyroid-related traits reveals novel loci and gender- specific differences in the regulation of thyroid function. *PLoS Genetics 2013;9(2): e1003266*.

7. TIM Korevaar*, **M Medici***, YB de Rijke, W Visser, SMPF de Muinck Keizer-Schrama, VWV Jaddoe, A Hofman, HA Ross, WE Visser, H Hooijkaas, EAP Steegers, H Tiemeier, JJ Bongers-Schokking, TJ Visser, RP Peeters. Ethnic differences in maternal thyroid parameters during pregnancy: the Generation R Study. *Journal of Clinical Endocrinology and Metabolism 2013;98(9):3678-3686*.

8. **M Medici**, A Ghassabian, W Visser, SM de Muinck Keizer-Schrama, VWV Jaddoe, WE Visser, H Hooijkaas, A Hofman, EA Steegers, JJ Bongers-Schokking, HA Ross, H Tiemeier, TJ Visser, YB de Rijke, RP Peeters. Women with high early pregnancy urinary iodine levels have an increased risk of hyperthyroid newborns: the population-based Generation R Study. *Clinical Endocrinology 2014;80(4):598-606*.

9. **M Medici***, E Porcu*, G Pistis*, A Teumer, SJ Brown, RA Jensen, R Rawal, GL Roef, TS Plantinga, SH Vermeulen, J Lahti, MJ Simmonds, LLN Husemoen, RM Freathy, BM Shields, D Pietzner, R Nagy, L Broer, L Chaker, TIM Korevaar, MG Plia, C Sala, U Völker, JB Richards, FC Sweep, C Gieger, T Corre, E Kajantie, B Thuesen, YE Taes, WE Visser, AT Hattersley, J Kratzsch, A Hamilton, W Li, G Homuth, M Lobina, S Mariotti, N Soranzo, M Cocca, M Nauck, C Spielhagen, A Ross, A Arnold, M van de Bunt, S Liyanarachchi, M Heier, HJ Grabe, C Masciullo, TE Galesloot, EM Lim, E Reischl, PJ Leedman, S Lai, A Delitala, AP Bremner, DIW Philips, JP Beilby, A Mulas, M Vocale, G Abecasis, T Forsen, A James, E Widen, J Hui, H Prokisch, EE Rietzschel, A Palotie, P Feddema, SJ Fletcher, K Schramm, JI Rotter, A Kluttig, D Radke, M Traglia, GL Surdulescu, H He, JA Franklyn, D Tiller, B Vaidya, T de Meyer, T Jørgensen, JG Eriksson, PC O'Leary, E Wichmann, AR Hermus, BM Psaty, T Ittermann, A Hofman, E Bosi, D Schlessinger, H Wallaschofski, N Pirastu, YS Aulchenko, A de la Chapelle, RT Netea- Maier, SCL Gough, H Meyer zu Schwabedissen, TM Frayling, J Kaufman, A Linneberg, K Räikkönen, JWA Smit, LA Kiemeney, F Rivadeneira, AG Uitterlinden, JP Walsh, C Meisinger, M den Heijer, TJ Visser, TD Spector, SG Wilson, H Völzke, AR Cappola, D Toniolo, S Sanna#, S Naitza#, RP Peeters#. Identification of novel genetic loci associated with thyroid peroxidase antibodies and clinical thyroid disease. *PLoS Genetics 2014;10(2): e1004123*.

10. **M Medici**, N Direk, WE Visser , TIM Korevaar, A Hofman, TJ Visser, H Tiemeier, RP Peeters. Thyroid Function within the Normal Range and the Risk of Depression: A Population-Based Cohort Study. *Journal of Clinical Endocrinology and Metabolism 2014;99(4):1213-1219.*

11. **M Medici***, TIM Korevaar*, S Schalekamp-Timmermans, R Gaillard, YB de Rijke, WE Visser, W Visser, SMPF de Muinck Keizer-Schrama, A Hofman, H Hooijkaas, JJ Bongers-Schokking, H Tiemeier, VWV Jaddoe, TJ Visser, RP Peeters , EAP Steegers Maternal early-pregnancy thyroid function is associated with subsequent hypertensive disorders of pregnancy: the Generation R Study. *Pending major revisions.*

12. **M Medici***, TIM Korevaar*, WE Visser, TJ Visser, RP Peeters. Thyroid function in pregnancy: What is normal? *Submitted.*

13. **M Medici**, WE Visser, TJ Visser, RP Peeters. Genetic determination of the hypothalamic- pituitary-thyroid axis: where do we stand? *Submitted.*

OTHER PUBLICATIONS

1. **M Medici**, JBJ van Meurs, F Rivadeneira, H Zhao, PP Arp, A Hofman, HAP Pols, A Uitterlinden. BMP-2 gene polymorphisms and osteoporosis: the Rotterdam Study. *Journal of Bone and Mineral Research 2006;21(6):845-854.*

2. R Cooper-Kazaz, WM van der Deure, **M Medici**, TJ Visser, A Alkelai, B Glaser, RP Peeters, B Lerer. Preliminary evidence that a functional polymorphism in type 1 deiodinase is associated with enhanced potentiation of the antidepressant effect of sertraline by triiodothyronine. *Journal of Affective Disorders 2009;116(1-2):113-116.*

3. WM van der Deure, **M Medici,** RP Peeters, TJ Visser. Genetic influences on thyroid function tests. *In: Brent GA, ed. Thyroid Function Testing, 1st ed. 2010, Springer- Verlag;21-37.*

4. AC van de Ven, RT Netea-Maier, **M Medici**, FC Sweep, HA Ross, A Hofman, J de Graaf, LA Kiemeney, AR Hermus, RP Peeters, TJ Visser, M den Heijer. Underestimation of effect of thyroid function parameters on morbidity and mortality due to intra-individual variation. *Journal of Clinical Endocrinology and Metabolism 2011;96(12):E2014-2017.*

5. A van Mullem, R van Heerebeek, D Chrysis, E Visser, **M Medici**, M Andrikoula, A Tsatsoulis, R Peeters, TJ Visser. Clinical phenotype and mutant TRα1. *New England Journal of Medicine 2012;366(15):1451-1453.*

6. M Louwerens*, BC Appelhof*, H Verloop*, **M Medici**, RP Peeters, TJ Visser, A Boelen, E Fliers, JW Smit, OM Dekkers. Fatigue and fatigue-related symptoms in patients treated for different causes of hypothyroidism. *European Journal of Endocrinology 2012;167(6): 809-815.*

7. TIM Korevaar, S Schalekamp-Timmermans, YB de Rijke, WE Visser, W Visser, SMPF de Muinck Keizer-Schrama, A Hofman, HA Ross, H Hooijkaas, H Tiemeier, JJ Bongers- Schokking, VWV Jaddoe, TJ Visser, EAP Steegers, **M Medici**, RP Peeters. Hypothyroxinemia and TPO-antibody positivity are risk factors for premature delivery: results from the prospective, population-based Generation R study. *Journal of Clinical Endocrinology and Metabolism 2013;98(11):4382-4390.*

8. L Chaker, C Baumgartner, MA Ikram, A Dehghan, **M Medici**, WE Visser, A Hofman, TJ Visser, N Rodondi, RP Peeters, OH Franco. Subclinical thyroid function and the risk of stroke: a systematic review and meta-analysis. *Submitted.*

9. L Chaker, C Baumgartner, WJP den Elzen, AM Ikram, M Blum, S Bakker, A Dehghan, C Drechsler, RN Luben, A Hofman, MLP Portegies, **M Medici**, G Iervasi, TH Collet, A Brenmer, C Wanner, M Iacoviello, R Dullaart, J Sgarbi, G Ceresini, R Westendorp, W Jukema, M Imaizumi, J Franklyn, D Bauer, A Cappola, J Walsh, S Razvi, KT Khaw, H Völzke, OH Franco, J Gussekloo, N Rodondi, RP Peeters. Subclinical hypothyroidism and the risk of stroke and stroke mortality: an individual participant-based meta-analysis. *Pending major revisions.*

10. TIM Korevaar, EAP Steegers, S Schalekamp-Timmermans, YB de Rijke, WE Visser, W Visser, SMPF de Muinck Keizer-Schrama, A Hofman, H Hooijkaas, H Tiemeier, JJ Bongers-Schokking, VWV Jaddoe, TJ Visser, **M Medici**, RP Peeters. Soluble Flt1 and Placental Growth Factor are novel determinants of newborn thyroid (dys)function: the Generation R study. *Accepted in the Journal of Clinical Endocrinology and Metabolism.*

11. DJ Gottlieb, K Hek, T Chen, NF Watson, G Eiriksdottir, M Cornelis, SC Warby, S Bandinelli, EM Byrne, L Cherkas, DS Evans, HJ Grabe, J Lahti, M Li, T Lehtimäki, T Lumley, KD Marciante, L Pérusse, BM Psaty, J Robbins, GJ Tranah, JM Vink, JB Wilk, JS Andrews, C Bellis, R Biffar, C Bouchard, B Cade, GC Curhan, JG Eriksson, R Ewert, L Ferrucci, T Fülöp, PR Gehrman, R Goodloe, TB Harris, AC Heath, D Hernandez, A Hofman, J Hottenga, D.

Hunter, MK Jensen, AD Johnson, M Kähönen, L Kao, P Kraft, EK Larkin, DS Lauderdale, AI Luik, **M Medici**, GW Montgomery, A Palotie, SR Patel, G Pistis, E Porcu, L Quaye, O Raitakari, S Redline, EB Rimm, JI Rotter, AV Smith, T Spector, A Teumer, AG Uitterlinden, M Vohl, E Widen, G Willemsen, T Young, X Zhang, Y Liu, J Blangero, DI Boomsma, V Gudnason, F Hu, M Mangino, NG Martin, GT O'Connor, KL Stone, T Tanaka, J Viikari, SA Gharib, NM Punjabi, K Räikkönen, H Völzke, E Mignot, H Tiemeier. Novel loci associated with usual sleep duration: The CHARGE Consortium genome-wide association study. *Pending major revisions*.

12. TIM Korevaar, EAP Steegers , S Schalekamp-Timmermans, YB de Rijke, WE Visser, A Hofman, H Tiemeier, VWV Jaddoe, TJ Visser, **M Medici**, RP Peeters. Reference ranges and determinants of total hCG levels during pregnancy: the Generation R study. *Submitted*.

13. TIM Korevaar, EAP Steegers, **M Medici**, YB de Rijke, WE Visser, A Hofman, H Hooijkaas, VWV Jaddoe, TJ Visser, H Tiemeier, RP Peeters. The effects of hCG on gestational thyroid (dys)function and associations with subsequent adverse pregnancy or child outcomes: the Generation R study. *Submitted*.

14. C Zevenbergen, W Klootwijk, RP Peeters, **M Medici**, YB de Rijke, S Huisman, H Goeman, G de Kuijper, H de Waal, ME Meima, PR Larsen, TJ Visser, WE Visser. Functional analysis of novel genetic variation in the thyroid hormone activating type 2 deiodinase. *Submitted*.

15. CM Beukhof, **M Medici**, AW van den Beld, WE Visser, WW de Herder, TJ Visser, L Schomberg, RP Peeters. Selenium status is positively correlated with bone mineral density in healthy aging man. *Submitted*.

16. CD Wirth, MR Blum, BR da Costa, C Baumgartner, T Collet, **M Medici**, RP Peeters, D Aujesky, DC Bauer, N Rodondi. Subclinical Thyroid Dysfunction and the Risk of Fractures: A Meta-Analysis. *Accepted in Annals of Internal Medicine*.

PRESS RELEASE

2014 "Active Thyroid May Raise Risk of Depression in Older Individuals"
Endocrine Society, February 2014.
Published on various websites, including: sciencedaily.com, health.usnews.com, theusatodaynews.com, medicinenet.com, clinicalresearch.com, nlm.nih.gov, ese-hormones.org, press-news.org, dailypioneer.com, newswise.com.

GRANTS/AWARDS:

2008 Jan Dekkerstichting/Ludgardine Bouwmanstichting grant

2009 Travel grant Annual Meeting American Thyroid Association, Chicago, USA

2009 Jan Dekkerstichting/Ludgardine Bouwmanstichting grant

2010 Goodlife Healthcare travel grant, ENDO 2010, San Diego, USA

2010 Travel grant International Thyroid Congress, Paris, France

2011 Journal of Endocrinology travel grant Annual meeting European Society for Endocrinology, Rotterdam, the Netherlands

2011 Goodlife Healthcare travel grant, Annual meeting of the European Society for Endocrinology, Rotterdam, the Netherlands

2011 Young Investigator Award European Society for Endocrinology

2011 Travel grant Annual meeting European Thyroid Association, Krakow, Poland

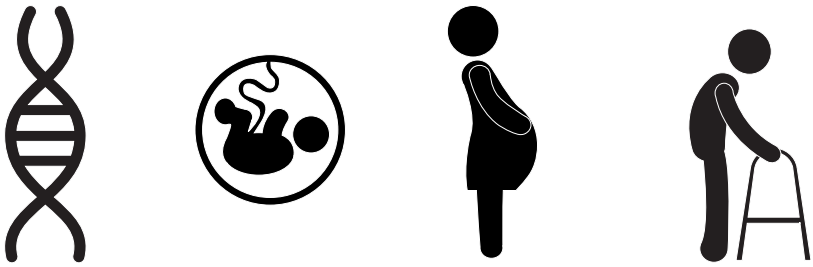
2011 Young Investigator Award European Thyroid Association

2012 Travel grant ENDO 2012, Houston, USA

2013 MSD Thyroid Award 2013




Curriculum vitae



CURRICULUM VITAE

Marco Medici was born on April 18th 1983 in Rotterdam, the Netherlands. After completing secondary school *cum laude* at the Regionale Scholengemeenschap Hoeksche Waard, he studied Medicine at the Erasmus University, Rotterdam. During the second year, he was invited to participate in the Master of Science in Clinical Epidemiology program by the Netherlands Institute of Health Sciences. In 2005, he obtained both his MSc degree as well as his doctoral degree, followed by completing his medical degree *cum laude* in 2008.

In 2008 he started a PhD project at the department of Endocrinology of the Erasmus MC under the supervision of Prof.dr.ir. T.J. Visser and Dr. R.P. Peeters. The results of these exciting years are presented in this thesis. For these studies he received six travel grant awards (ATA 2009, ENDO 2010, ITC 2010, ESE 2010, ETA 2011 and ENDO 2012), two young investigator awards (ESE 2011 and ETA 2011), and he was honoured as a MSD Thyroid Award 2013 winner. In 2012 he started his residency in Internal Medicine at the Erasmus MC, under the supervision of Prof.dr. J.L.C.M. van Saase. In 2013, he continued his residency at the Sint Franciscus Gasthuis, Rotterdam, under the supervision of Dr. A.P. Rietveld.

In the summer of 2014, he will start a postdoctoral fellowship at the Brigham and Women's hospital and Harvard Medical School, Boston, USA, and in 2016 he will start his fellowship in Clinical Endocrinology at the Erasmus MC.