Early Life Impacts of Thyroid Function and Human Chorionic Gonadotropin (hCG)

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Early Life Impacts of Thyroid Function and Human Chorionic Gonadotropin (hCG)

De invloed van schildklierfunctie en humaan choriongonadotrofine (hCG) in het vroege leven

Thesis

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MANUSCRIPTS THAT FORM THE BASIS OF THIS THESIS

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Chapter 2.2
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Chapter 4.1
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Chapter 4.2
Barjaktarovic M, Korevaar TI, Gaillard R, de Rijke YB, Visser TJ, Jaddoe VWV, Peeters RP. Childhood thyroid function, body composition and cardiovascular function.
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Chapter 4.3
Veldscholte K, **Barjaktarovic M**, Trajanoska K, Jaddoe VWV, Visser TJ, de Rijke YB, Peeters RP, Rivadeneira F, Korevaar TI. The association of thyroid function with bone density during childhood. *Submitted*

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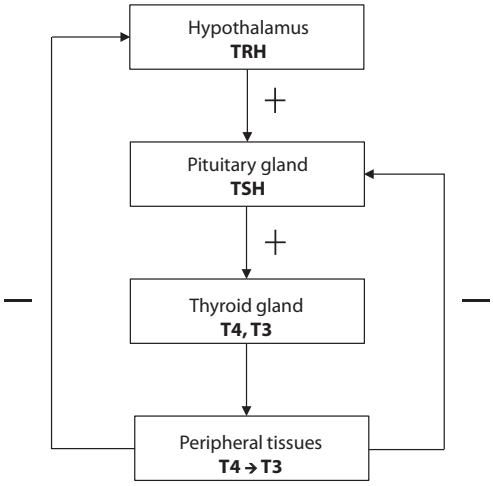
CHAPTER 1

General introduction

Background

Thyroid hormone is produced by the thyroid gland and its concentration is regulated by a negative feedback system known as the hypothalamic-pituitary-thyroid axis (depicted in Figure 1) [1]. Thyroid hormone regulates metabolism and is essential for the development and differentiation of practically all cells in the human body [1]. For that reason, growth and differentiation of almost all tissues, especially those belonging to the central nervous system, are dependent on adequate thyroid function. [1-3]. This is particularly important during the first half of pregnancy when placental development and fetal growth and differentiation completely depend on the maternal supply of thyroid hormone. Deficiency of thyroid hormone is associated with the impaired neural development and subsequent cognitive impairment [1-3]. Thyroid dysfunction during pregnancy may have important consequences; first of all because low thyroid hormone availability is associated with the risk of premature delivery [4,5], and secondly because high thyroid hormone availability may have adverse effects on fetal development, increasing the risk of pre-eclampsia, low birth weight and suboptimal brain development [3,6-8]. For that reason, great effort has been put into investigation and explanation of pregnancy-specific changes, as well as into understanding of the pathophysiology of thyroid dysfunction in pregnancy, with the ultimate aim to improve both maternal and fetal care.

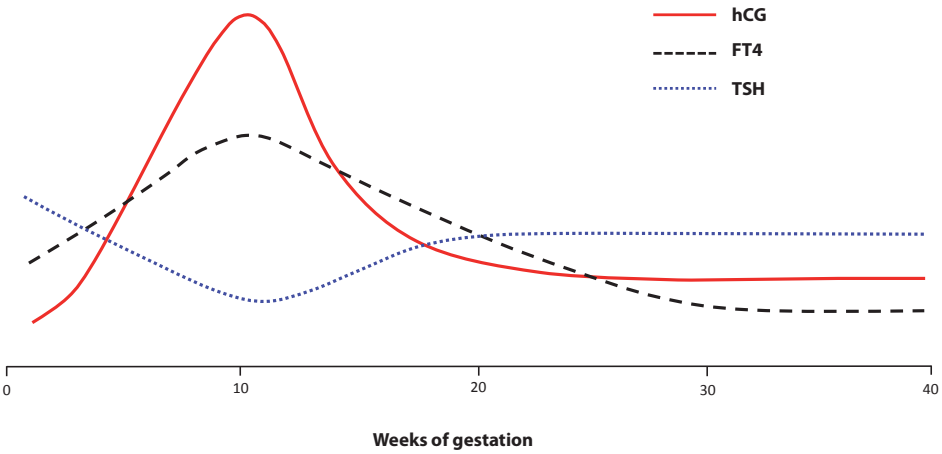
Figure 1. Hypothalamic-pituitary-thyroid axis



During pregnancy, an increased metabolic demand and hormonal alterations result in several adaptive changes in thyroid physiology [9] (depicted in Figure 2). First of all, the progression of pregnancy comes with an increase in concentration of thyroid hormone binding proteins (most notable thyroxine-binding globulin), which, together with the action of placental type 3 iodothyronine deiodinase, a thyroid hormone inactivating

enzyme, increases the need for thyroid hormone [9]. Second, there is an increased need for maternal thyroid hormone availability for the fetus, as the fetal thyroid gland is not fully functional until the second half of pregnancy [10]. Third, human chorionic gonadotropin concentration (hCG), due to its ability to stimulate the thyroid-stimulating hormone (TSH) receptor [11], induces an increase in free thyroxine (FT4) concentration and a decrease in TSH at the end of the first trimester [9]. This complicated, multifaceted mechanism that ensures the supply of thyroid hormone to the fetus, emphasizes the importance of optimal thyroid hormone regulation for proper fetal growth and development.

Figure 2. Physiology of thyroid function during pregnancy



Thyroid function and the placenta

A successful pregnancy requires optimal placental function, as the fetus completely depends on an optimally functioning placental barrier for its supply of nutrients, respiratory gas exchange and elimination of metabolic waste products [12,13]. Furthermore, the placenta is a prime endocrine organ producing hormones that are crucial for maintaining the pregnancy, including hCG, estrogen, progesterone and prostaglandins [14]. Placental dysfunction may result in pregnancy complications, such as pre-eclampsia, fetal growth restriction and premature delivery, which are the major causes of maternal and perinatal morbidity and mortality worldwide [15-18].

Trophoblast cells express thyroid hormone transporters and receptors [19-22] and a successful placentation requires optimal thyroid hormone concentration [23]. Placentation is a complex process that consists of interstitial invasion of trophoblast cells into maternal decidua and endovascular trophoblast (EVT) invasion into maternal spiral arteries [24]. This is, in part, regulated by pro- and anti-angiogenic factors and cytokines [24]. Thyroid hormone regulates the secretion of several growth factors and cytokines that are critical for EVT invasion and angiogenesis of maternal and fetal placental vessels, including

angiogenin, angiopoietin 2 , vascular endothelial growth factor-A, interleukin 10 and tumor necrosis factor alpha [25]. Furthermore, thyroid hormone may attenuate proliferation and invasion of trophoblast [20,26].

Interestingly, placental dysfunction and thyroid dysfunction are associated with the same pregnancy complications, such as pre-eclampsia and fetal growth restriction [24,27]. As these pregnancy complications may arise from impaired placentation [24] and given that placental tissue is responsive to thyroid hormone [19-22], part of this thesis focuses on how early-pregnancy thyroid function could regulate placental function and could potentially mediate the associations of thyroid function with pregnancy complications. Despite the existing experimental data suggesting a role of thyroid hormone in placentation, there is a paucity of population-based studies investigating these effects. One of the aims of this thesis was to translate and quantify the experimental findings on the effects of thyroid hormone on placental function into a clinical context.

The role of hCG

hCG is a pregnancy-specific hormone, produced by the trophoblast cells from implantation of the embryo onwards, throughout the whole pregnancy [28]. hCG plays a key role in promoting progesterone production by the corpus luteum during early pregnancy, as well as in the differentiation of trophoblast, placental development and angiogenesis [28,29]. The latter is partly mediated via regulating effects of hCG on vascular endothelial growth factors [30-34]. By co-regulating angiogenesis in a timely manner, hCG ensures a proper placental development which is crucial for the outcome of pregnancy [31,33]. Because of the structural homology with TSH, hCG is able to stimulate the TSH receptor thereby stimulating the thyroid gland [9,28]. The subsequent changes in thyroid physiology during pregnancy affect TSH and FT4 concentrations [9] (Figure 2), raising a question whether the effects of thyroid hormone on pregnancy-specific outcomes, such as pre-eclampsia or fetal growth restriction, might be initiated by hCG effects on the thyroid or if the effects of these two important hormones are linked at all.

Although clinical studies have shown that hCG is associated with adverse outcomes of pregnancy, these associations differ based on the gestational age at which hCG was measured or on the hCG isoform that was assessed. For example, while several studies suggest that in early and late pregnancy high hCG is associated with the risk of pre-eclampsia [35-38], studies also report an association of low early-pregnancy β-hCG [39] and low early-pregnancy hyperglycosylated hCG [40] with the risk of pre-eclampsia. Similarly, there are reports of an association of high hCG concentrations [41,42] but also of low hCG concentrations [43,44] with fetal growth restriction. Little is known on the potential mediators in these associations, for instance angiogenic factors, as well as on the potential gestational-age dependent variation in the effects.

Thyroid function and metabolism

While thyroid hormone regulates metabolism of practically the whole body, thyroid hormone may specifically regulate the metabolism of homocysteine. This may occur via two potential mechanisms: first of all, proliferative processes that depend on folate and vitamin B12 concentrations are stimulated by thyroid hormone [45-47]. A higher thyroid hormone concentration might therefore result in a higher homocysteine concentration, as this would lead to a lower availability of folate and vitamin B12 for re-methylation of homocysteine to methionine [47]. Secondly, reports from animal studies suggest that the activity of enzymes required for re-methylation of homocysteine, methylenetetrahydrofolate reductase (MTHFR) and methionine synthase may be thyroid hormone dependent [47]. Although human studies have shown that thyroid dysfunction is associated with higher homocysteine concentration [48-50], the direction and consistency of the association are not clear [48-52]. This can be clinically relevant since high homocysteine concentrations are associated with adverse cerebrovascular, pregnancy-specific and neonatal outcomes [53-56]. To date, population-based data investigating the association of thyroid function with homocysteine concentration are sparse and therefore it remains unknown to what extent these potential pathophysiological mechanisms altogether are of clinical relevance.

Thyroid function in childhood

Thyroid hormone is important for optimal childhood growth and development. This is for example reflected by the fact that even mild forms of thyroid dysfunction are associated with suboptimal developmental outcomes, such as weight gain, impaired growth, hyperlipidemia and impaired cognitive development [57]. Interestingly, the effects of thyroid hormone on multiple organ systems have been extensively studied in adults, whereas population-based data on the effects in early childhood are sparse. For instance, the effects of thyroid (dys)function on the cardiovascular system are well-known in the adult population, as is exhibited by the effects on vascular resistance, heart rate, cardiac contractility and mass [58], yet little is known about these effects during childhood when cardiac growth and development take place. Different types of evidence support the regulating role of thyroid hormone in cardiac development and function in early life [59]. In line with this, cardiomyocytes express thyroid hormone receptors during fetal and postnatal life [60]. Similarly, thyroid hormone has a critical role in the linear growth and bone maturation, and thyroid hormone receptors are expressed at the sites of bone formation [61]. During childhood, hyperthyroidism is associated with premature accretion of growth plates and cranial sutures, resulting in short stature, whereas hypothyroidism is associated with impaired bone ossification, also resulting in short stature [61,62]. In adults, high thyroid function is associated with high bone resorption and low bone mineral density, leading to osteoporosis [61,62]. Although the effects on the bone occur

via thyroid hormone, there are reports suggesting a direct role of TSH on osteoblasts and osteoclasts [63], however, this remains debated. Thus far, only few studies have investigated the effects of variation of thyroid function on the cardiovascular development, as well as on the potential association of body composition and/or its mediating role in the association of thyroid function with cardiovascular function during childhood. In addition, there is a paucity of data on the effects of variation of thyroid function on the bone development during childhood. Using an epidemiological approach and a population-based setting, part of these associations is investigated in this thesis.

Proper TSH and FT4 reference ranges are essential for an adequate diagnosis of thyroid disease. Apart from the recommendation of the European Thyroid Association of using age-adjusted values [64], no further consensus has been reached on the definition of TSH and FT4 reference ranges during childhood, complicating the clinical diagnosis of thyroid dysfunction. The existing literature on childhood TSH and FT4 reference ranges is hampered by the heterogeneity in terms of age range, ethnicity and assay use. It is not known to what extent differences in the methodology and population-specific factors add to the overall heterogeneity. Furthermore, a proper understanding of the thyroid function determinants is crucial in order to identify and/or exclude a cause of an abnormal thyroid function test. This requires a thorough investigation, most importantly so that physicians may assess whether a reference range is generalizable to a specific patient population, but also that future studies, examining the association of thyroid function with various outcomes would take into account important confounding and mediating factors.

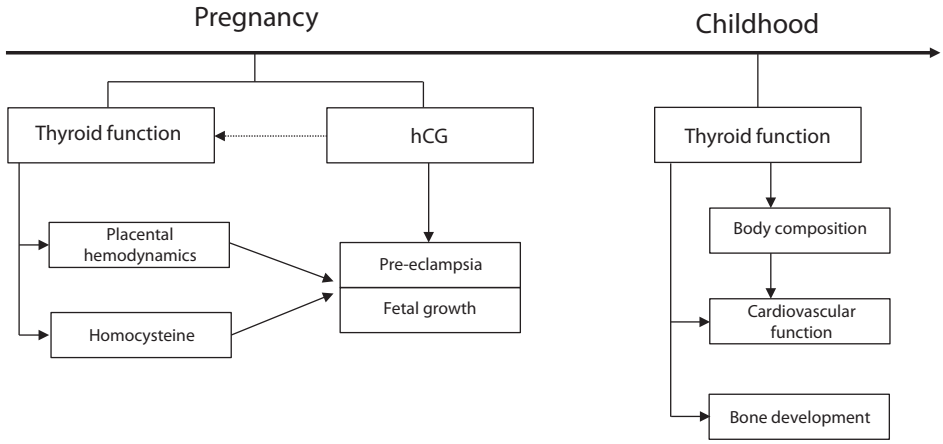
Aims

This thesis aims to examine the developmental and metabolic influence of endocrine factors, in particular related to thyroid function and hCG, on the outcomes of pregnancy and early childhood development. The studies described here investigate the associations of gestational thyroid function with placental hemodynamic function, homocysteine concentration and its potential mediating role in pregnancy complications. In order to delineate to what extent there is a contributory role of hCG in the pathophysiology of pregnancy complications, the associations of hCG with pre-eclampsia and fetal growth are investigated. Furthermore, the determinants of childhood thyroid function and the associations of childhood thyroid function with the important target organs are investigated along with the potential mediating role of body composition. The concept of the associations examined in this thesis is depicted in Figure 3.

Setting

The studies presented in this thesis are embedded in Generation R, a prospective population-based cohort from early fetal life onwards, in Rotterdam, the Netherlands [65].

Figure 3. The associations examined in this thesis



This cohort was designed to study early environmental and genetic determinants of growth, development and health during fetal and postnatal life [65]. Eligible participants for the study were 8879 pregnant women with an expected delivery date between April 2002 and January 2006 that were enrolled in the cohort during pregnancy [65]. Blood samples for TSH, FT4 and hCG measurements were obtained at inclusion in the study. Fetal weight and placental ultrasound measurements were performed during prenatal visits at mid- (18-25 week) and late (after 25th week) pregnancy [65]. Pre-eclampsia diagnosis was confirmed by certified medical doctors by reviewing hospital charts. At birth, cord blood samples were drawn and TSH and FT4 concentrations were measured. At the median age of 6 years, children were invited to a dedicated research center where detailed body composition, bone mineral density and cardiovascular measurements were performed.

Outline of the thesis

Chapter 2 focuses on the associations of thyroid function in pregnancy with placental hemodynamics and homocysteine concentrations. In **Chapter 2.1** the associations of gestational thyroid function with placental vascular resistance in mid- and late pregnancy were investigated, as well as the potential mediating role of placental function in the previously described associations of thyroid function with the outcomes of pregnancy. In **Chapter 2.2** the associations of thyroid function with maternal and neonatal homocysteine concentrations were investigated. **Chapter 3** focuses on the associations of hCG concentrations with the outcomes of pregnancy. **Chapter 3.1** focuses on the association of hCG with the risk of pre-eclampsia and in **Chapter 3.2** the association of hCG with fetal growth trajectory is presented. **Chapter 4** focuses on the determinants of childhood thyroid function and the associations of childhood thyroid function with tar-

get organs. **Chapter 4.1** provides a detailed overview of the existing literature on childhood reference ranges for TSH and FT4, and shows the determinants of these reference ranges in a population-based cohort. **Chapter 4.2** focuses on the association of thyroid function with cardiovascular function and investigates the role of body composition in this association. **Chapter 4.3** describes the association of childhood thyroid function with bone mineral density.

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CHAPTER 2

Thyroid function and pregnancy

CHAPTER 2.1

The association of maternal thyroid function with placental hemodynamics

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ABSTRACT

Study question: What is the clinical association of maternal thyroid function with placental hemodynamic function?

Summary answer: Higher FT4 concentration in early pregnancy is associated with higher placental vascular resistance.

What is known already: Suboptimal placental function is associated with pre-eclampsia (which, in turn, further deteriorates placental hemodynamics and impairs fetal blood supply), fetal growth restriction and premature delivery. Studies have suggested that thyroid hormone has a role in placental development through the effects on trophoblast proliferation and invasion.

Study design, size, duration: This study was embedded in The Generation R cohort, a population-based prospective study from early fetal life onwards in Rotterdam, the Netherlands. In total, 7069 mothers with expected delivery date between April 2002 and January 2006 were enrolled during early pregnancy.

Participants/materials, setting, methods: Thyroid stimulating hormone (TSH) and free thyroxine **(**FT4) concentrations were measured during early pregnancy (median 13.4 weeks, 95% range 9.7-17.6 weeks). Placental function was assessed by Doppler ultrasound via measurement of arterial vascular resistance, i.e. umbilical artery pulsatility index (PI) and uterine artery resistance index (RI) (both measured twice, between 18-25th and after 25th gestational weeks) and the presence of uterine artery notching (once after the 25th gestational week) in 5184 pregnant women.

Main results and the role of chance: FT4 was positively linearly associated with umbilical artery PI in the second and third trimester as well as with uterine artery RI in the second trimester and the risk of uterine artery notching in the third trimester ($P<0.05$ for all). The association of thyroid function with preeclampsia and birth weight was partially mediated through changes in placental function, the percentages of mediated effects being 10.4% and 12.5%, respectively.

Limitations, reasons for caution: A potential limitation is the availability of a single TH measurement and differential missingness of placental ultrasound measurements for the adverse outcomes.

Wider implications of the findings: Higher FT4 concentration in early pregnancy is associated with higher vascular resistance in the second and third trimester in both the maternal and fetal placental compartment. These effects on placental function might explain the association of FT4 with adverse pregnancy outcomes, including preeclampsia and fetal growth restriction.

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Key words: thyroid function, placenta, placental hemodynamics, pregnancy

INTRODUCTION

Adequate placental function is essential for an uncomplicated pregnancy and optimal fetal development as it enables fetal nutrient supply, respiratory gas exchange and elimination of metabolic waste products [1,2]. Furthermore, the placenta produces hormones that are crucial for maintaining pregnancy including human chorionic gonadotropin (hCG), estrogen, progesterone and prostaglandins [3]. A suboptimal placental function is associated with pregnancy complications, including pre-eclampsia (which, in turn, further deteriorates placental hemodynamics and impairs fetal blood supply), fetal growth restriction and premature delivery, which are the major causes of maternal and perinatal morbidity and mortality worldwide [4-7].

Thyroid hormone (TH) transporters and receptors are expressed in the trophoblast cells [8-11] and optimal TH concentration is necessary to ensure appropriate placentation [12]. Placentation is a complex process that requires proper interstitial invasion of fetal trophoblast cells into maternal decidua and endovascular trophoblast (EVT) invasion into maternal spiral arteries [13]. This is, in part, regulated by pro- and anti-angiogenic factors and cytokines [13]. TH regulates secretion of several growth factors and cytokines that are critical for EVT invasion and angiogenesis of maternal and fetal placental vessels, including angiogenin, angiopoietin 2 (Ang-2), vascular endothelial growth factor-A (VEGF-A), interleukin 10 (IL-10) and tumor necrosis factor alpha (TNF-α) [14]. Furthermore, TH attenuates epidermal growth factor (EGF)-initiated trophoblast proliferation [11], motility [15] and invasion [16].

Low thyroid function has been associated with premature delivery [17,18] and high thyroid function has been associated with pre-eclampsia [19,20] and fetal growth restriction [21,22], adverse pregnancy outcomes that could be arising from impaired placentation in early gestation [13]. Given that placental tissue is responsive to TH [8-11], we hypothesized that early maternal thyroid function is a regulator of placentation. Despite the increasing body of basic evidence suggesting that TH plays a role in regulation of placental development, there is a lack of data that translate these findings to clinical outcomes. Moreover, as thyroid dysfunction [17,19,21] and placental dysfunction [4] are associated with the same pregnancy complications, the clinical association of thyroid function with adverse pregnancy outcomes might be mediated via changes in placental function or vice versa.

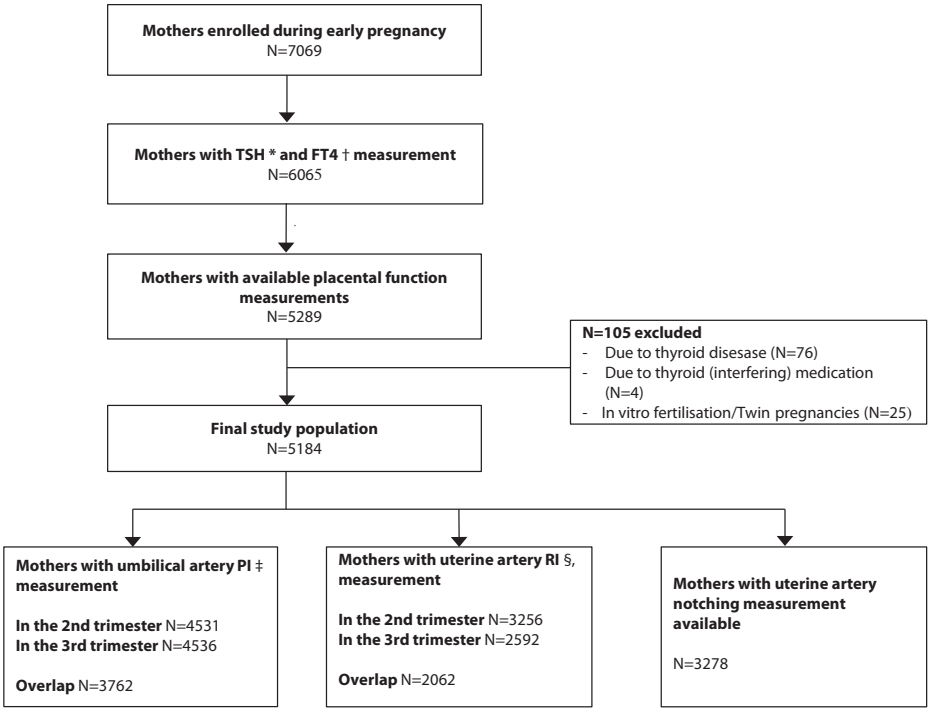
Therefore, the aim of this study was to translate and quantify experimental findings on the link between thyroid hormone and placental function into a clinical context within a large prospective population-based cohort. In addition, we aimed to examine the mediating role of the placental function in the association of TH with birthweight, pre-eclampsia and premature delivery.

MATERIALS AND METHODS

Study population

This study was embedded in The Generation R cohort, a population-based prospective study from early fetal life onwards in Rotterdam, the Netherlands [23]. The study was designed to identify early environmental and genetic causes leading to normal and abnormal growth, development and health during fetal life and childhood [23]. In total, 7069 mothers with expected delivery date between April 2002 and January 2006 were enrolled during early pregnancy. Thyroid-stimulating hormone (TSH) and free thyroxine (FT4) were determined in the first available serum sample during early pregnancy (<18 weeks) and were available in 6065 women, from which 5289 had available placental function measurements. Women with thyroid disease, thyroid (interfering) medication, in vitro fertilization and/or twin pregnancies, were excluded from the analysis (N=76, N=4, N=25, respectively). The final population of women included in the analysis comprised 5184 women (Figure 1). Written informed consent was obtained from all participants. The study has been approved by the local Medical Ethics Committee.

Figure 1. Flowchart showing selection procedure of the study population



* TSH – thyroid stimulating hormone, † FT4 – free thyroxine, ‡ PI –pulsatility index, § RI – uterine artery resistance index

Thyroid measurements

Maternal serum samples were obtained in early pregnancy (median 13.4 weeks, 95% range 9.7 -17.6 weeks). Plain tubes were centrifuged and serum was stored at -80°C. TSH and FT4 concentrations in maternal serum samples were determined using chemiluminescence assays (Vitros ECi; Ortho Clinical Diagnostics). Maternal thyroid peroxidase antibodies (TPOAbs) were measured using the Phadia 250 immunoassay (Phadia AB) and were regarded as positive when greater than 60 IU/ml [24]. Euthyroidism was defined according to the 2.5th – 97.5th percentile reference range for the study population [24].

Placental function measurements

Measurements of placental vascular resistance were used as a reflection of placental function and a proxy measure of the placentation success [25]. Placental vascular resistance was evaluated with recorded flow-velocity waveforms from the umbilical (representing the fetal vascular compartment) and uterine (representing the maternal vascular compartment) arteries in the second trimester (median 20.5 weeks, 95% range 18.7-23.1 weeks) and third trimester (median 30.4 weeks, 95% range 28.6-32.8 weeks) [26], with the median time of 9.9 weeks between the two measurements. The median time intervals between blood sampling and placental hemodynamic measurements in the 2nd trimester was 7.1 weeks and in the 3rd trimester was 17 weeks. A raised umbilical artery pulsatility index (PI) and uterine artery resistance index (RI) indicate increased placental vascular resistance which is a sign of (subsequent) placental insufficiency, that may occur as a result of impaired placentation [27,28]. Umbilical artery PI was measured in a free-floating loop of the umbilical cord. Uterine artery RI was measured in the uterine arteries near the crossover with the external iliac artery. For each measurement, three consecutive uniform waveforms were recorded by pulsed Doppler ultrasound, during fetal apnea and without fetal movement. The mean of three measurements was used for further analysis. The presence of notching in the third trimester was assessed in the uterine arteries and reflects an abnormal waveform resulting from increased blood flow resistance, which is a sign of placental insufficiency [29]. Ultrasound measurements and analyses were performed in a blinded fashion with regards to the previous measurements and pregnancy outcome.

Outcomes of pregnancy

Information on birth weight was obtained from hospital registries. Birth weight standard deviation scores adjusted for gestational age were constructed using the Niklasson percentile growth curves [30]. Premature delivery was defined as a gestational age at birth <37 weeks. Gestational hypertension was defined as development of systolic blood pressure ≥ 140 mmHg and/or diastolic blood pressure ≥90mmHg after 20 weeks of gestation in previously normotensive women. These criteria plus the presence of

proteinuria (defined as 2 or more dipstick readings of 2+ or greater, 1 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 pre-eclampsia [31].

Covariates

Information on maternal ethnicity and smoking status was obtained by questionnaires during pregnancy. Ethnicity was determined by the country of origin and was defined according to the classification of Statistics Netherlands [23]. Maternal smoking was classified as no smoking, smoking until known pregnancy and continued smoking during pregnancy. Information on parity and sex of the child was obtained from hospital registries. Body mass index (BMI) was measured at inclusion in the study.

Statistical analysis

We investigated the associations of TSH and FT4 with umbilical artery PI and uterine artery RI by using multiple linear regression analyses with restricted cubic splines utilizing three knots, to account for possible non-linear associations [32]. To study the association of TSH and FT4 with the risk of notching in the uterine arteries, we used multiple logistic regression models with restricted cubic splines utilizing three knots. TSH and FT4 values were logarithmically transformed to allow for a better model fit. Multivariable associations were graphically depicted by plots (main manuscript) and β estimates/ odds ratios with 95% confidence intervals are shown in Supplemental Tables 2 and 3). In order to properly investigate placental function as a mediator in the associations of thyroid function with pregnancy outcomes, we investigated the prerequisite associations: the association of thyroid function and placental function was examined in this study, the associations of thyroid function and pregnancy outcomes were examined and described previously [17,19,21] as well as was the association of placental vascular resistance indices with pregnancy outcomes [33]. To examine the mediating role of placental function in the associations of TH with pregnancy outcomes and vice versa, we analyzed the direct and indirect causal mediation effects by performing mediation analyses using Imai et al. approach [34].

All model covariates were selected based on biological plausibility based on the previous studies, change of the effect estimate of interest and/or residual variability of the model in this study. The analyses were adjusted for gestational age at blood sampling, smoking, maternal age, parity, ethnicity, BMI, fetal sex and gestational age at ultrasound measurement. We performed sensitivity analyses in order to examine whether additional adjustment to hCG, placental angiogenic factors (placental growth factor and soluble FMS-like tyrosine kinase, previously described as the determinants of thyroid function [35]), maternal blood pressure or presence of TPOAbs would affect the effect estimates. We also performed sensitivity analyses by selecting euthyroid women only.

We accounted for the high number of statistical tests (38 in total) by controlling the false discovery rate (Benjamini and Hochberg) using the fdrtool package [36,37]. This method allows for tailored identification of the expected proportion of false positive results among all rejected null hypotheses. We identified that a q-value of 0.045 (i.e. the cut-off for a 4.5% chance of having a type I error) was similar to a *P*-value of 0.05, therefore a *P*-value threshold of <0.05 was considered for statistical significance.

In the first part of the analysis, where we studied the association of TSH and FT4 with placental vascular resistance, we performed multiple imputation according to Markov Chain Monte Carlo method, for covariates with missing data [38]. Before imputation of the missing values, we performed exploratory analyses by investigating the pattern of missingness for each variable. All variables showed random missingness patterns and the missingness was fully accounted for by complete variables rendering us to conclude the data was M(C)AR. The percentage of missing data was less than 1% for sex, parity, BMI and gestational age at birth variables. Furthermore, the percentage of missing data was 10.8% for smoking, 6.4% for education and 3.4% for ethnicity variables. Twenty imputed data sets were created and pooled for the analysis. Maternal smoking, education, ethnicity, parity, BMI and fetal sex were then added to the model. Furthermore, we added umbilical artery PI, uterine artery RI, uterine artery notching, maternal TSH and FT4 concentrations as prediction variables only. No statistically significant differences in descriptive statistics were found between the original and imputed datasets. For mediation sub-analyses, differential missingness for data on placental hemodynamics on outcomes in datasets that were previously used to study adverse outcomes was coped with by performing multiple imputation for data on umbilical artery PI, uterine artery RI and uterine artery notching values [39] of placental function data. Twenty imputed data sets were created and pooled for the analysis. TSH and FT4 concentration, as well as pregnancy outcomes were used as predictor variables only and were not imputed. No statistically significant differences in descriptive statistics were found between the imputed datasets.

Statistical analyses were performed using Statistical Package of Social Sciences version 21.0 for Windows (SPSS Inc. Armonk, NY) and R statistical software version 3.2.0 (package *rms*, *mediation* and *fdrtool*).

RESULTS

The final study population consisted of 5184 pregnant women (Figure 1) for which at least one placental function measurement was available. The concurrent measurements of second- and third-trimester umbilical artery PI and uterine artery RI were available for 3762 and 2062 women, respectively (Figure 1). Descriptive statistics of the study popu-

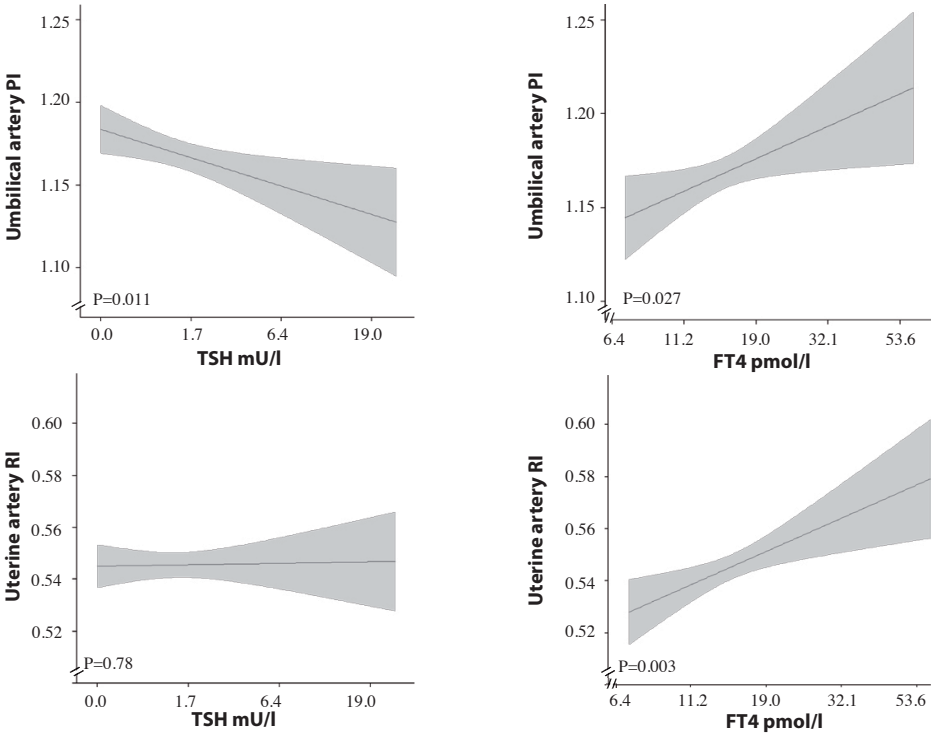
lation are shown in Table 1. As compared to women for which the placental function measurements were available, women without placental function measurements had blood samples drawn at slightly later gestational age, had higher BMI and were of lower educational level (Supplemental Table 1).

The association of thyroid function with placental vascular resistance indices

As is shown in Figure 2, TSH was negatively linearly associated with umbilical artery PI (*P*=0.011) but not with uterine artery RI (*P*=0.78) in the second trimester. Furthermore, FT4 was positively linearly associated with umbilical artery PI (*P*=0.027) and uterine artery RI (*P*=0.003) in the second trimester (Figure 2).

As Figure 3 shows, TSH was not associated with umbilical artery PI (*P*=0.18) and uterine artery RI (*P*=0.75) in the third trimester. FT4 was positively linearly associated with umbilical artery PI (*P*=0.015) but not with uterine artery RI (*P*=0.91) in the third trimester (Figure 3).

Figure 2. The association of maternal thyroid function with the 2nd trimester placental function

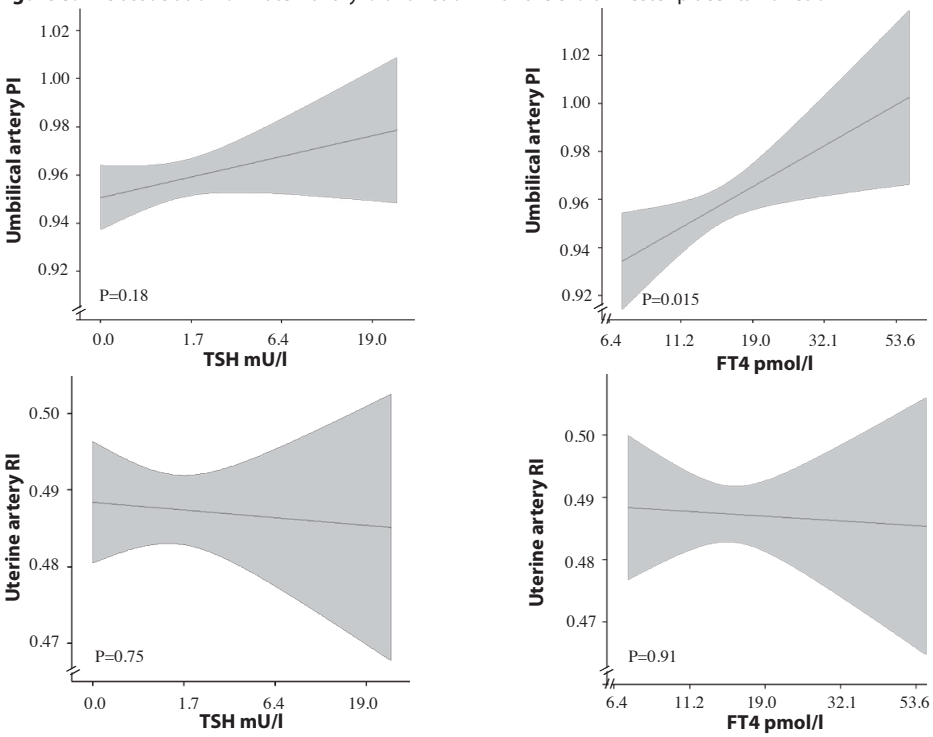


Plots show the linear regression models for TSH and FT4 and the resistance indices of the umbilical and uterine artery in the second trimester of pregnancy, as predicted mean with 95 percent confidence interval. Analyses were adjusted for gestational age at blood sampling, gestational age at ultrasound, smoking, BMI and fetal sex.

Table 1. Descriptive Statistics of the Participants

Characteristic	Value
TSH, median (95%range), mU/l,	1.34 (0.04-4.49)
FT4, median (95%range), pmol/l	14.7 (10.2-22.2)
Gestational age at blood sampling, median (95%range)	13.4 (9.7-17.6)
Umbilical artery PI, mean ±sd	
Second trimester	1.20 ±0.18
Third trimester	0.98 ± 0.17
Uterine artery RI, mean ±sd	
Second trimester	0.54 ±0.09
Third trimester	0.48 ±0.08
Uterine artery notching, n (%)	
yes	326 (9.9)
no	2952 (90.1)
Age, mean ±sd , years	29.7 ±5.0
BMI, median, (95% range), kg/m2	23.5 (18.5-35.6)
Parity, n (%)	
Nullipara	3057 (57.5)
Primipara	1589 (29.9)
Multipara	669 (12.6)
Smoking status, n (%)	
Non smokers	3821 (71.9)
Stopped smokers	505 (9.5)
Smokers	989 (18.6)
Educational level, n (%)	
No education or primary education	587 (11.0)
Secondary education	2429 (45.7)
Higher education	2299 (43.3)
Ethnicity, n (%)	
Dutch	2726 (51.3)
Moroccan	350 (6.6)
Turkish	425 (8.0)
Surinam	477 (9.0)
Dutch Antilles	173 (3.3)
Asian	148 (2.8)
Other – Western	477 (9.0)
Other – Non-Western	539 (10.1)
Fetal sex, n (%)	
male	2667 (50.2)
female	2648 (49.8)
TPO antibody positivity, n (%)	
yes	274 (5.3)
no	4557 (87.9)

Figure 3. The association of maternal thyroid function with the 3rd trimester placental function



Plots show the linear regression models for TSH and FT4 and the resistance indices of the umbilical and uterine artery in the third trimester of pregnancy, as predicted mean with 95 percent confidence interval. Analyses were adjusted for gestational age at blood sampling, gestational age at ultrasound, smoking, BMI and fetal sex.

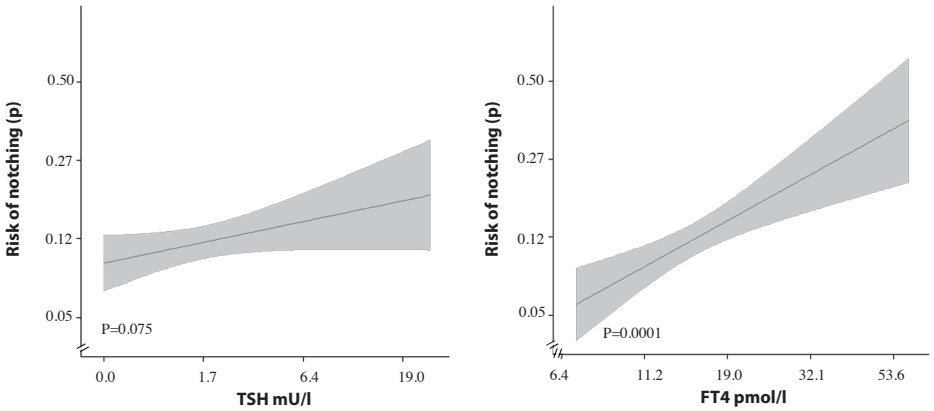
Figure 4 shows thyroid function and the risk of uterine artery notching during the third trimester. Higher concentrations of FT4 were associated with a higher risk of notching (*P*=0.0001), whereas TSH was not associated with the risk of notching (*P*=0.075).

Sensitivity analyses showed no change in the results after adjusting for hCG, placental angiogenic factors, maternal blood pressure and TPOAbs. All reported associations remained similar when analyzed in euthyroid women only, except for the association of FT4 with umbilical artery PI measured in the second trimester that was not statistically significant (Supplemental Figures 1-3).

Placental function as a potential mediator in the association of thyroid function with adverse pregnancy outcomes

In Supplemental Table 4, the results of mediation analysis for different outcomes (pre-eclampsia, birth weight and premature delivery) are shown. There was no mediation by placental function in the association of thyroid function with the risk of pre-eclampsia except for second-trimester uterine artery RI (*P* for mediation = 0.02, percentage of me-

Figure 4. The association of maternal thyroid function with the risk of uterine artery notching in the 3rd trimester



Plots show the logistic regression models for TSH and FT4 and the risk of 3rd trimester notching in the uterine artery, as predicted mean with 95 percent confidence interval. Analyses were adjusted for gestational age at blood sampling, smoking, BMI, gestational age at ultrasound, maternal age, ethnicity, parity and fetal sex.

diated effect: 10.4%). Similarly, in the association of thyroid function with birth weight, there was no mediating role of placental function except for the second-trimester uterine artery RI (*P* for mediation <0.01, percentage of mediated effect: 12.5%). There was no mediating role of placental function in the association of thyroid function and the risk of premature delivery.

DISCUSSION

Our data show an association of early gestational thyroid function with measures of placental vascular function in pregnancy. To our knowledge, this is the first study that investigates the association of gestational thyroid function with the placental function in a clinical context. Higher maternal FT4 concentration during early pregnancy was associated with higher placental vascular resistance in the period of 18.7-23.1 weeks and 28.6-32.8 weeks of gestation. Taken together, these results suggest that high thyroid function during early pregnancy may influence placental function during the second half of pregnancy, most likely through impaired placentation. Our results also suggest that 10.4% and 12.5% of the association of high thyroid function with pre-eclampsia and birth weight could be occurring through changes in placental hemodynamics, respectively.

In the current study, higher FT4 concentration was associated with higher vascular resistance in the second trimester, in both fetal and maternal compartment of the

placenta. This suggests that TH affects the formation of the placenta as a whole, i.e. both feto-placental and utero-placental circulation, and that high thyroid function in early pregnancy is a risk factor for impaired placentation and vascularization. This could be explained by a combination of TH-mediated effects; first of all, TH mediates down-regulation of VEGF-A, a factor promoting maternal and fetal angiogenesis and increasing trophoblast motility [40,41]. Secondly, TH attenuates EGF-related actions on trophoblast motility and invasion [11]. Furthermore, TH induces down-regulation of IL-10, necessary for vascular development, and up-regulates TNF-α, which is known to inhibit EVT invasion and trophoblast proliferation [14,42].

Higher FT4 was associated with placental outcomes in the third trimester, namely a higher umbilical PI, as well as with a higher risk of uterine artery notching. This suggests that the effects of TH on placental development during early pregnancy may have a persistent impact on the quality of placental vessels and function in both fetal and maternal compartment. On the other hand, FT4 was not associated with third-trimester uterine artery RI (a measure of placental function on the maternal side). This may suggest that, although the placental development as a whole is affected by exposure to THs, the effects on the fetal side are more persistent, possibly due to the active placental transfer of maternal thyroxine to the fetus or due to gestational age-specific changes in the TH effects on the angiogenic factors and cytokines secretion [14]. Alternatively, this might be explained by the second wave of EVT invasion which occurs during the second trimester [43]. This process may remodel spiral arteries and thus improve placental angiogenesis and blood flow during later gestation, yet be differently affected by THs as compared to early trophoblast invasion.

The exact origin of impaired placentation has not been clarified completely. Placentation is a very precise process and impairment of any stage can result in pregnancy complications [13]. The fact that we observed similar associations also within the euthyroid subgroup of women, suggests that even "high-normal" FT4 values might be leading to a certain impairment in the placental function.

TSH may have transient and limited effects on the placentation, compared to FT4 effects. High TSH concentration was associated with lower vascular resistance in the umbilical artery during the second trimester, in line with the opposing effects of FT4 at that time point. However, there was no association of TSH with umbilical artery resistance in the third trimester, nor with uterine artery vascular resistance in second or third gestational trimester. Similarly, other gestational outcomes determined by thyroid function, such as fetal brain development and pre-eclampsia, are shown to be associated with FT4 concentration and not with TSH concentration [19,44]. Furthermore, TH production during pregnancy is considerably stimulated by hCG and the increase in FT4 subsequently causes a transient decrease in TSH concentration [45]. This might suggest that FT4 better reflects thyroid function during gestation and that the role of TSH as a

unique indicator of thyroid function is potentially less pertinent during pregnancy, compared to a non-pregnant state. Together with the placental transfer of FT4 but not TSH, this could explain stronger associations of FT4 with placental hemodynamics compared to the associations with TSH observed in this study.

Alternatively, we can speculate that the small effects of TSH we observed could be a physiological reflection of thyrotropin releasing hormone effects, which is shown to be present in substances released by placental tissue [46,47]. Those effects might be timely regulated and pointed towards the control of the fetal hypothalamo-pituitary-thyroid axis formation occurring in early pregnancy. The potential transient nature of this phenomenon could also explain the transient associations we observed between TSH and placental function measurements.

It is known that pregnancies with overt hyperthyroidism, mostly caused by TSH receptor stimulating antibodies in the context of Graves' disease, are more likely to be complicated with both maternal and fetal morbidity, including premature delivery, placenta abruptio and fetal growth restriction [48]. Furthermore, even high-normal FT4 concentration is shown to be associated with higher risk of pre-eclampsia [19] and fetal growth restriction [21,22]. Our results might suggest that the effects of TH on these outcomes are partially explained by the effects of high thyroid function on the placental function. Based on the performed mediation analysis, we observed that 10.4% and 12.5% of the association of TH with pre-eclampsia and birth weight could be mediated via changes in placental function, respectively. However, these effects are relatively small and were not consistent across pregnancy outcomes or ultrasound measurements. This suggests that the association of TH with pregnancy outcomes is largely explained by either (1) direct effects of TH, or (2) through effects on potential mediators other than placental function, such as effects on fetal growth and development or more generalized effects on overall metabolic state. On one hand, this could be expected since TH responsive tissues are widespread and the range of TH effects includes regulation of many maternal and fetal tissues. Nonetheless, the analyses in the current study should not yet overrule a potentially important role of the placenta as an influential mediator, as the methods used in this study might be insufficient to accurately examine and estimate potential effects. Further studies are needed to verify our results. Of particular interest would be to re-analyze clinical trials that have investigated the effects of levothyroxine treatment. Little is known about the effects of TH on the placentation in vivo and/or in humans. To our knowledge, no study has investigated the association of gestational thyroid function with a measure of placental function in a clinical setting. We were able to study the association of maternal thyroid function in early pregnancy with subsequent measures of placental function prospectively, in a large number of women and also at the two different time-points of gestation.

A potential limitation of this study is that only a single TH measurement was available and therefore we were not able to assess the association of TH changes during gestation with placental function. However, longitudinal studies in pregnant women have shown a relatively low intra-individual variation of TH during the course of gestation [49]. Another potential limitation is differential missingness of placental ultrasound measurements, which may have introduced bias. This was particularly the case for the mediation analyses on pre-eclampsia for which the missingness led to a large proportion of missing data of the outcome. However, we coped with this by imputing variables with missing data in the datasets used for mediation analyses. Finally, the observational nature of this study does not allow for inference of causality and does not preclude the existence of residual confounding.

In conclusion, our data demonstrate that high thyroid function in early pregnancy is associated with measures of placental vascular function in both maternal and fetal compartment during the second and third trimester. The underlying mechanism of these associations may involve TH effects on the key growth factors and cytokines included in early placentation. Further research is necessary to investigate the biological mechanism by which maternal TH affects placental function and to further translate the findings from in vitro studies into clinically relevant associations.

Supplemental Table 1. Descriptive Statistics of the Participants – Non response analysis

Characteristic	Value (response)	Value (non-response)	P value
TSH, median (95%range), mU/l,	1.34 (0.04-4.49)	1.47 (0.06-4.52)	0.2
FT4, median (95%range), pmol/l	14.7 (10.2–22.2)	14.6 (9.4-22.6)	0.7
Gestational age at blood sampling, media median (95%range) median (95% range), weeks	13.4 (9.7-17.6)	14.2 (10.5-17.8)	<0.001
Age, mean ±sd, years	29.7±5.0	29.2 ±5.2	0.02
BMI, median, (95% range), kg/m2	23.5 (18.5-35.6)	24.2 (18.6-38.3)	<0.001
Parity, n (%)			0.12
Nullipara	3057 (57.5)	270 (53.9)	
Primipara	1589 (29.9)	147 (29.3)	
Multipara	669 (12.6)	61 (12.2)	
Smoking status, n (%)			0.4
Non smokers	3821 (71.9)	360 (71.9)	
Stopped smokers	505 (9.5)	37 (7.4)	
Smokers	989 (18.6)	104 (20.8)	
Educational level, n (%)			<0.01
No education or primary education	587 (11.0)	91 (18.2)	
Secondary education	2429 (45.7)	227 (45.3)	
Higher education	2299 (43.3)	153 (36.6)	
Ethnicity, n (%)			<0.01
Dutch	2726 (51.3)	238 (47.5)	
Moroccan	350 (6.6)	37 (7.4)	
Turkish	425 (8.0)	24 (4.8)	
Surinam	477 (9.0)	53 (10.6)	
Dutch Antilles	173 (3.3)	18 (3.6)	
Asian	148 (2.8)	15 (3.0)	
Other – Western	477 (9.0)	65 (13.0)	
Other – Non-Western	539 (10.1)	51 (10.2)	
Fetal sex, n (%)			0.7
Male	2667 (50.2)	250 (49.9)	
Female	2648 (49.8)	251 (50.1)	

Supplemental Table 2. Association of maternal thyroid function with placental hemodynamic function

	Umbilical artery PI in the 2nd trimester	Uterine artery RI in the 2nd trimester	Umbilical artery PI in the 3rd trimester	Uterine artery RI in the 3rd trimester
log TSH	-0.017 (-0.030, -0.004) *	0.667 (-0.007, 0.008)	0.008 (-0.004, 0.021)	-0.001 (-0.016, 0.014))
log FT4	0.035 (0.005, 0.065) *	0.026 (0.009, 0.043) *	0.034 (0.006, 0.060)*	0.001 (-0.006, 0.008)

Values are regression coefficients (95% confidence intervals) from the multivariate linear regression analyses. Models were adjusted for gestational age at blood sampling, gestational age at ultrasound, smoking, BMI and fetal sex

* P<0.05

† P<0.001

Supplemental Table 3. Association of maternal thyroid function with the risk of notching in the 3rd trimester

	OR (95% CI)
log TSH	1.062 (0.978, 1.153)
log FT4	1.050 (1.021, 1.080) †

Values are odds ratios (95% confidence intervals) from the multivariate logistic regression analyses. Models were adjusted for gestational age at blood sampling, gestational age at ultrasound, smoking, BMI, maternal age, ethnicity, parity and fetal sex

* P<0.05

† P<0.001

Supplemental Table 4. The mediation role of placental function in the association of thyroid function with gestational outcomes (imputed data)

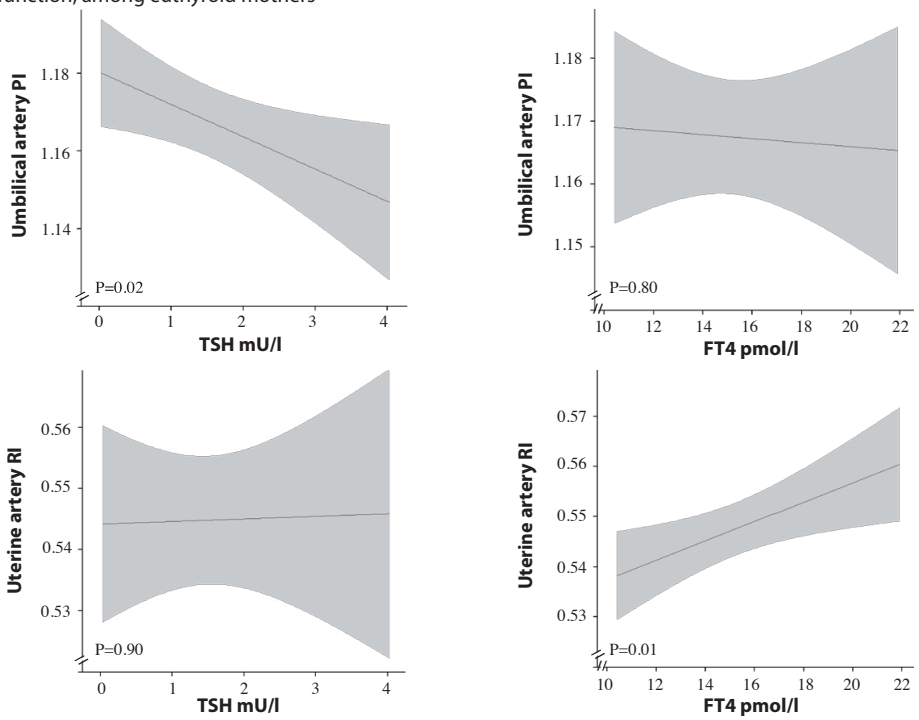
Preeclampsia (FT4)	Total effect	P value	Direct effect	**Mediated effect**	P value	Percentage of mediated effect
UMPI 3 †	0.0143	0.05	0.0141	0.0002	0.53	2.0
UTRI 2 ‡	0.0149	0.03	0.0138	0.0020	0.02	10.4
Birth weight (FT4)	Total effect	P value	Direct effect	**Mediated effect**	P value	Percentage of mediated effect
UMPI 2*	-0.0143	0.00	-0.0140	-0.0003	0.51	2.0
UMPI 3	-0.0146	0.00	-0.0139	-0.0008	0.26	5.0
UTRI 2	-0.0143	0.00	-0.0125	-0.0018	0.00	12.5
Notching	-0.0149	0.01	-0.0139	-0.0010	0.68	6.8
Premature delivery (Hypothyroxinemia)	Total effect	P value	Direct effect	**Mediated effect**	P value	Percentage of mediated effect
UTRI 2	0.0824	0.02	0.0811	0.0033	0.66	2.8

* UMPI 2 – umbilical artery pulsatility index in the 2nd trimester

† UMPI 3 – umbilical artery pulsatility index in the 3rd trimester

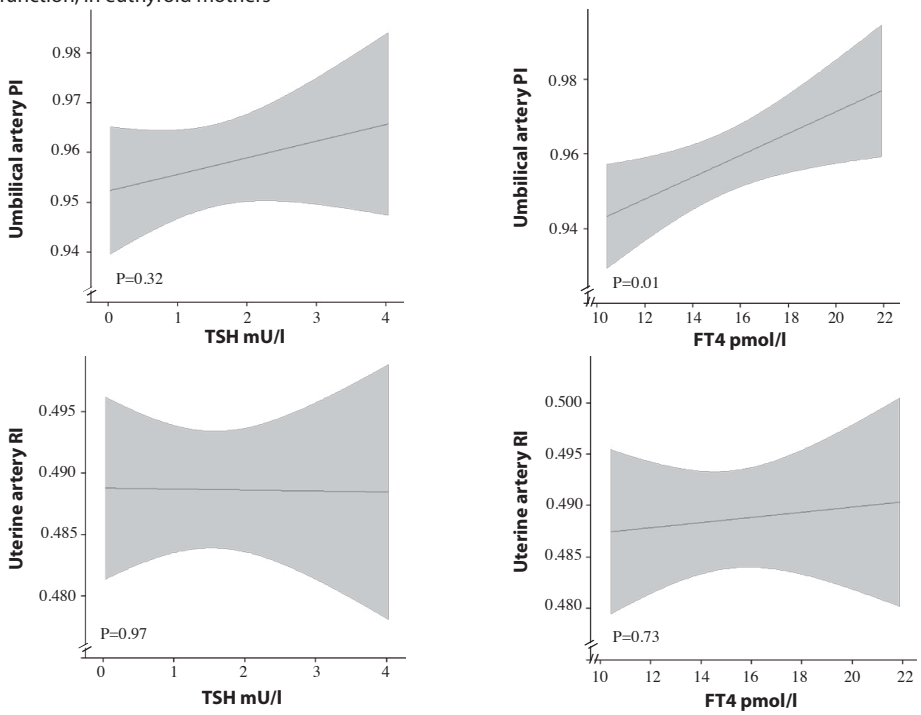
‡ UTRI 2 – uterine artery resistance index in the 2nd trimester

Supplemental Figure 1. The association of maternal thyroid function with the 2nd trimester placental function, among euthyroid mothers



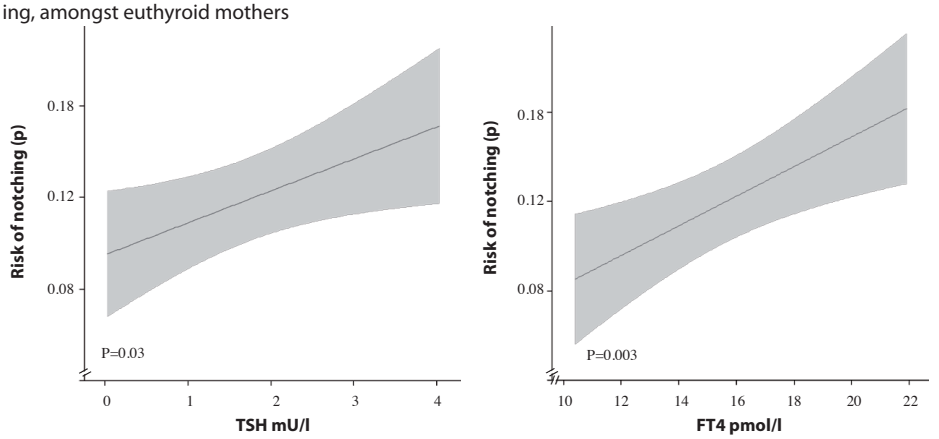
Plots show the linear regression models for FT4 and TSH and the resistance indices of the umbilical and uterine artery in the second trimester of pregnancy, as predicted mean with 95 percent confidence interval. Analyses were adjusted for gestational age at blood sampling, gestational age at ultrasound, smoking, BMI and fetal sex.

Supplemental Figure 2. The association of maternal thyroid function with the 3rd trimester placental function, in euthyroid mothers



Plots show the linear regression models for FT4/TSH and uterine artery RI, as predicted mean with 95 percent confidence interval. Analyses were adjusted for gestational age at blood sampling, gestational age at ultrasound, smoking, BMI and fetal sex.

Supplemental Figure 3. The association of maternal thyroid function with the risk of uterine artery notching, amongst euthyroid mothers



Plots show the logistic regression models for TSH and FT4 and the risk of 3rd trimester notching in the uterine artery, as predicted mean with 95 percent confidence interval. Analyses were adjusted for gestational age at blood sampling, gestational age at ultrasound, smoking, BMI, ethnicity, parity and fetal sex.

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CHAPTER 2.2

The association of thyroid function with maternal and neonatal homocysteine concentrations

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ABSTRACT

Context: High homocysteine concentrations are associated with maternal pregnancy complications and low birth weight, jaundice and cerebrovascular accidents in neonates. Thyroid hormone may interfere with homocysteine metabolism via stimulation of vitamin B12- and folate-dependent processes and via effects on enzymes of the remethylation pathway.

Objective: Investigating the associations of maternal and neonatal thyroid function with homocysteine during pregnancy and after delivery, respectively.

Design, setting and participants: Within Generation R study, a population-based prospective cohort, we studied the associations of maternal and neonatal TSH and FT4 with homocysteine, folate and vitamin B12 concentrations using multiple linear regression analyses.

Main outcome measures: Thyroid stimulating hormone (TSH), free thyroxine (FT4), homocysteine, folate and vitamin B12 concentrations were determined in early pregnancy (<18 weeks; N=1094 women without folic acid supplementation) and in cord blood of 4475 neonates.

Results: In neonates, there was a positive association of FT4 with homocysteine and an inverse association of TSH with homocysteine. The associations attenuated after adjustment for folate and vitamin B12 concentration (β change for FT4: 0.00559 ±0.001, $P<0.0001$ to 0.00310 ±0.001, $P=0.015$; and for TSH: -0.00165 ±0.001, $P=0.005$ to -0.00086 ±0.001, $P=0.11$). In mothers, there was a positive association of FT4 with homocysteine ($P=0.026$) but no association of FT4 with folate or vitamin B12 ($P\geq0.08$).

Conclusion: Higher thyroid function is associated with higher homocysteine concentrations in pregnant women and in neonates. These data provide new insights into the effects of thyroid hormone on folate- and vitamin B12-dependent processes during early growth and development.

INTRODUCTION

High concentrations of homocysteine, a non-protein amino acid, are associated with adverse coronary, cerebrovascular and maternal pregnancy-specific outcomes [1-6]. In neonates, high homocysteine is associated with low birth weight, jaundice and cerebrovascular accidents [7-9]. In adults, high homocysteine may affect reproductive function and fertility [10] and is associated with neurological disorders [11]. High homocysteine concentrations may have a role in early pregnancy loss, preeclampsia, premature delivery, placental abruption, delayed embryonic development and congenital birth defects [2-6,12].

Homocysteine also serves as a marker of folate (vitamin B9) and vitamin B12 (cobalamin) since conversion of homocysteine to methionine occurs through a re-methylation pathway which requires folate and vitamin B12 [13]. A deficiency of these vitamins negatively affects re-methylation cycle resulting in higher homocysteine concentrations [13-16]. Folate and vitamin B12 play a key role in important metabolic processes, including DNA and RNA synthesis, amino acid metabolism, cell proliferation and erythropoiesis [14,15,17]. During fetal development and in the neonatal period, these metabolic and proliferative processes are particularly intense [18].

Thyroid hormone may affect homocysteine metabolism via two potential mechanisms. First of all, thyroid hormone stimulates folate- and vitamin B12-dependent biochemical processes, including DNA synthesis [19,20], amino acid metabolism [21], cell proliferation [22,23] and erythroblast differentiation [24-26]. A higher thyroid function and a consequent stimulation of these folate- and vitamin B12-consuming metabolic processes may lead to higher homocysteine concentrations, as has been suggested previously [27,28]. Secondly, animal studies indicate that thyroid hormone affects the activity of methylenetetrahydrofolate reductase (MTHFR) and methionine synthase, enzymes required for re-methylation of homocysteine [28]. Although some studies in humans showed that thyroid dysfunction is associated with high homocysteine concentrations [29-31], others did not [32,33] and population-based studies are lacking.

There is a high demand for folate and vitamin B12 in the developing fetus [18]. For that reason, folic acid/multivitamin supplementation is advised from the periconceptional period onwards to prevent neural tube defects [34]. Still, high demands for folate and vitamin B12 continue in the neonatal period [18] and determinants of homocysteine metabolism in this period might indirectly have a role in the pathophysiology of clinical events associated with high homocysteine [8,9]. We hypothesized that thyroid function may be a determinant of homocysteine concentration during gestation and in the neonatal period.

The aim of this study was to investigate the association of thyroid function with homocysteine, folate and vitamin B12 concentrations in pregnant women and in neonates and to explore which of the two potential pathways may play a role.

METHODS

Study population

This study was embedded in Generation R, a population-based prospective cohort from early fetal life onwards, in Rotterdam, the Netherlands [35]. The study was designed to identify early environmental and genetic causes leading to normal and abnormal growth, development, and health during fetal life and childhood [35]. In total, 7069 mothers with expected delivery date between April 2002 and January 2006 were enrolled during early pregnancy. Thyroid stimulating hormone (TSH) and free thyroxine (FT4) were determined in the first available serum sample during early pregnancy (<18 weeks) in 5859 women, from which women with thyroid disease or thyroid (interfering) medication, twin pregnancies and /or IVF were excluded from the analysis (N=88, N=64, N=24, respectively; Figure 1). From these women, 5683 had homocysteine, folate or vitamin B12 concentration measurements available (Figure 1). Furthermore, a subgroup of women (N=1094; Figure 1) did not take folic acid supplementation during pregnancy. From the total population of women, 4968 neonates had available cord blood TSH and FT4 measurements available, from which 4475 had data on cord blood homocysteine, folate or vitamin B12 concentration available (Figure 1). Written informed consent was obtained from all participants. The general design, research aims and specific measurements in The Generation R study have been approved by the Medical Ethical Committee of the Erasmus Medical Center, Rotterdam.

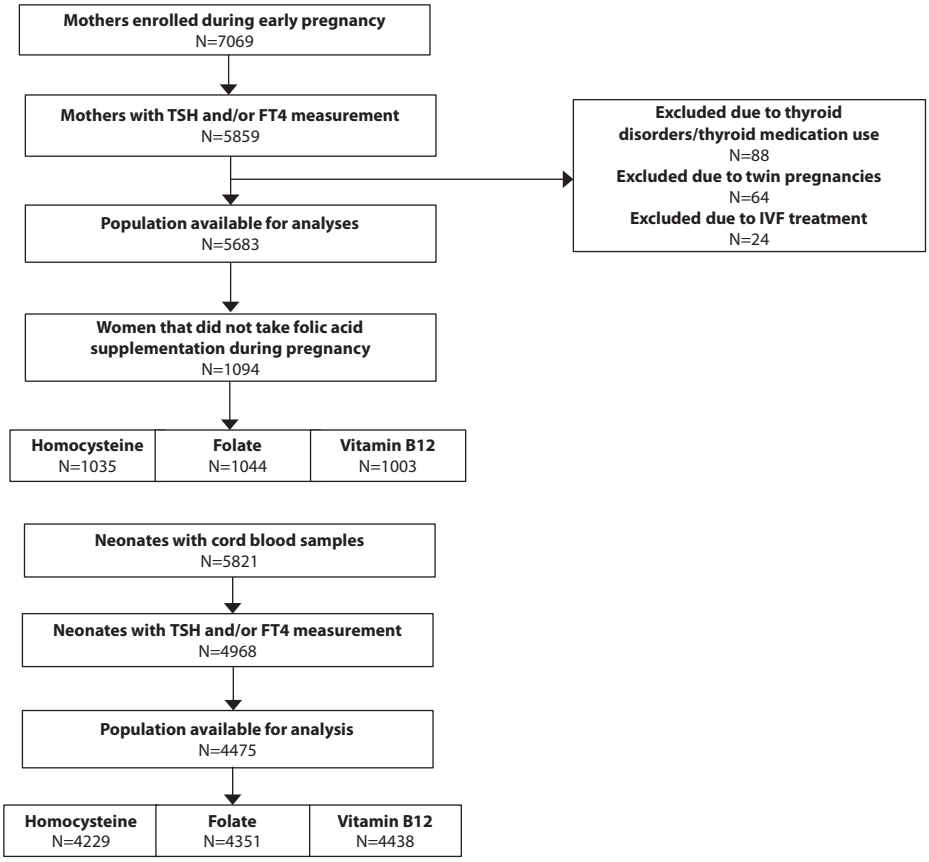
Thyroid function measurements

Maternal serum samples were obtained in early pregnancy (median 13.2 weeks, 95% range 9.7 – 17.6 weeks) and cord blood samples were obtained directly after birth (median gestation age at birth 40.1 week, 95% range 36.6 –42.3 weeks). Plain tubes were centrifuged and serum was stored at -80°C. TSH and FT4 concentrations in maternal and cord blood serum samples were determined using chemiluminiscence assays (Vitros ECI; Ortho Clinical Diagnostics). The intra- and interassay variation coefficients were < 4.1% for TSH at a range of 3.97–22.7 mU/ml and <5.4% for FT4 at range of 14.3 – 25.0 pmol/l.

Homocysteine, folate and vitamin B12 measurements

Maternal and cord blood samples were obtained at the same time as for thyroid function measurements. Total homocysteine, folate and total vitamin B12 concentrations were measured using a chemiluminiscence immunoassay on the Architect System (Abbott Diagnostics B.V.) with a between-run variation coefficient ranging from 1.5 to 8.9%. The analytic ranges for homocysteine, folate, and vitamin B12 were 1–50 µmol/l, 1.8–45.3 nmol/l and 44–1476 pmol/l, respectively.

Figure 1. Flowchart showing selection procedure of the study population



Folic acid supplement intake during pregnancy

Information on folic acid supplement use (0.4 – 0.5 mg) and the time of supplement initiation was obtained by questionnaires at study enrollment. We categorized women into 3 groups: (1) women with no use of supplements, (2) women that started the use during first 10 weeks of pregnancy and (3) women that started the use periconceptually. The percentage of available data for folic acid supplementation during pregnancy was 75.1%.

MTHFR single nucleotide polymorphism (SNPs)

DNA was extracted from white blood cells obtained from early pregnancy blood samples in mothers and neonates at birth and C677T (rs1801133) and A1298C (rs1801131) were genotyped. In mothers, genotyping was performed using a Taqman allelic discrimination assay (Applied Biosystems) and Abgene QPCR Rox mix (Abgene, Hamburg, Germany). The genotyping reaction was amplified by using the GeneAmp PCR system 9600

(95°C for 15 min), then 40 cycles of 94°C (15s) and 60°C (1 min). The fluorescence was detected on 7900 HT Fast Real-Time PCR System (Applied Biosystems) and individual genotypes were determined by using SDS software (version 2.3; Applied Biosystems)[36]. The genotype frequencies of MTHFR C677T were 31.8% (CC), 25.1% (TC) and 5.9% (TT) and A1298C were 30.3% (AA), 26.6% (CA) and 5.9% (CC). In neonates, genotype data were extracted from an imputed genomewide association scan (HapMap phase II release 22). The genotype frequencies of MTHFR C677T were 47.0% (CC), 38.2% (TC) and 8.3% (TT) and A1298C were 47.1% (AA), 37.5% (CA) and 9.7% (CC). The percentage of missing data for MTHFR SNPs was 47% in the maternal data (subgroup of women that did not take folic acid supplements) and 6.4% in the neonatal data.

Covariates

Information on ethnicity, educational level and smoking status was obtained by questionnaires at study enrollment. Ethnicity was determined by the country of origin and was defined according to the classification of Statistics Netherlands [35]. Maternal smoking was defined as no smoking, smoking until known pregnancy and continued smoking during pregnancy. Information on parity and sex of the child was obtained from hospital registries. BMI was measured at study enrollment.

Statistical analyses

The association of maternal and neonatal TSH and FT4 with homocysteine, folate and vitamin B12 concentrations was investigated using multiple linear regression analyses with restricted cubic splines utilizing three knots, to account for possible non-linear associations. Homocysteine, folate and vitamin B12 values were logarithmically transformed in order to achieve normal distribution of the residuals of the regression models. Multivariable associations were graphically depicted by plots and β estimates with 95% confidence intervals are shown in Supplemental Tables 4 and 5. In order to identify associations that are more likely to reflect normal biology and to exclude the effect of folic acid supplements on the associations, main analysis with maternal data was performed in the subgroup of women that did not take any folic acid supplements during pregnancy. In order to examine whether the association of thyroid function with homocysteine, folate or vitamin B12 differs based on MTHFR enzyme activity, we tested for interaction by adding product terms of TSH or FT4 and single nucleotide polymorphism (SNP) of MTHFR (rs1801133 and 1801131) to the models studying both mothers and neonates.

The mediator role of gestational homocysteine concentration in the association of thyroid function with birth weight (described previously [37]) was investigated by performing a mediation analysis using the approach of Imai et al. [38].

All model covariates were selected based on biological plausibility, change of the effect estimate of interest or residual variability of the model. The analyses were adjusted

for gestational age at blood sampling, ethnicity, maternal age, educational level, parity and smoking status in the maternal models and for birth weight standardized to gestational age, ethnicity, maternal age, educational level, parity and smoking in the neonatal models.

We performed multiple imputation according to Markov Chain Monte Carlo method for covariates with missing data [39]. Before imputation of the missing values, we performed exploratory analyses by investigating the pattern of missingness for each variable. All variables showed random missingness pattern. In the maternal data, the percentage of missing data was less than 1% for BMI and parity, 4% for ethnicity, 7.1% for education level and 11.4% for smoking. In the neonatal data, the percentage of missing data was less than 1% for parity and maternal age, 3.6% for ethnicity, 6.7% for maternal education, 2.0% for birthweight and 10.8% for smoking. Five imputed datasets were created and pooled for analysis. Maternal BMI, parity, educational level, ethnicity and smoking were then added to the maternal models and maternal age, parity, smoking, education level, neonatal birth weight and ethnicity were added to the neonatal models. Furthermore, we added TSH, FT4 concentrations as well as homocysteine, folate, and vitamin B12 concentrations as prediction variables only. There were no statistically significant differences in descriptive statistics between the original and imputed datasets.

Statistical analyses were performed using Statistical Package of Social Sciences version 21.0 for Windows (SPSS Inc. Armonk, NY) and R statistical software version 3.2.1 (package 'rms' and 'mediation').

RESULTS

After exclusions, the final study population consisted of 5683 women, of which 1094 women did not use pre- or postconceptional folic acid supplements, and 4475 neonates (Figure 1). Descriptive statistics are shown in Table 1 (descriptives for the total group of women are shown in Supplemental Table 1).

Mothers (non-supplemented)

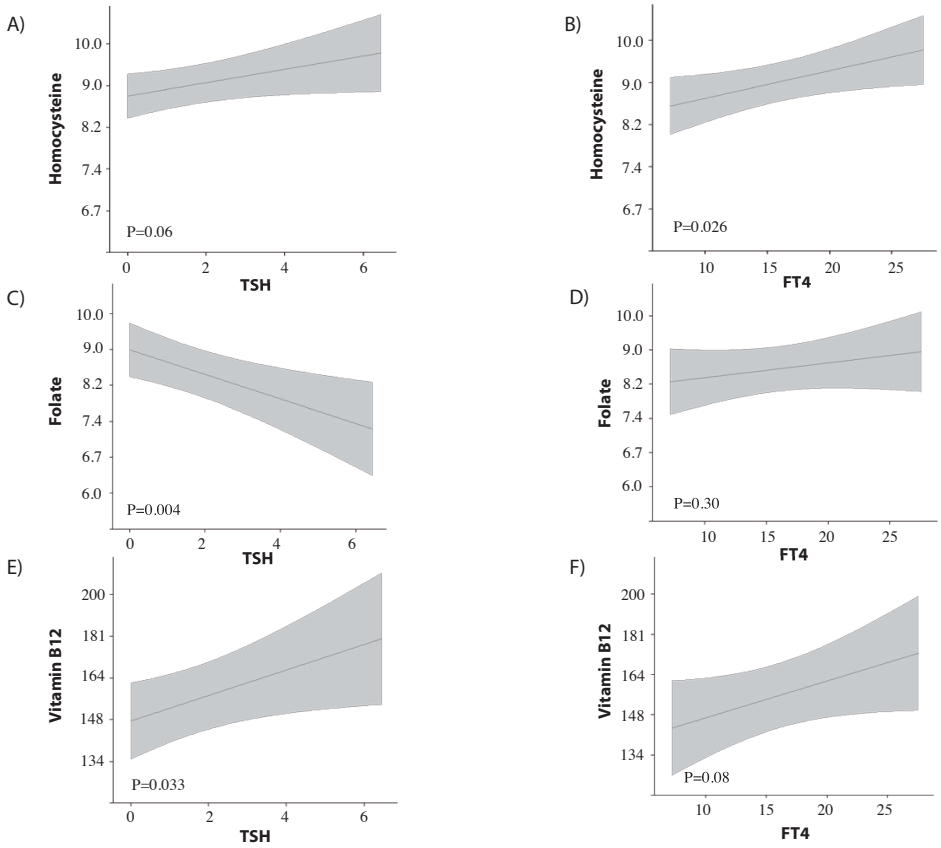
There was no statistically significant association of TSH with homocysteine (Figure 2A, *P*=0.06), whereas there was a positive association of FT4 with homocysteine (Figure 2B, *P*=0.026). There was an inverse association of TSH with folate (Figure 2C, *P*=0.004), whereas there was no association of FT4 with folate (Figure 2D, *P*=0.30). There was a positive association of TSH with vitamin B12 (Figure 2E, *P*=0.033) whereas there was no statistically significant association of FT4 with vitamin B12 (Figure 2F, *P*=0.08). The association of thyroid function with homocysteine did not change after adjustment for folate and vitamin B12 concentrations (data not shown). The results remained similar

after adjustment for TPO antibodies or hCG concentrations and did not differ according to MTHFR SNPs (Supplemental Table 2).

Table 1. Descriptive Statistics of the Participants

Characteristic - mothers	Value	Characteristic - neonates	Value
TSH, median (95%range), mU/l	1.3 (0.03-3.96)	**TSH, median (95% range), mU/l**	9.12 (3.35-32.9)
FT4, median (95%range), pmol/l	14.5 (10.2-22.6)	**FT4, median (95% range), pmol/l**	20.5 (15.4-28.5)
Homocysteine, median, (95%range), µmol/l	7.4 (4.6-15.0)	**Homocysteine, median (95% range) µmol/l**	9.1 (5.2-16.5)
Folate, median, (95%range), nmol/l	8.6 (4.7-21.3)	**Folate, median (95% range), nmol/l**	20.4 (10.4-38.4)
Vitamin B12, median, (95%range), pmol/l	157.0 (61.1-421.3)	**Vitamin B12, median (95% range), pmol/l**	300.0 (120.0-900.0)
Gestational age at blood sampling, median (95%range), weeks	13.9 (9.6-17.8)	**Gestational age at birth, median (95% range), weeks**	40.1 (36.6-42.3)
Age, mean ±sd , years	27.8 ±5.6	**Birth weight, mean ± sd, g**	3463.9 ±502.9
BMI, median, (95% range), kg/m2	23.5 (18.5-35.7)	**Gender, n (%)**	
Parity, n (%)		Males	2272 (50.8)
Nullipara	499 (45.6)	Females	2202 (49.2)
Primipara	334 (30.5)	**Ethnicity**	
Multipara	261 (23.9)	Dutch	2704 (55.0)
Smoking status, n (%)		Moroccan	354 (7.2)
Non smokers	756 (69.1)	Turkish	400 (8.1)
Stopped smokers	77 (7.0)	Surinamese	377 (7.7)
Smokers	261(23.9)	Asian	150 (3.1)
Educational level, n (%)		Other - European	355 (7.2)
No education or primary education	273 (25.0)	Other – Non-European	573 (11.7)
Secondary education	615 (56.2)		
Higher education	206 (18.8)		
Ethnicity, n (%)			
Dutch	231 (21.1)		
Moroccan	163 (14.9)		
Turkish	188 (17.2)		
Surinamese	156 (14.3)		
Asian	51 (4.7)		
Other – European	82 (7.5)		
Other – Non-European	223 (20.4)		

Figure 2. The association of maternal thyroid function with homocysteine, folate and vitamin B12 concentrations



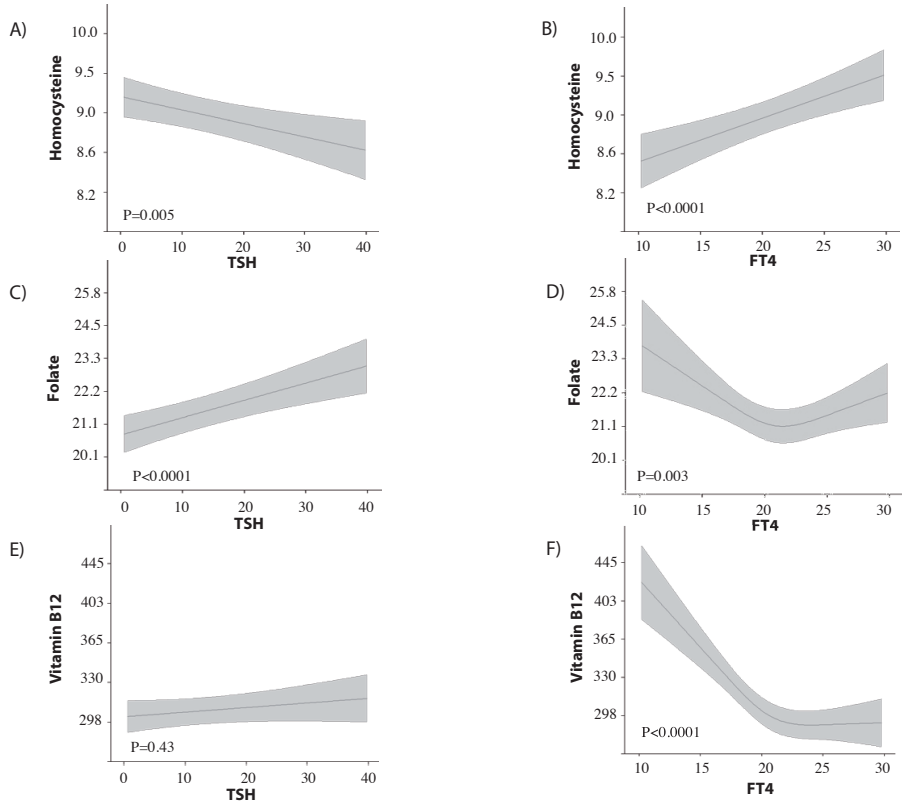
Plots show the linear regression models for the association of TSH and FT4 with folic acid, vitamin B12 and homocysteine concentrations, as predicted mean with 95 percent confidence interval in the group of mothers that did not take folic acid supplements anytime during pregnancy. Analyses were adjusted for gestational age at blood sampling, fetal sex, ethnicity, maternal age, educational level, parity and smoking status.

The associations of TSH or FT4 with homocysteine, folate and vitamin B12 for the total group of women, irrespective of folic acid supplementation status, are shown in Supplemental Figure 1.

Neonates

There was an inverse association of TSH with homocysteine (Figure 3A, P=0.005) and a positive association of FT4 with homocysteine (Figure 3B, P<0.0001). There was a positive association of TSH with folate (Figure 3C, P<0.0001) and an inverse association of FT4 with folate (Figure 3D, P=0.003). There was no association of TSH with vitamin B12 (Figure 3E, P=0.43) and there was an inverse association of FT4 with vitamin B12 (Figure 3F, P<0.0001).

Figure 3. The association of neonatal thyroid function with homocysteine, folate and vitamin B12 concentrations



Plots show the linear regression models for the association of TSH and FT4 with folic acid, vitamin B12 and homocysteine concentrations, as predicted mean with 95 percent confidence interval in the neonates. Analyses were adjusted for birth weight standardized for gestational age, fetal sex, ethnicity, maternal age, educational level, parity and smoking status.

The association of TSH with homocysteine attenuated after adjustment for folate and vitamin B12 (beta estimate change: -0.00165 ±0.00058 (*P*=0.005) to -0.00086 ±0.00054 (*P*=0.11)). Similarly, the association of FT4 with homocysteine attenuated after adjustment for folate and vitamin B12 (beta estimate change: 0.00559 ±0.00138 (*P*<0.0001) to 0.00310 ±0.00127 (*P*=0.015)). Taken together, this indicates that in neonates, roughly 48-55% of the association of thyroid function with homocysteine is mediated through changes in folate and vitamin B12.

Although there was a positive association of maternal homocysteine concentrations with neonatal homocysteine concentrations (data not shown), the results remained similar after adjustment for maternal homocysteine, folate or vitamin B12 concentrations, maternal folate supplementation status and did not differ according to differences in neonate MTHFR SNPs (Supplemental Table 2).

Of all covariates in the models, gestational age at blood measurement and ethnicity had the largest effect on the association studied (up to 36% and 27% change in the effect estimate, respectively).

The mediator role of homocysteine in the association of maternal thyroid function with birth weight

Mediation analysis showed that the effect of FT4 on birth weight was in part mediated through effects of FT4 on homocysteine concentrations (13.3% of mediated effect, $P<0.001$; Supplemental Table 3).

DISCUSSION

Our data show an association of thyroid function with homocysteine concentrations in pregnant women and in neonates. To our knowledge, this is the first study that investigates the associations of both maternal and neonatal thyroid function with homocysteine, folate and vitamin B12 concentrations in a population-based setting. In pregnant women, higher FT4 concentrations were associated with higher homocysteine. In neonates, higher FT4 and lower TSH were associated with higher homocysteine concentrations and this was mediated by the effects of thyroid function on folate and vitamin B12 concentrations.

There is a paucity of population-based studies investigating the association of thyroid function with homocysteine. Some clinical studies suggest that hypothyroid patients have higher homocysteine concentration compared to euthyroid controls [29,30]. Conversely, in patients with thyrotoxicosis, a positive association of FT4 with homocysteine is reported [31]. However, due to differences in determinants of homocysteine as well as potential pathological influences in diseased and/or elderly populations, the previous findings are ungeneralizable to women in pregnancy or to children.

We demonstrate that in neonates, higher FT4 and lower TSH concentrations are associated with higher homocysteine concentrations. This suggests that a thyroid hormone-mediated stimulation of metabolic effects may lead to lower availability and/or activity of factors necessary for homocysteine conversion or degradation. In neonates, higher thyroid function was also associated with a lower folate and vitamin B12 concentration, in line with the notion of depleting effects of higher thyroid function on folate and vitamin B12 concentrations [27,28,31]. This hypothesis is further supported by the attenuation of the association of thyroid function with homocysteine by 48-55% after adjustment for folate and vitamin B12 concentration. The growth of fetal tissues, especially in the last trimester, requires intense cell proliferation that is dependent on folate and vitamin B12 concentrations [18]. The most important processes that depend on folate and vitamin

B12 consumption include DNA synthesis, amino acid metabolism and erythroblast differentiation [19-26]. Animal data show that thyroid hormone is essential for adequate erythropoiesis especially at birth [40], in line with a progressive increase in erythrocyte content throughout gestation and the highest values at birth [41]. This is in line with the timing of our thyroid function measurement and might in part explain the observed differences between maternal and neonatal analyses.

Animal studies show that hyperthyroidism leads to higher homocysteine concentrations [42-44] and thyroid hormone dependent effects on methionine synthase activity have been suggested [28]. In turn, this enzyme is vitamin B12 dependent [28]. As such, higher thyroid hormone concentrations may affect the re-methylation cycle via lowering of vitamin B12 and or/folate availability, as well as via direct interference with the enzymes of the re-methylation cycle. However, our analyses regarding genetic variance in the MTHFR gene did not suggest that MTHFR enzyme is implicated in such mechanisms. Future studies are required to further elucidate pathways through which thyroid hormone affects the metabolic cycle of homocysteine.

In mothers, higher FT4 concentrations were also associated with higher homocysteine concentrations. However, opposite to the results in neonates, this association could not be explained by the relation of FT4 with folate and vitamin B12. This difference between pregnant women and neonates could be explained by various factors. Firstly, diet and vitamin supplements during pregnancy may interfere with homocysteine metabolism [13]. To minimize the latter effect, we focused only on women that were not taking folic acid supplements. However, no data were available on vitamin B12 supplementation or other vitamin supplementation. Furthermore, it is likely that some women would use combined (pre)conceptional multivitamin formulas and the availability of our data on supplement usage leaves the possibility of data misclassification. In contrast, the associations in neonates might potentially be less affected by such factors and might represent a better model for studying this association. Secondly, beside the re-methylation to methionine, a significant portion of homocysteine is actually converted to cystathionine via vitamin B6-dependent process [13]. The positive association of FT4 with homocysteine in mothers could potentially be due to a greater effect of thyroid hormone on homocysteine conversion to cystathionine than on re-methylation to methionine. In line with this, lower vitamin B6 concentrations were observed in hyperthyroidism compared to euthyroid state [45]. Unfortunately, we do not have vitamin B6 concentrations available to further investigate this hypothesis. In addition, women with a higher general susceptibility to autoimmune disease may have both TPOAbs and gastric auto-antibodies, and the latter could affect vitamin B12 concentrations [46]. Therefore, we adjusted all models for TPOAbs, which did not result in a change in the effect estimates. This suggests that the observed association is unlikely to be confounded by or mediated via higher susceptibility to autoimmunity in general. Finally, hemodilution and hormonal changes in pregnancy

might complicate the interpretation of the studied associations with homocysteine [47,48]. Nonetheless, the positive association of maternal thyroid hormone with homocysteine concentration is worth of further investigation, especially considering its potential mediating role in the association of high thyroid hormone with lower birth weight we observed. Therefore, due to potential harmful effects of high homocysteine concentrations that may contribute to adverse pregnancy outcomes, clinical determinants of homocysteine concentrations may be helpful in predicting the risk of pregnancy complications. Future studies should confirm our findings regarding the association of high gestational thyroid hormone with high homocysteine concentrations and further investigate the extent of this effect and its potential role in pregnancy complications.

Our analyses showed that gestational age at blood sampling and ethnicity were the most important confounding variables, which indicates that future studies should take into account gestational age-related changes as well as potential genetic differences in thyroid function and homocysteine concentration.

A potential limitation of our study is the availability of a single TSH, FT4 and homocysteine measurement only. This did not enable us to investigate whether the observed associations change in time, particularly in later stages of gestation. However, during pregnancy, both thyroid function [49] and homocysteine concentrations [48] are subject to a relatively low intra-individual change, indicating that no large changes in the current associations can be expected. Another potential limitation of the current study is the fact that a relatively large proportion of pregnant women (47%) had missing data on MTHFR SNPs. Therefore, analyses might have been underpowered to detect potential relevant MTHFR-dependent differences in the association of thyroid function with homocysteine. However, MTHFR-dependent differences were not observed in neonates either, for which data on MTHFR SNPs were available in 93.6%. In addition, we cannot exclude the possibility that the difference in results between mothers and neonates is due to the lack of power to detect similar effects in our subset of women non-supplemented with folic acid. Finally, the observational nature of this study does not allow for inference of causality and does not exclude the existence of residual confounding.

In conclusion, our study demonstrates that higher thyroid function is associated with higher homocysteine concentration in pregnant women and in neonates. In neonates, the underlying mechanism of this association is likely to involve thyroid hormone mediated stimulation of metabolic processes that require folate and vitamin B12. In pregnant women, different mechanisms seem to underlie the effects of thyroid function on homocysteine metabolism. These data provide new insights into the effects of thyroid hormone on folate and vitamin B12-dependent biochemical processes during early growth and development. Further research is required to investigate the clinical significance of thyroid hormone dependent folate and/or vitamin B12 utilization, and to elucidate the potential role of thyroid function on vitamin B6 dependent pathway of homocysteine catabolism.

Supplemental Table 1. Descriptive Statistics of the Participants

Characteristic – mothers total group	Value
TSH, median (95%range), mU/l	1.35 (0.05-4.51)
FT4, median (95%range), pmol/l	14.7 (10.3-22.1)
Homocysteine, median, (95%range), µmol/l	6.9 (4.6-12.1)
Folate, median, (95%range), nmol/l	15.7 (5.5-37.3)
Vitamin B12, median, (95%range), pmol/l	169.0 (72.0-417.7)
Gestational age at blood sampling, median (95%range), weeks	13.2 (9.7-17.6)
Age, mean ±sd , years	29.7 ±5.0
BMI, median, (95% range), kg/m2	23.5 (18.5-35.7)
Parity, n (%)	
Nullipara	3096 (57.9)
Primipara	1574 (29.5)
Multipara	673 (12.6)
Smoking status, n (%)	
Non smokers	3821 (71.9)
Stopped smokers	505 (9.5)
Smokers	989 (18.6)
Educational level, n (%)	
No education or primary education	543 (10.2)
Secondary education	2254 (45.9)
Higher education	2346 (43.9)
Ethnicity, n (%)	
Dutch	2791 (52.2)
Moroccan	319 (6.0)
Turkish	429 (8.0)
Surinamese	480 (9.0)
Asian	138 (2.6)
Other – European	449 (8.4)
Other – Non-European	737 (13.8)
Folic acid supplement use, n (%)	
Not used	1094 (19.3)
Started during first 10 weeks	1386 (24.4)
Started periconceptionally	1844 (32.4)

Supplemental Table 2. Interaction terms of TSH and FT4 with MTHFR SNPs

	Interaction term	*P* value		
		Homocysteine	*Folate*	*Vitamin B12*
Mothers	TSH*rs1801133	0.05	0.47	0.83
	TSH*rs1801131	0.74	0.93	0.69
	FT4*rs1801133	0.42	0.59	0.88
	FT4*rs1801131	0.75	0.69	0.99
Newborns	TSH*rs1801133	0.86	0.78	0.21
	TSH*rs1801131	0.51	0.97	0.73
	FT4*rs1801133	0.81	0.03	0.92
	FT4*rs1801131	0.41	0.08	0.58

Supplemental Table 3. The role of homocysteine in the association of maternal thyroid function with birth weight.

Total effect	P value	Direct effect	Mediated effect	P value	Percentage of mediated effect
-0.036	0.00	-0.031	0.005	0.00	13.3

Supplemental Table 4. The association of maternal thyroid function with homocysteine, folate and vitamin B12 concentrations

	Log (homocysteine)	Log (folate)	Log (vitamin B12)
TSH	0.016 (-0.001, 0.032)	-0.034 (-0.057, -0.010)*	0.031 (0.003, 0.059)*
FT4	0.007 (0.001, 0.012)*	0.004 (-0.004, 0.012)	0.009 (-0.001, 0.019)

Values are beta coefficents (95% confidence intervals) from the multivariate linear regression analyses. Models were adjusted for gestational age at blood sampling, ethnicity, fetal sex, maternal age, educational level, parity and smoking status. Analyses show the associations

* P<0.05

** P<0.001

The estimated coefficients should be interpreted as:

1. An increase in one unit of TSH would result in 3.4% decrease in folate and 3.1% increase in vitamin B12
2. An increase in one unit of FT4 would result in 0.7% increase in homocysteine

Supplemental Table 5. The association of neonatal thyroid function with homocysteine, folates and vitamin B12 concentrations

	Log (homocysteine)	Log (folate)	Log (vitamin B12)
TSH	-0.002 (-0.004, -0.001)**	0.003 (0.001, 0.004)**	0.001 (-0.002, 0.003)
FT4	0.007 (0.004, 0.010)**	-0.011 (-0.003, -0.050)* 0.012 (0.000, 0.015)*	-0.041 (-0.063, -0.025)** 0.036 (-0.014, 0.004)

Values are beta coefficents (95% confidence intervals) from the multivariate linear regression analyses. Models were adjusted for for birth weight standardized for gestational age, fetal sex, ethnicity, maternal age, educational level, parity and smoking status.

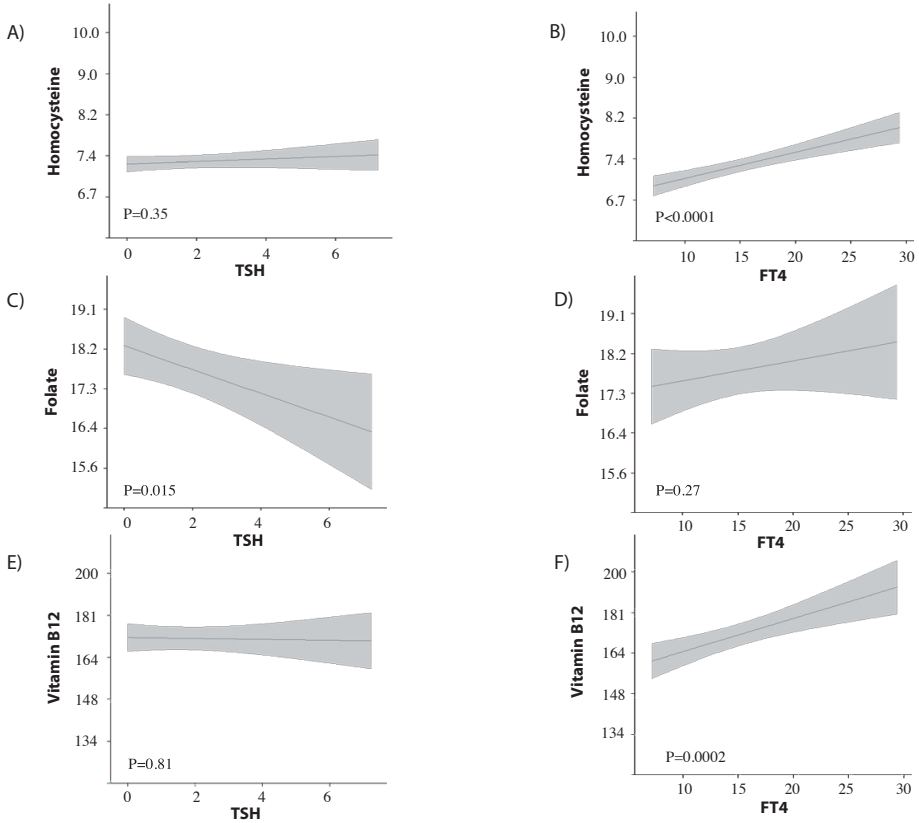
* P<0.05

** P<0.001

The estimated coefficients should be interpreted as:

1. An increase in one unit of TSH would result in 0.2% decrease in homocysteine and 0.3% increase in folate
2. An increase in one unit of FT4 would result in 0.7% increase in homocysteine and 1.1% decrease in folate and 4.1% decrease in vitamin B12

Supplemental Figure 1. The association of maternal thyroid function with homocysteine, folate and vitamin B12 concentrations in all mothers.



Plots show the linear regression models for the association of TSH and FT4 with folic acid, vitamin B12 and homocysteine concentrations, as predicted mean with 95 percent confidence interval irrespective of folic acid intake during pregnancy. Analyses were adjusted for gestational age at blood sampling, ethnicity, fetal sex, maternal age, educational level, parity and smoking status.

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CHAPTER 3

hCG and pregnancy outcomes

CHAPTER 3.1

Human chorionic gonadotropin (hCG) and the risk of pre-eclampsia

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Submitted

ABSTRACT

Background: Abnormal placentation in early pregnancy and disturbance in the balance between pro- and anti-angiogenic factors play a role in the pathogenesis of pre-eclampsia. Human chorionic gonadotropin **(**hCG) regulates placental development and angiogenesis and may affect the ratio of soluble fms-like tyrosine kinase 1 (sFlt-1) and placental growth factor (PlGF) in the serum. We investigated the association of total hCG concentration with the risk of pre-eclampsia.

Methods: In a large, population-based prospective cohort (N=7754), we measured total hCG and sFlt-1 and PlGF concentrations at 14.4 weeks (95% range 10.1 – 26.1 weeks); in mid-pregnancy (median 20.4 weeks, 95% range 18.5 – 23.5 weeks) a second measurement of sFlt-1 and PlGF was performed. Pre-eclampsia diagnosis was confirmed by a certified medical doctor reviewing hospital charts. We used multiple linear and logistic regression to study the association of hCG with sFlt-1/PlGF ratio and the risk of pre-eclampsia, respectively.

Results: A high hCG concentration was associated with a 1.5-2.7-fold higher risk of pre-eclampsia (P=0.0001), and with a higher sFlt-1/PlGF ratio during early pregnancy (P<0.0001). The first association attenuated by 40% after adjustment for early pregnancy sFlt-1/PlGF ratio (β estimate change: from 0.19 ±0.10; P=0.052, to: 0.12 ±0.10; P=0.22). The association of high hCG with the risk of pre-eclampsia was more dominant in pregnancies with a male fetus compared to pregnancies with a female fetus (4.0-fold higher risk vs 2.2-fold higher risk, respectively) .

Conclusion: High total hCG concentrations in early pregnancy are associated with a higher risk of pre-eclampsia. The effects of high hCG concentration on the balance between pro- and anti-angiogenic factors during pregnancy may have a role in the pathophysiology involved.

INTRODUCTION

Pre-eclampsia is a multisystem disorder defined by new-onset hypertension and proteinuria after the 20th week of pregnancy [1]. Pre-eclampsia occurs in 2-8% of pregnancies and is a major cause of maternal morbidity and adverse perinatal outcomes, accounting for 10-15% cases of maternal deaths worldwide [2]. In this life-threatening condition, maternal complications arise from excessive inflammation and endothelial damage to multiple organs, including the placenta [2]. The known risk factors for pre-eclampsia include nulliparity, age over 40 years and pre-existing conditions such as hypertension, renal diseases or diabetes [2].

Although the exact pathophysiological mechanism is unclear, it is postulated that an abnormal placentation in early pregnancy predisposes to pre-eclampsia [2] and that a disturbance in the balance between pro- and anti-angiogenic factors plays an important role [3,4]. Placental growth factor (PlGF), a member of vascular endothelial growth factor (VEGF) family and a pro-angiogenic factor, is expressed in the placenta throughout pregnancy [5]. Soluble fms-like tyrosine kinase 1 (sFlt-1) is a potent anti-angiogenic factor that binds VEGFs including PlGF, thereby inhibiting angiogenesis during pregnancy [6]. An abnormal serum sFlt-1/PlGF ratio, with low concentrations of PlGF and elevated concentrations of sFlt-1, often occurs in pre-eclamptic women and may be a marker of impaired placentation [7,8].

Human chorionic gonadotropin (hCG), a pregnancy-specific hormone produced by trophoblast cells, regulates progesterone production, implantation, uterine growth and immune cell function [9]. hCG also regulates placental development, angiogenesis and vasculogenesis, partially via effects on VEGFs [10-14]. Several studies suggest that high and/or low hCG concentration is a marker of subsequent clinical manifestation of pre-eclampsia [15-18]. However, considerable between-study discrepancies exist, such as gestational age of hCG measurement, specific hCG isoform that was assessed and specific pre-eclampsia phenotype analyzed [15-20]. To our knowledge, no study has investigated the potential role of early placental function markers that may predispose to pre-eclampsia, such as the sFlt-1/PlGF ratio, in the association of hCG with the risk of pre-eclampsia.

In the current study, we aimed to investigate the associations of total hCG concentrations with sFlt-1 and PlGF ratio, and the risk of subsequent pre-eclampsia, in a large population-based prospective cohort.

MATERIALS AND METHODS

Study population

This study was embedded in Generation R, a population-based prospective cohort from early fetal life onwards, in Rotterdam, the Netherlands [21]. This cohort was designed to study early environmental and genetic determinants of growth, development and health during fetal and postnatal life [21]. Eligible participants for this study were 8879 pregnant women with an expected delivery date between April 2002 and January 2006 that were enrolled in the cohort during pregnancy [21]. Women with in vitro fertilization treatment (N=38), twin pregnancies (N=91) or pre-existing hypertension (N=75) were excluded from the analysis. Total hCG was determined in all first available serum samples and these data were available in 7754 women [22].

hCG measurements

Total hCG (at 14.4 weeks, 95% range 10.1 – 26.1 weeks) was measured using a solid-phase two-site chemiluminescent immunometric assay, calibrated against WHO 3rd IS 75/537, on an Immulite 2000 XPi system (Siemens Healthcare Diagnostics, Deerfield, IL, USA). The assay detects serum intact hCG, hyperglycosylated hCG, serum nicked hCG, serum nicked hyperglycosylated hCG, serum asialo hCG, serum hCG free β-subunit and serum nicked hCG β. The inter assay variation coefficient was 8.0, 6.3 and 5.1% at the concentration of 9.7, 53.1 and 821.5 IU/L, respectively. hCG concentrations were standardized by calculating standard deviation scores adjusted to gestational age at blood sampling as described previously [22].

Pre-eclampsia

Pre-eclampsia diagnosis was confirmed by a certified medical doctor by reviewing hospital charts [23] and was defined according to the criteria of the International Society for the Study of Hypertension in Pregnancy: the development of a systolic blood pressure (BP) of 140 mm Hg or greater and/or a diastolic BP of 90 mm Hg or greater (at least two BP readings) after 20 weeks of gestation in a previously normotensive woman 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 24h urine collection containing at least 300 mg of protein) [1]

Angiogenic factor measurements

sFlt-1 and PlGF concentrations were obtained from EDTA samples drawn in early pregnancy (median 13.2 weeks, 95% range 9.6 – 17.6 weeks) and in mid-pregnancy (median 20.4 weeks, 95% range 18.5 – 23.5 weeks) and analyzed using a microparticle-enhanced immunoassay on the Architect System (Abbott Diagnostics BV, Hoofddorp). The

between-run coefficients of variation for sFlt-1 were 2.8% at 5.5ng/ml and 2.3% at 34.0 ng/ml. The coefficients for PlGF were 4.7% at 24 pg/ml and 3.8% at 113 pg/ml. Measurements were available for 70.8% and 87.5% women for the first and second time-point, respectively. For each measurement, we constructed a ratio of sFlt-1 and PlGF, defined as a clinical predictor factor of the risk of pre-eclampsia [7,8].

Covariates

Information on maternal age, educational level, ethnicity and smoking status was obtained by questionnaires during pregnancy. Ethnicity was determined according to the country of origin and defined according to the classification of Statistics Netherlands and maternal smoking was classified as no smoking, smoking until known pregnancy and continued smoking during pregnancy [21]. Information on in vitro fertilization treatment, parity and sex of the child was obtained from community midwifery and hospital registries.

Statistical analysis

The association of hCG with sFlt-1/PlGF ratio was investigated using multiple linear regression models. The association of hCG with pre-eclampsia (and pregnancy-induced hypertension) was investigated using multiple logistic regression analyses. We used restricted cubic splines utilizing three or four knots to assess possible nonlinearity. Subsequently, to further quantify the risk of pre-eclampsia, we calculated odds ratios for the highest and the lowest 5th, 10th and 15th percentile cutoffs for hCG concentration. Multivariable associations were graphically depicted by plots and β estimates with 95% confidence intervals are shown in Supplemental Table 2. We tested for effect modification by gestational age at blood sampling or fetal sex by introducing a product interaction term of hCG * gestational age at blood sampling or hCG * fetal sex to the model and a *P* value of <0.15 was considered for stratification.

All model covariates were selected based on biological plausibility, change in the effect estimate or residual variability of the model. All analyses were adjusted for maternal age, ethnicity, BMI, parity, educational level, smoking status and fetal sex.

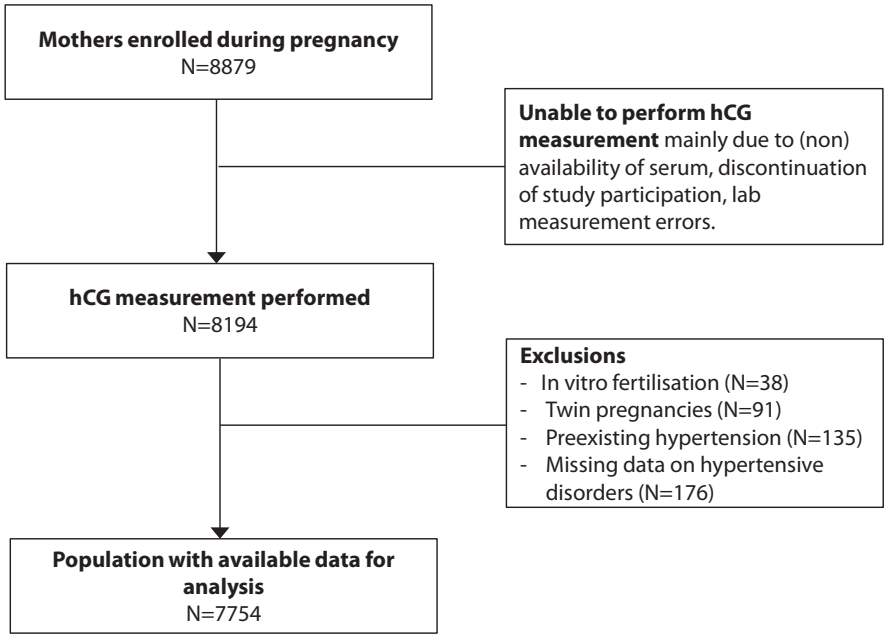
In order to cope with missing data on the covariates, we performed multiple imputation according to the Markov Chain Monte Carlo method [24]. Before imputation, exploratory analyses were performed by investigating the pattern of missingness for each variable. All variables showed a random missingness pattern. The percentage of missing data was less than 1% for maternal age, BMI, parity and fetal sex, 5.3% for ethnicity, 8.8% for education level and 12.6% for smoking. Five imputed datasets were created and pooled for analysis. hCG concentration adjusted for gestational age at the measurement and the risk of pre-eclampsia were used as predictor variables only. There were no differences in descriptive characteristics between the original and imputed datasets.

Statistical analyses were performed using Statistical Package of Social Sciences version 21.0 for Windows (SPSS Inc. Armonk, NY) and R statistical software version 3.2.1 (package 'rms').

RESULTS

After exclusions, the final population comprised 7754 women (Figure 1), descriptives of which are shown in Table 1. There were 165 (2.2%) women who developed pre-eclampsia; 4075 (54.6%) women were nulliparous and the majority of the population was of Dutch origin (3574; 47.9%) (Table 1).

Figure 1. Flowchart showing selection procedure of study population



The association of hCG with angiogenic factors

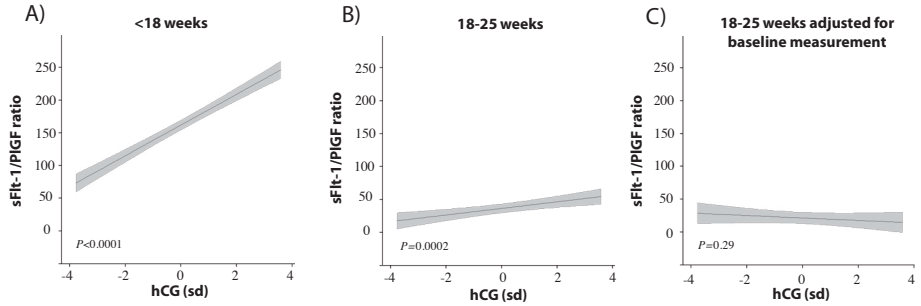
There was a positive association of hCG with sFlt-1/PlGF ratio during early pregnancy ($P<0.0001$; Figure 2A). The positive association of hCG with sFlt-1/PlGF during mid-pregnancy ($P=0.0002$, Figure 2B) attenuated after adjustment for sFlt-1/PlGF measurement at inclusion ($P=0.29$; Figure 2C). There was no association of hCG with the delta (the difference between first and second measurement) sFlt-1/PlGF ratio after adjustment

Table 1. Descriptive Statistics

Characteristic	Value
hCG, median (95%range), IU/l	35522.0 (6074.9-99890.3)
Gestational age at blood sampling, median (95% range), weeks	14.4 (10.1 – 26.1)
Hypertensive disorders in pregnancy, n (%)	
Pre-eclampsia	165 (2.2)
Pregnancy-induced hypertension	286 (3.7)
No disorder	7303 (94.2)
sFlt-1/PlGF ratio <18 weeks, median, (95% range)	117.0 (19.5 – 432.6)
sFlt-1/PlGF ratio 18-25 weeks, median, (95% range)	24.4 (5.3 – 106.8)
Age, mean (sd), years	29.6 (5.3)
BMI, mean (sd), kg/m2	24.7 (4.4)
Parity, n(%)	
Nullipara	4075 (54.6)
Primipara	2260 (30.3)
Multipara	1133 (15.2)
Smoking status, n(%)	
Non smokers	5466 (73.2)
Stopped smokers	632 (8.5)
Smokers	1370 (18.3)
Educational level, n(%)	
No education or primary education	933 (12.5)
Secondary education	3510 (47.0)
Higher education	3025 (40.5)
Ethnicity, n(%)	
Dutch	3574 (47.9)
Moroccan	556 (7.4)
Turkish	736 (9.9)
Surinam	686 (9.2)
Asian	437 (5.9)
Other – European	585 (7.8)
Other – Non-european	894 (12.0)
Fetal sex, n(%)	
Male	3763 (50.4)
Female	3705 (49.6)

for the sFlt-1/PlGF measurement at inclusion (P=0.29; Supplemental Figure 1). The associations of hCG with PlGF and sFlt-1, separately, are shown is Supplemental Figures 2 and 3, respectively.

Figure 2. The association of maternal hCG concentration with sFlt-1/PlGF ratio

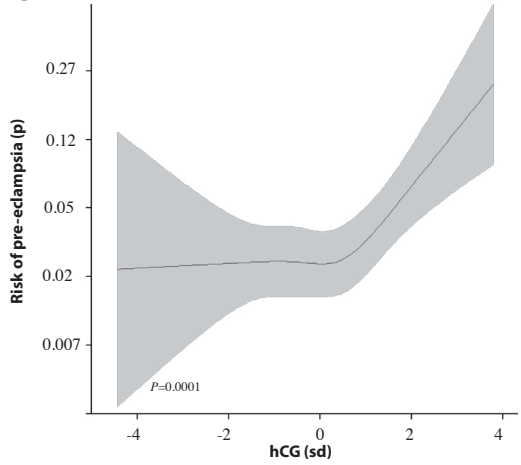


Plots show linear regression models for the association of hCG with the sFlt1/PlGF ratio, as predicted mean with 95 percent confidence interval. Analyses were adjusted for ethnicity, BMI, maternal age, fetal sex, educational level, parity and smoking status. The latter association was associated for the measurement at baseline.

The association of hCG with pre-eclampsia

Figure 3 shows the association of hCG with the risk of pre-eclampsia. A higher hCG concentration was associated with a 1.5-2.7-fold higher risk of pre-eclampsia, depending on the hCG cutoff value (Figure 3). After addition of a product interaction term to the model (hCG*fetal sex, *P*=0.020), we stratified the association of hCG with the risk of pre-eclampsia for fetal sex, which showed a predominant association of high hCG with the risk of pre-eclampsia in pregnancies with male fetuses (4.0-fold higher risk in males vs 2.2-fold higher risk in females, respectively; Supplemental Figure 4). We did not observe effect modification by gestational age (hCG*gestational age, *P*=0.73; data not shown).

Figure 3. The association of maternal hCG concentration with the risk of pre-eclampsia



hCG cutoff	OR	(95%CI)	*P*-value
<5	0.98	(0.47-2.03)	0.95
<10	0.83	(0.47-1.46)	0.51
<15	0.89	(0.57-1.41)	0.64
>85	1.50	(1.00-2.23)	0.046
>90	2.09	(1.37-3.18)	0.001
>95	2.73	(1.65-4.50)	<0.001

Plot shows regression model for the association of hCG with the risk of preeclampsia, as predicted mean with 95 percent confidence interval. Analyses were adjusted for ethnicity, BMI, maternal age, fetal sex, educational level, parity and smoking status.

Furthermore, when we adjusted the association of hCG with the risk of pre-eclampsia to sFlt-1/PlGF ratio (in the subgroup of women with available data on angiogenic factors), the association attenuated by 40% (β estimate change: from 0.19 ±0.10; *P*=0.052, to 0.12 ±0.10; *P*=0.22). The association of hCG with the risk of pregnancy-induced hypertension is shown in Supplemental Figure 5.

DISCUSSION

In this study, we show associations of high total hCG concentrations with high sFlt-1/PlGF ratio and high risk of pre-eclampsia. Our results suggest that up to 40% of the association of hCG with pre-eclampsia might be mediated via effects of hCG on the angiogenic factors sFlt-1 and PlGF.

Pre-eclampsia is an important cause of maternal morbidity and mortality as well as premature deliveries worldwide [1,2,25,26]. A great effort has been put into investigating the pathophysiological mechanisms leading to pre-eclampsia and the clinical markers of this condition [2]. Although the exact mechanism remains to be elucidated, abnormal placentation and a subsequent hyperinflammatory response characterized by a disturbance in pro- and anti-angiogenic factors play a major role [2-4]. Multiple studies have investigated the association of hCG with the risk of pre-eclampsia, comprising data on different hCG isoforms [15-20,27]. Although in early and in late pregnancy high total hCG and high β-hCG concentrations are suggested as a marker of (subsequent) pre-eclampsia [15-18], some studies report an association of low β-hCG in early pregnancy with pre-eclampsia [19,27] and one study suggests that a lower hyperglycosylated hCG in early pregnancy predisposes to pre-eclampsia [20]. The interpretation and comparison of these studies are hampered by the differences in hCG measurement, given that different hCG isoforms predominate during distinct phases of gestation [28,29]. Future studies that would meta-analyze individual participant data could particularly prove useful in elucidating the extent of these differences in the association with pre-eclampsia.

In pre-eclamptic women, the sFlt-1/PlGF ratio is higher compared to healthy women [7,30,31]. It is considered that high production of sFlt-1in the placenta largely counteracts the effects of PlGF and thereby contributes to the development of hypertension and proteinuria [6]. In pathological states characterized by high hCG concentrations, such as molar pregnancy, which predisposes to pre-eclampsia, the expression of placental sFlt-1 is higher compared to PlGF expression [32,33]. As hCG may play a role in the regulation of placental angiogenesis via effects on the production of VEGFs [10-14], we hypothesized that abnormal hCG concentrations may be a contributing factor for the unfavorable balance in the expression of placental pro- and anti-angiogenic factors in pre-eclampsia.

There may be an additional underlying mechanism involved contributing to the association of high hCG concentration with the higher risk of pre-eclampsia such as hCG-modulating effects on immune cells, that may respond differently depending on the hCG concentration [34]. In general, hCG has a protective effect on pregnancy and promotes fetal survival by promoting immune tolerance via interaction with macrophages, natural killer cells, B- and T-lymphocytes [34]. However, in higher doses, hCG may exhibit harmful effects on the fetal development [35,36] and may induce an immune cell response with altered cytokine and reactive oxygen species production [37], triggering an exaggerated sFlt-1 production and thereby reinforcing the cumulative effect of factors predisposing to pre-eclampsia [38]. Alternatively, higher availability of sFlt-1 and anti-angiogenic effects characterizing pre-eclampsia might be due to higher hCG-stimulated binding of PlGF to membrane receptors and local circulating receptors, because of the modulating activity of hCG on VEGFs [4,10-14]. Moreover, it has been proposed that dysregulation of not only concentrations of total hCG but also glycosylation patterns of hCG might underlie the pathophysiological events and impaired angiogenic and immune response in pre-eclampsia [39]. Despite its known pro-angiogenic effects in a normal pregnancy, the effects of altered hCG concentrations on the cross-talk between trophoblast, endothelial cells and immune system in pre-eclampsia are poorly understood [39]. Therefore, future studies should further elucidate the multifactorial risk factor profile for the association of hCG with pre-eclampsia.

In an attempt to diminish the adverse effects of oxidative stress and hypertension occurring in pre-eclampsia, a series of maternal compensatory responses may occur [40,41]. The association of a high hCG concentration with a higher risk of pre-eclampsia could therefore also represent a compensatory response of the trophoblast cells to feto-placental hypoxia, in which high hCG concentrations are supposed to stimulate angiogenic factors expression and improve placental function [5,6,10,14]. Another relevant determinant of adaptive mechanisms in pregnancy is fetal sex, as hCG concentrations are dependent on fetal sex [42,43]. The exact mechanism underlying the fetal sex-dependent variation in hCG concentration is not clear, although it is suggested that it originates from the hypothalamo-pityitary-gonadal axis and higher testosterone concentrations in the presence of Y chromosome [22,42,43]. Although fetal sex-specific differences in placental function are shown between pre-eclamptic and healthy women [44], a recent meta-analysis did not detect a fetal sex-specific difference in the risk of overall pre-eclampsia [45]. We demonstrate that women with high hCG concentrations and a male fetus have a higher risk of pre-eclampsia compared to women with a female fetus. Taken together, the sex-specific differences observed in our study might be explained by a potentially greater susceptibility of male fetuses to the impact of hCG variation, or fetal-sex specific differences in the variation of hCG-isoforms could mediate these differences [22]. In addition, the hypothesis of fetal sex-specific differences is further supported by our previous

study showing that the association of low hCG with lower fetal growth is stronger in male fetus pregnancies [46].

We were able to perform analyses using prospectively collected data on total hCG measurements, rather than only a single isoform, and also had detailed data available on pre-eclampsia and relevant covariates available in a large population. We were limited by the fact that we only had a single hCG measurement available, rendering us unable to examine inter-individual differences in hCG variation during pregnancy. Future studies may want to assess whether hCG trajectories are associated with pre-eclampsia. Another potential limitation could be the availability of data on PlGF and sFlt-1 in 70.8% of the study population, which could potentially introduce bias. However, a non-response analysis showed no difference in mean hCG concentrations or in the frequency of pre-eclampsia between participants with and without available data PlGF and sFlt-1. Finally, the current study is observational and thus does not allow for inference about causality and does not exclude residual confounding.

In conclusion, this study demonstrates that high hCG concentrations in early pregnancy are associated with a higher risk of pre-eclampsia. The effects of high hCG concentration on the balance between pro- and anti-angiogenic factors during pregnancy might partially explain this finding.

Supplemental Table 1. Descriptive Statistics – Non response for sFlt-1/PlGF

Characteristic	Value (response)	Value (non response)	P value
hCG standardized for gestational age, mean (sd)	0.008 (1.000)	-0.002 (0.989)	0.68
Hypertensive disorders in pregnancy, n (%)			0.051
Preeclampsia	96 (1.7)	41 (1.8)	
Pregnancy induced hypertension	221 (4.0)	65 (2.9)	
No disorder	5151 (93.8)	2152 (95.1)	
Age, mean (sd), years	29.7 (5.0)	29.5 (5.8)	0.035
BMI, mean (sd), kg/m2	24.4 (4.4)	25.7 (4.7)	<0.001
Parity, n(%)			<0.001
Nulipara	3128 (57.0)	1159 (51.2)	
Primipara	1653 (30.1)	672 (29.7)	
multipara	711 (12.9)	431 (19.1)	
Smoking status, n(%)			
Non smokers	3974 (72.4)	1685 (74.5)	
Stopped smokers	510 (9.3)	166 (7.3)	
Smokers	1008 (18.4)	411 (18.2)	
Educational level, n(%)			<0.001
No education or primary education	577 (10.5)	375 (16.6)	
Secondary education	2534 (46.1)	1117 (49.4)	
Higher education	2381 (43.4)	770 (34.0)	
Ethnicity, n(%)			<0.001
Dutch	2879 (52.4)	883 (39.0)	
Moroccan	330 (6.0)	227 (10.0)	
Turkish	473 (8.6)	290 (12.8)	
Surinam	463 (8.4)	242 (10.7)	
Asian	299 (5.4)	142 (6.3)	
Other – European	449 (8.2)	149 (6.6)	
Other – Non-european	599 (10.9)	329 (14.5)	
Fetal sex, n(%)			0.27
Male	2792 (50.8)	1119 (49.5)	
Female	2700 (49.2)	1143 (50.5)	

Supplemental Table 2. The association of maternal hCG (sd) with sFlt-1/PlGF ratio

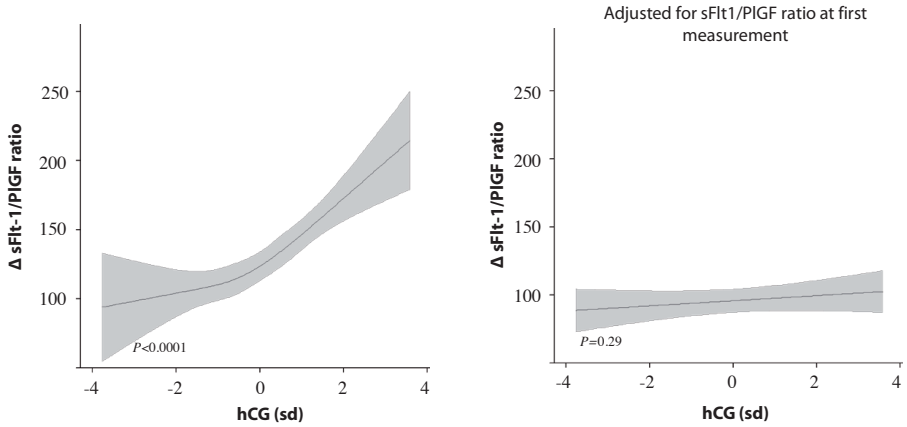
	Beta (95% CI)
sFlt-1/PlGF <18 weeks	23.47 (20.46, 26.49)**
sFlt-1/PlGF 18-25 weeks	5.01 (2.34, 7.68)**
sFlt-1/PlGF 18-25 weeks (adjusted for the measurement at baseline)	-1.88 (-5.41, 1.65)
Δ sFlt-1/PlGF	0.020 (0.018, 0.023) **
Δ sFlt-1/PlGF (adjusted for the measurement at baseline)	0.0002 (-0.0007, 0.0011)

Values are beta coefficents (95% confidence intervals) from the multivariate linear regression analyses. Models were adjusted for ethnicity, BMI, fetal sex, maternal age, educational level, parity and smoking status. hCG concentrations were transformed to standard deviation scores adjusted to gestational age at blood sampling.

* P<0.05

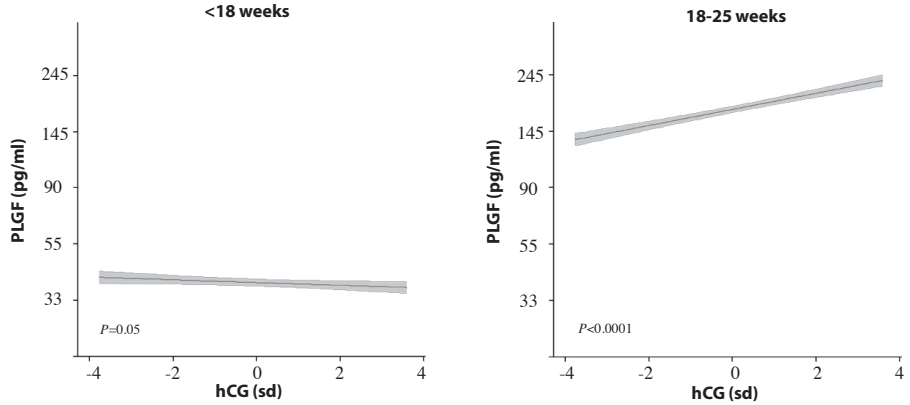
** P<0.001

Supplemental Figure 1. The association of maternal hCG concentration with ΔsFlt-1/PlGF ratio



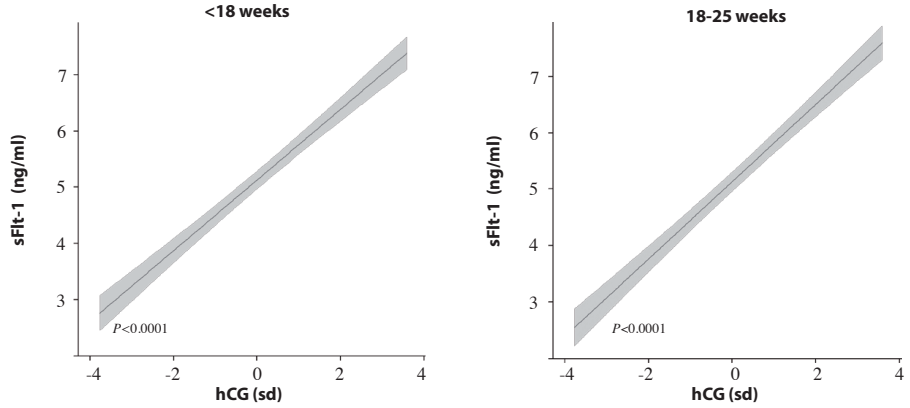
Plots show regression model for the association of hCG with Δ sFlt1/PlGF ratio, as predicted mean with 95 percent confidence interval. Analyses were adjusted for ethnicity, BMI, maternal age, fetal sex, educational level, parity and smoking status.

Supplemental Figure 2. The association of maternal hCG concentration with PLGF concentration



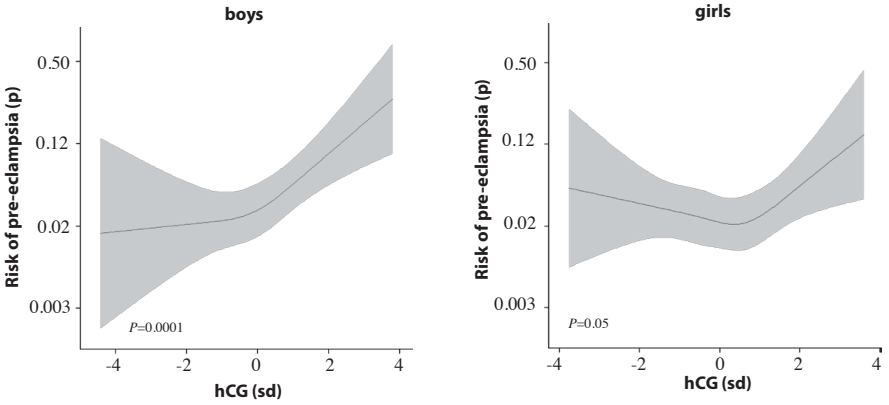
Plots show regression model for the association of hCG with PLGF, as predicted mean with 95 percent confidence interval. Analyses were adjusted for gestational age at blood sampling, ethnicity, BMI, maternal age, fetal sex, educational level, parity and smoking status.

Supplemental Figure 3. The association of maternal hCG concentration with sFlt-1 concentration



Plots show regression model for the association of hCG with sFlt1, as predicted mean with 95 percent confidence interval. Analyses were adjusted for gestational age at blood sampling, ethnicity, BMI, maternal age, educational level, fetal sex, parity and smoking status.

Supplemental Figure 4. The association of maternal hCG concentration with the risk of preeclampsia stratified for fetal sex

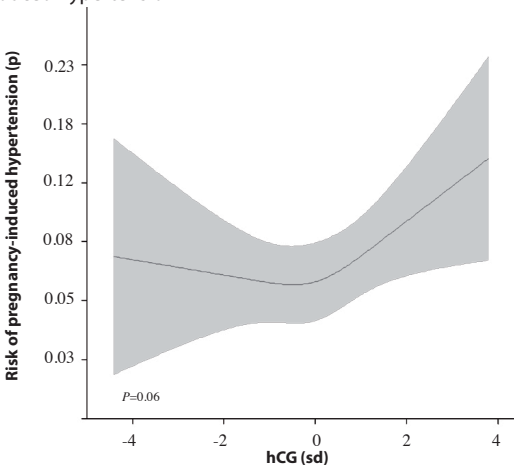


hCG cutoff	OR	(95%CI)	*P*-value
<5	0.87	(0.31-2.44)	0.79
<10	0.79	(0.36-1.76)	0.57
<15	0.64	(0.31-1.31)	0.23
>85	2.05	(1.17-3.60)	0.012
>90	3.06	(1.71-5.47)	<0.001
>95	4.04	(1.98-8.26)	<0.001

hCG cutoff	OR	(95%CI)	*P*-value
<5	1.13	(0.40-3.23)	0.82
<10	0.87	(0.39-1.94)	0.73
<15	1.17	(0.64-2.12)	0.62
>85	1.15	(0.66-2.01)	0.63
>90	1.48	(0.80-2.72)	0.21
>95	2.22	(1.07-4.59)	0.032

Plots show regression model for the association of hCG with the risk of preeclampsia, as predicted mean with 95 percent confidence interval. Analyses were adjusted for ethnicity, BMI, maternal age, educational level, parity and smoking status.

Supplemental Figure 5. The association of maternal hCG concentration with the risk of pregnancy-induced hypertension



hCG cutoff	OR	(95%CI)	*P*-value
<5	1.16	(0.72-1.89)	0.54
<10	1.00	(0.68-1.47)	0.99
<15	0.87	(0.87-0.62)	0.44
>85	1.33	(0.96-1.86)	0.09
>90	1.36	(0.92-2.01)	0.12
>95	0.97	(0.52-1.80)	0.92

Plots show regression model for the association of hCG with the risk of pregnancy induced hypertension, as predicted mean with 95 percent confidence interval. Analyses were adjusted for, ethnicity, BMI, maternal age, fetal sex, educational level, parity and smoking status.

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CHAPTER 3.2

Human chorionic gonadotropin (hCG) concentrations during the late first trimester are associated with fetal growth in a fetal sex-specific manner

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ABSTRACT

Background: Human chorionic gonadotropin (hCG) is a pregnancy-specific hormone that regulates placental development. hCG concentrations vary widely throughout gestation and differ based on fetal sex. Abnormal hCG concentrations are associated with adverse pregnancy outcomes including fetal growth restriction. We studied the association of hCG concentrations with fetal growth and birth weight. In addition, we investigated effect modification by gestational age of hCG measurement and fetal sex.

Methods: Total serum hCG (median 14.4 weeks, 95% range 10.1 – 26.2), estimated fetal weight (measured by ultrasound during 18-25th weeks and >25th weeks) and birth weight were measured in 7987 mother-child pairs from the Generation R cohort and used to establish fetal growth. Small for gestational age (SGA) was defined as a standardized birth weight lower than the 10th percentile of the study population.

Results: There was a non-linear association of hCG with birth weight (*P*=0.009). However, only low hCG concentrations measured during the late first trimester (11th and 12th week) were associated with birth weight and SGA. Low hCG concentrations measured in the late first trimester were also associated with decreased fetal growth (*P*=0.0002). This was the case for both male and female fetuses. In contrast, high hCG concentrations during the late first trimester were associated with increased fetal growth amongst female, but not male fetuses.

Conclusion: Low hCG in the late first trimester is associated with lower birth weight due to a decrease in fetal growth. Fetal sex differences exist in the association of hCG concentrations with fetal growth.

INTRODUCTION

Optimal intrauterine conditions are essential for proper fetal development and growth. Intrauterine conditions are highly dependent on the placental function since the placenta is the main source of fetal nourishment and the main regulator of intrauterine environment [1,2]. A suboptimal intrauterine environment leads to fetal adaptations that may affect fetal growth and thereby lead to lower birth weight [3,4]. Low birth weight is an important determinant of child's health and a major risk factor for several noncommunicable diseases later in life including coronary heart disease, stroke, hypertension and type 2 diabetes [5,6].

Human chorionic gonadotropin (hCG) is a pregnancy-specific hormone that is produced by trophoblast cells from the time of embryo implantation onwards [7]. Besides promoting progesterone production by corpus luteal cells, hCG has been shown to regulate many processes that are related to fetal growth including trophoblast differentiation, uterine growth, various aspects of placentation as well as uterine angiogenesis and vasculogenesis [7,8]. More specifically, hCG has also been shown to stimulate the production of endocrine gland-derived vascular endothelial growth factor (EG-VEGF), which is involved in the physiology of placental development [9,10]. By acting on cytotrophoblast cells, EG-VEGF is involved in the process of trophoblast shell and arterial plugs formation, necessary for preventing maternal blood flow into the intervillous space during early pregnancy [9]. hCG may also directly influence uterine and fetal growth by acting on gonadotropin receptors present in the uterine tissue and fetal membranes [8,11,12].

Although clinical studies have shown that hCG is associated with adverse outcomes, this association seems to differ according to gestational age at which hCG is measured. Low hCG concentrations in the first trimester as well as high hCG concentrations in the second trimester have both been associated with pregnancy loss and pre-eclampsia [13-16]. High hCG concentrations in the second trimester have also been associated with gestational hypertension, fetal growth restriction, fetal death and preterm delivery [16,17]. Given that hCG concentrations vary throughout gestation, it is remarkable that very little is known about potential gestational time-dependent effects of hCG on adverse pregnancy outcomes.

Given the important role of hCG in many fetal growth-related processes, we examined the overall and gestational age-dependent associations of hCG with fetal growth, as well as possible fetal sex-specific differences, in a large population-based prospective cohort study.

MATERIALS AND METHODS

Study population

This study was embedded in The Generation R cohort, a population-based prospective study from early fetal life onwards in Rotterdam, the Netherlands [18]. The study was designed to identify early environmental and genetic causes leading to normal and abnormal growth, development and health during fetal life and childhood [18]. In total, 8879 mothers with expected delivery date between April 2002 and January 2006 were enrolled during pregnancy. Total hCG was determined in all first available serum samples and this data, together with data on birth weight, was available in 7987 mother-child pairs. Women with twin pregnancies (N=90) or in vitro fertilization treatment (N=38) were excluded from the analysis.

hCG measurements

Total hCG was measured in serum using a solid-phase two-site chemiluminiscent immunometric assay, calibrated against WHO 3rd IS 75/537, on an Immulite 2000 XPi system (Siemens Healthcare Diagnostics, Deerfield, IL, USA). The assay detects serum intact hCG, hyperglycosated hCG, serum nicked hCG, serum nicked hyperglycosated hCG, serum asialo hCG, serum hCG free β-subunit and serum nicked hCG β. hCG concentrations were transformed to standard deviation scores adjusted to gestational age at blood sampling [19].

Fetal growth and birth weight

Early fetal growth was estimated by ultrasound measurement of crown-rump length (CRL) in a subset of women (N=1526) which had a reliable and regular menstrual cycle [18,19]. CRL values were transformed to standard deviation scores adjusted to gestational age of pregnancy determined according to the last menstrual period (LMP). CRL was measured by ultrasound in early pregnancy, in a true mid-sagittal plane with the genital tubercle and the fetal spine longitudinally in view [20]. The maximum length from cranium to the caudal rump was measured as a straight line [20].

Fetal weight was estimated by ultrasound measurements in the period of 18-25th week of pregnancy (median=20.5 week, 95% range 18.5 -23.4 week; N=7471) and after the 25th week of pregnancy (median=30.3 week, 95% range 28.3 - 33.0 week; N=7641) and estimations for fetal weight were transformed to standard deviation scores adjusted to gestational age of pregnancy determined by crown-rump length and biparietal diameter, as has been described previously [20]. Information on birth weight was obtained from community midwives, obstetricians and hospital registries. Birth weight standard deviations scores, adjusted for gestational age, were constructed using the Niklasson percentile growth curves [21]. Small for gestational age at birth (SGA) was defined as a standardized birth weight lower than the 10th percentile of the study population.

Covariates

Information on maternal age, smoking status, educational level and ethnicity was obtained by questionnaires during pregnancy. Ethnicity was determined by the country of origin and was defined according to the classification of Statistics Netherlands [18]. Maternal smoking was classified as no smoking, smoking until known pregnancy and continued smoking during pregnancy. Information on fertility treatment, parity, placental weight at birth and sex of the child was obtained from community midwives, obstetricians, and hospital registries. Gestational weight gain was defined as the difference between self-reported maternal weight before pregnancy and maternal weight measured in the third trimester (a sensitivity analysis using maternal weight measured in early pregnancy and in the third trimester did not reveal more confounding potential or explained variability of the model). Free thyroxine (FT4) and thyroid stimulating hormone (TSH) were available in a subset of 5498 pregnant women during early pregnancy [22].

Statistical analysis

We investigated the association of hCG concentrations with CRL within a subset group of women (with regular cycles and known last menstrual period) and birth weight within the whole group by using multiple linear regression analysis with restricted cubic splines utilizing three knots. Multivariable associations were graphically depicted by plots (main manuscript) and the key β estimates with 95% confidence intervals are shown in Supplemental Table 3. We tested for effect modification with gestational age at blood measurement and fetal sex by introducing a product interaction term of hCG and gestational age at blood sampling or fetal sex to the model. Given the fetal growth differences across gestational age and between male and female fetuses, a *P*-value of <0.15 was considered for stratification. We subsequently stratified the analyses by quintiles of gestational age at blood sampling and in case of a difference between these time points, further stratification was performed per one or more gestational weeks. To study the association of hCG with the risk of SGA we used multiple logistic regression models with restricted cubic splines utilizing three knots. The association of hCG concentrations with fetal growth throughout gestation was analyzed using unbalanced repeated measurement regression models for which the outcome consisted of standardized estimated fetal weight in the second and third trimester and standardized birth weight. These models take the correlation between repeated measurements of the same subject into account and allow for incomplete outcome data [23]. We used an unstructured covariance matrix with fixed effects, and added the interaction term of hCG with the time component (gestational age) in the models, and adjusted for covariates. Based on the size of effect estimate differences and biological plausibility extracted from the literature, we stratified these analyses in a similar manner as previously described analyses on birth

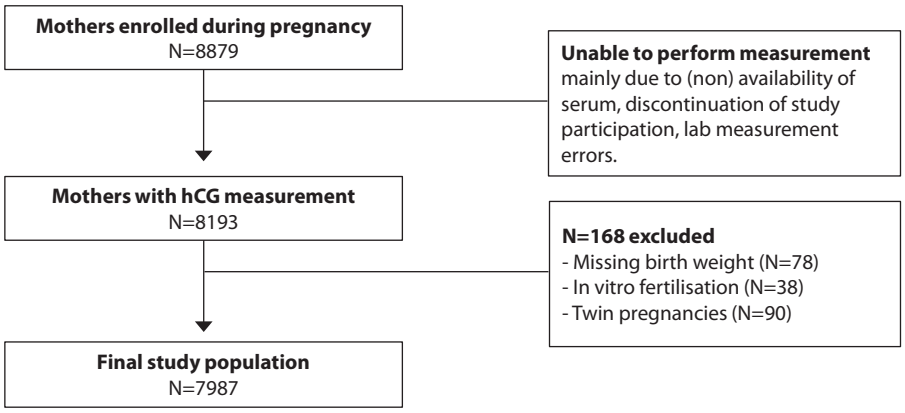
weight. Lower statistical power in subsequent subset analyses and the need for testing three-way interactions were deemed statistically not viable. The hCG cutoff in the repeated measurement analyses was chosen based on the optimal power necessary for the biologically plausible reference group.

All model covariates were selected based on biological plausibility, change in effect estimate of interest or residual variability of the model. All analyses were adjusted for maternal age, smoking status, BMI, parity, educational level, ethnicity, fetal sex, placental weight at birth, gestational age at blood sampling and gestational weight gain of the mother. Placental weight, as a marker for trophoblast cell mass, may be an important determinant of both hCG concentrations [19] and fetal growth and from that reason was adjusted for in the models. Maternal weight gain was taken as a proxy for the potential confounding effects of hyperemesis gravidarum (HG). Gestational weight change is an important clinical marker of the HG effects [24] and the same goes for other symptoms of HG such as reflux/belching, nausea and vomiting – but addition of these factors did not change our models.

For covariates with missing data, multiple imputation according to the Markov Chain Monte Carlo method was used [25]. Five imputed data sets were created and pooled for the analysis. Maternal smoking, education, ethnicity, BMI, parity, placental weight, gestational weight gain and fetal sex were added to the model. Furthermore, we added gestational age at blood sampling, hCG level and maternal FT4 level as prediction variables only. No statistically significant differences in descriptive statistics were found between the original and imputed datasets.

Statistical analyses were performed using Statistical Package of Social Sciences version 21.0 for Windows (SPSS Inc. Armonk, NY), R statistical software with RMS package version 3.2.0 and SAS software for Windows version 9.3.

Figure 1. Flowchart showing selection procedure of study population



RESULTS

The final study population consisted of 7987 pregnant women (Figure 1), descriptive statistics of which are shown in Table 1. Maternal hCG concentrations were measured at the moment of inclusion in the study (median 14.4 weeks, 95% range 10.1 – 26.2 weeks). In the study population, mean (±SD) birth weight was 3412.0 (±559.7) grams, mean gestational age at birth was 39.8 (±1.9) weeks, mean maternal age was 29.6 (±5.3) years, women were predominantly nulliparous (55.3%), non-smokers (72.7%) and of Dutch origin (46.6%).

Table 1. Descriptive Statistics of Mother and Child Pairs

Characteristic	Value
hCG, median (95%range), IU/l,	35559.0 (6106.8-101808.5)
Birth weight, mean (sd), g	3412.0 (559.7)
Gestational age at blood sampling, median (95% range), weeks	14.4 (10.1-26.2)
Gestational age at birth, mean (sd), weeks	39.8 (1.9)
Age, mean (sd), years	29.6 (5.3)
BMI, median (95% range), kg/m2	23.9 (18.7-36.5)
Parity, n(%)	
Nullipara	4531 (55.3)
Primipara	2475(30.2)
Multipara	1187 (14.5)
Smoking status, n(%)	
Non smokers	5953 (72.7)
Stopped smokers	691 (8.4)
Smokers	1549 (18.9)
Educational level, n(%)	
No education or primary education	1046 (12.8)
Secondary education	3844 (46.9)
Higher education	3303 (40.3)
Ethnicity, n(%)	
Dutch	3742 (46.6)
Moroccan	530 (6.6)
Turkish	710 (8.8)
Surinam	686 (8.5)
Other – European	598 (7.5)
Other – Non-european	1758 (21.9)
Fetal sex, n(%)	
Male	4124 (50.3)
Female	4069 (49.7)
Gestational weight gain, mean (sd), kg	10.0 (5.1)
Placental weight at birth (g), median, (95% range)	620.0 (390.0-950.0)

The association of maternal hCG with birth weight

In the whole population, there was a non-linear association of maternal hCG concentrations with birth weight (Supplemental Figure 1, *P*=0.009) and the risk of SGA (Supplemental Figure 1, Supplemental Table 1, *P*=0.028). Considering that hCG concentrations vary throughout gestation, we investigated whether gestational age at blood sampling modifies the association of hCG concentrations with birth weight. After addition of a product interaction term to the model (hCG*gestational age at blood sampling; *P*=0.10), we stratified the association of hCG concentration with birth weight by gestational age at blood sampling (Figure 2 and Supplemental Figure 2). The association of hCG with birth weight was present in the 11th week (Figure 2, *P*=0.03; beta estimates shown in Supplemental Table 3) and 12th week of pregnancy (Figure 2, *P*=0.002; Supplemental Figure 3), but not from the 13th week onwards (Figure 2; Supplemental Figure 3). The association of hCG with the risk of SGA showed a consistent trend with the results from linear regression analysis with birth weight, with a tendency of higher odds of SGA in low hCG concentrations in the 11th and 12th week (Figure 2 and Supplemental Figure 3). The odds of SGA in women with low hCG concentration measured in the 11th and 12th week (<5th to <15th percentile) were 1.80 - 2.21 fold higher than the reference group (Supplemental Table 2).

Subsequently, we set out to investigate if the association of hCG with birth weight occurs due to changes during early pregnancy (crown rump length) or also due to changes in the second half of pregnancy (fetal growth).

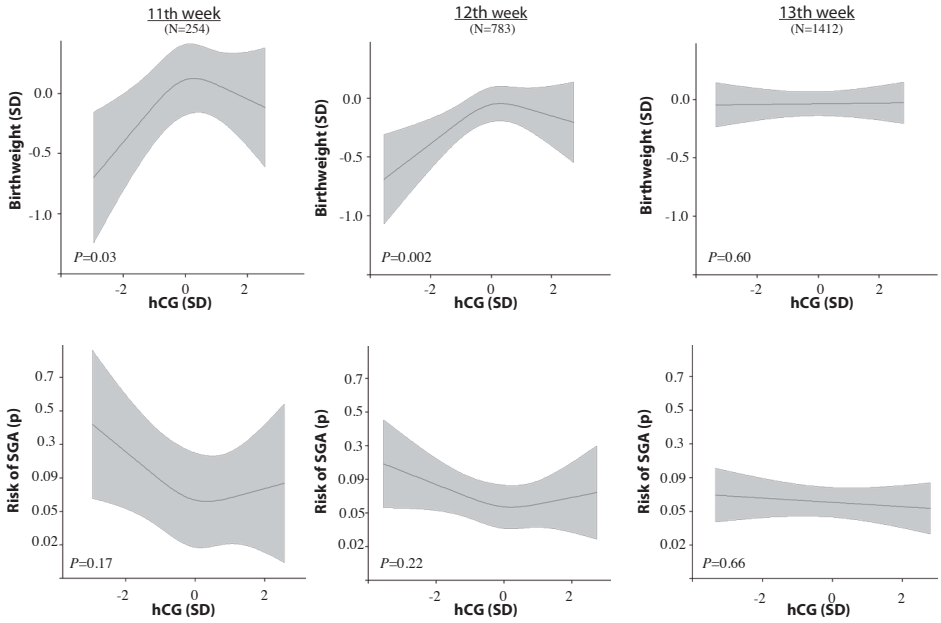
The association of maternal hCG with crown rump length

In the subset of women with regular cycles and known last menstrual period, in which CRL measurement was available, hCG concentrations during early pregnancy were negatively associated with CRL, but this analysis did not reach statistical significance (Supplemental Figure 4; N=1526).

The association of maternal hCG with fetal growth

Maternal hCG concentrations were associated with estimated fetal weight in the whole population, but this association was most prominent in pregnancies in which hCG was measured in the 12th week of gestation (Table 2). The association between hCG concentrations measured in the 12th week of pregnancy and estimated fetal weight is illustrated in Figure 3. The estimated fetal weight in women with a relatively high hCG concentration (Figure 3, 3rd tertile, depicted by the dotted line) was lower in mid pregnancy but accelerated throughout gestation, finally reaching a birth weight similar to the reference group (2nd tertile). In contrast, estimated fetal weight in women with low hCG concentration (Figure 3, 1st tertile, depicted by the solid line) was similar in mid-pregnancy, but was associated with lower birth weight than the reference group after a decrease of fetal growth in the second half of pregnancy.

Figure 2. The association of maternal hCG with birth weight and small for gestational age (SGA) stratified per week of hCG measurement



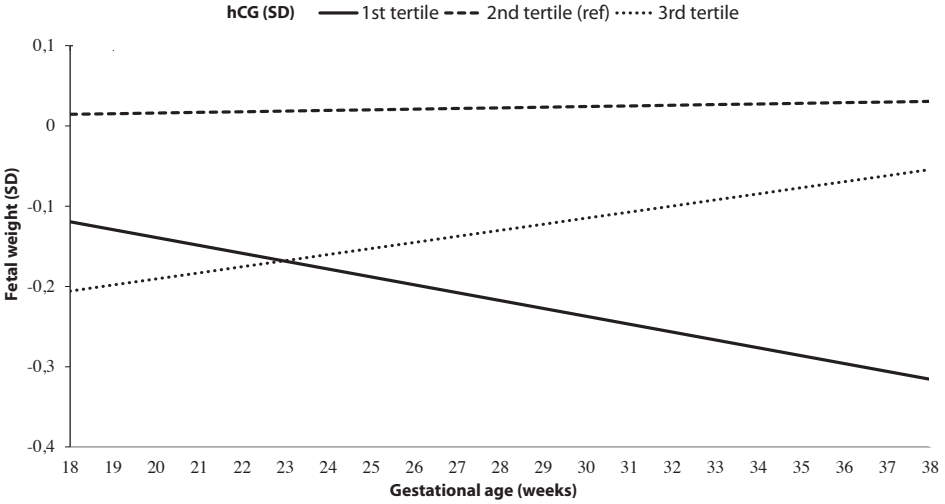
Plots show the linear regression models for total hCG (standardized according to gestational age at measurement; SD) and birthweight (standardized according to gestational age at birth; SD), as well as the logistic regression model for hCG and birthweight small for gestational age (defined as birthweight below 10th percentile for gestational age) as predicted mean with 95 percent confidence interval. Analyses were adjusted for maternal age, smoking, BMI, parity, placental weight at birth, education level, ethnicity, gestational weight gain and fetal sex.

Table 2. The association of maternal hCG with fetal growth throughout gestation.

	Beta	±SE	*P*-value	95% Confidence Interval	
hCG in all	0.002	±0.001	0.0017	(0.001, 0.003)	
hCG stratified per week of measurement					**Number of participants per week**
11th week	-0.001	±0.003	0.81	(-0.007, 0.005)	254
12th week	0.007	±0.002	0.0002	(0.003, 0.010)	783
13th week	0.000	±0.001	0.94	(-0.003, 0.003)	1412
14th week	0.000	±0.002	0.78	(-0.003, 0.004)	1060

Table shows the effects estimates of repeated measurement model for the association of total hCG (standardized according to gestational age at measurement; SD) with fetal growth (standardized estimated fetal weight measured using ultrasound in mid pregnancy (18-25 weeks), late pregnancy (>25 weeks) and birth weight), as beta estimate with standard error. Analyses were performed after exclusion of women with IVF treatment (N=38), twin pregnancy (N=90) and were adjusted for maternal age, smoking, BMI, parity, education level, ethnicity, gestational weight gain, placental weight at birth and fetal sex.

Figure 3. The association of maternal hCG measured in the 12th week of pregnancy with fetal growth throughout gestation



Graph depicts the beta estimates from a repeated measurement model of the association of total hCG (standardized according to gestational age at measurement; SD) with fetal growth (standardized estimated fetal weight measured using ultrasound in mid pregnancy (18-25 weeks), late pregnancy (>25 weeks) and birth weight). Analyses were adjusted for maternal age, smoking, BMI, parity, education level, ethnicity, gestational weight gain, placental weight at birth and fetal sex.

Sex-specific differences in the association of hCG with birth weight and fetal growth

Considering the biological differences in fetal growth and in hCG physiology between male and female fetuses, we tested for effect modification by fetal sex. After addition of a product interaction term to the model (hCG*fetal sex; *P*=0.10), we stratified analyses according to fetal sex. The association of hCG concentrations (measured in week 11-12) and birth weight did not differ for fetal sex (Supplemental Figure 5 and 6). However, in women with low hCG concentrations (<10th or <15th percentile), the risk of SGA was higher in male than in female fetal-sex (Supplemental Table 4).

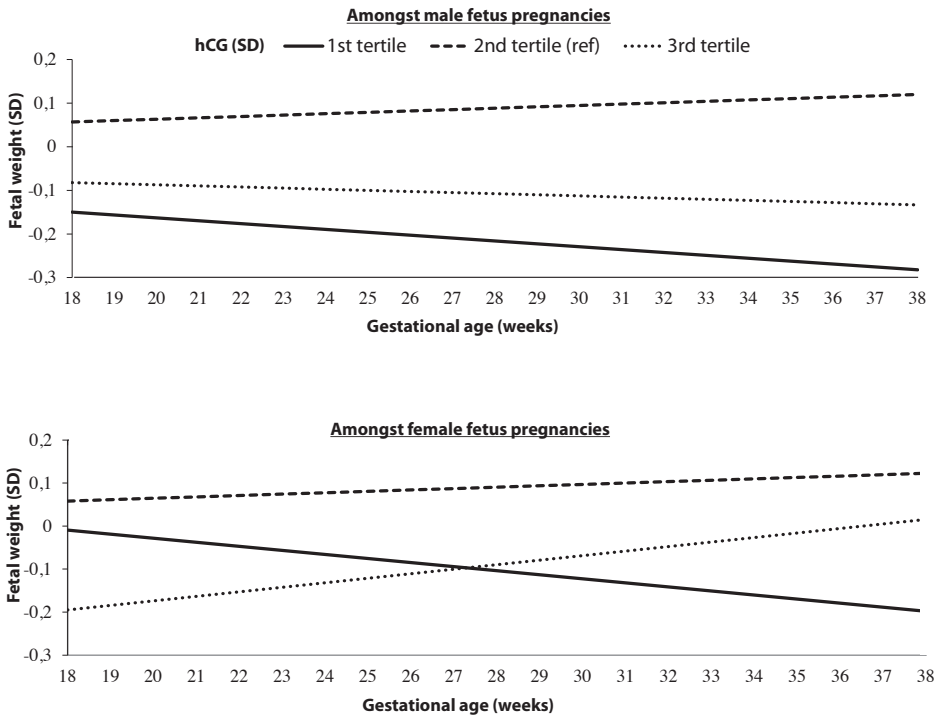
The association of hCG concentrations with estimated fetal weight differed according to fetal sex (Supplemental Table 5). In women with relatively low hCG during the late first trimester (Figure 4, 1st tertile, depicted by the solid lines), estimated fetal weight in mid-pregnancy was overall lower in male than in female fetuses. However, in female fetuses, low maternal hCG was associated with a greater deceleration of fetal growth than in male fetuses (-0.17 SD decrease in male fetus pregnancies versus -0.26 SD decrease in female fetus pregnancies; Figure 4).

In women with high hCG concentrations during the late first trimester (Figure 4, 3rd tertile, depicted by the dotted lines), estimated fetal weight during mid-pregnancy was

overall lower in female than in male fetuses. However, in female fetuses, high maternal hCG concentrations were associated with an acceleration of fetal growth as compared to a slight deceleration in male fetus pregnancies (-0.07 SD decrease in males versus +0.26 SD increase in females; Figure 4).

There was no effect modification by, or change in the results after adjustment for maternal FT4 and TSH concentrations (data not shown).

Figure 4. The association of maternal hCG in 11th or 12th week with fetal growth stratified by fetal sex



DISCUSSION

Our study shows that low hCG concentrations in the late first trimester are associated with lower birth weight and an increased risk of SGA. We demonstrate that the association of low hCG concentrations in the late first trimester with lower birth weight arises due to a decrease in fetal growth during the second half of pregnancy. In contrast, high concentrations of hCG in the late first trimester were associated with growth acceleration which resulted in a normal birth weight. A low hCG concentration was associated with a decrease in fetal growth, independent of fetal sex. However, high hCG concentrations were associated with an accelerated fetal growth in female, but not in male fetuses.

Fetal growth and birth weight reflect intrauterine conditions during pregnancy. Major healthcare problems such as cardiovascular diseases and type 2 diabetes are preordained in utero and low birth weight is one of the main determinants of those disorders [5,6]. Previous studies that investigated hCG as a potential determinant of fetal growth restriction mostly focused on either the first or second trimester. Furthermore, most of those studies examined only the association of the β-hCG isoform and fetal growth, meaning that non-measured differences in other important active hCG-isoforms could have influenced the results [26]. A large Danish study of 9450 women in the 8th -13th week of pregnancy reported that a low free β-hCG concentration is associated with SGA [27]. A study of 8012 pregnant women by Krantz et al. reported that a low free β-hCG concentration in the 10th-13th week of pregnancy is associated with an increased risk of fetal growth restriction (FGR) [28]. Amongst 100 women, Abdel Moety et al. also reported that low concentrations of free β-hCG measured in 11th to 14th week of gestation are associated with FGR [29]. In contrast, other studies showed that a high hCG concentration in the second trimester is associated with a decrease in fetal growth and/or FGR [16,17]. To date, no study had investigated the association of total hCG with fetal growth or investigated the role of gestational age of hCG measurement and/or fetal sex in this association.

In the current study, low hCG concentrations were only associated with low birth weight when hCG was measured in the 11th or 12th week of gestation, suggesting that hCG has a specific role in fetal growth during the transitional period from the first to the second trimester. This period marks the start of maternal blood supply in the intervillous space and the end of the hypoxic fetal environment [30]. Oxidative stress in early gestation is a risk factor for adverse pregnancy outcomes including fetal growth restriction, which might be due to a lack of antioxidant enzymes in fetal tissues at that time [31]. hCG is indirectly involved in the maintenance of early pregnancy hypoxia via regulating endocrine gland-derived vascular endothelial growth factor (EG-VEGF), a factor that at least partially ensures physiologically low oxygen concentrations during early pregnancy through stimulation of the arterial plugs formation [9]. The specific association in the current study of low hCG in the late first trimester with decreased fetal growth might be due to a suboptimal development of the trophoblast shell and arterial plugs, or an earlier release of the arterial plugs via lower levels of EG-VEGF. This could potentially expose the fetus to the harmful effects of O_2 free radicals. Further studies are needed to replicate our findings and investigate the association of repeatedly measured hCG concentrations with fetal growth.

Studies have shown that fetal growth and maternal hCG concentrations differ depending on fetal sex. hCG concentrations in pregnancies with a female fetal sex are higher from as early as the third post-fertilization week [32-35]. In this study, the continuous association of hCG concentrations measured at the end of the first trimester with birth weight did not differ between pregnancies with a male or female fetus. Nevertheless,

low hCG concentrations were associated with a higher risk of SGA in male fetal sex. This discrepancy already suggests that there is a fetal sex-specific association of hCG with fetal growth patterns. Moreover, further analyses revealed that the association of low hCG during the late first trimester with fetal growth deceleration was stronger in female fetal sex. Furthermore, high hCG concentrations during the late first trimester were associated with growth acceleration in the female, but not male fetuses.

Male fetuses may be more susceptible to the impact of relatively low hCG concentrations during the late first trimester than female fetuses, considering the fact that mean hCG concentrations are lower in pregnancies with a male fetus [32,33]. On the other hand, our findings show that the association of low hCG concentrations with decreased fetal growth is present in both female and male fetal sex. This suggests that low hCG concentrations during the first trimester have an impact on the fetal growth trajectory independent of fetal sex. Interestingly, the main difference between males and females in the current study was the association of high late first trimester hCG with growth acceleration during mid-pregnancy, which was only present in female fetuses. This might be due to the higher concentrations of hCG in female fetal sex, or due to fetal sex-specific differences in hCG isoforms [19]. Future studies are needed to investigate the mechanisms that underlie the differential effects of female fetal sex in the association of hCG and fetal growth in more depth. In addition, replication of fetal sex-specific differences in the association of hCG with fetal growth will be required, preferably in samples that have adequate power to test for higher order interactions.

The strengths of the current study include the availability of hCG concentrations as well as detailed fetal growth data with serial fetal weight measurements in a large population. The fact that hCG measurements were also available across a wide gestational time span, enabled us to observe a change in the association of hCG with fetal growth across gestational age. We were, however, limited by the fact that a single hCG measurement was available. As a consequence, we were not able to assess interindividual differences in the change of hCG concentrations during pregnancy. Also, the observational nature of this study does not allow for inference of causality and does not preclude the existence of residual confounding. As such, it is possible that it is the fetal size that affects maternal hCG concentration and/or that placental growth plays a confounding or mediating role. However, the latter seems less likely since the addition of placental weight to the model did not cause any meaningful changes in effect estimates.

In conclusion, we demonstrate that late first trimester hCG concentrations are associated with fetal growth and birth weight. In women with low hCG concentrations during the late first trimester, fetal growth is lower resulting in a lower birth weight. In female fetal-sex pregnancies, a high maternal hCG concentration at the end of the first trimester is associated with fetal growth acceleration. The underlying mechanism of these effects might involve a flawed protection from oxidative stress due to the effects of hCG on

arterial plug formation. Further research is necessary to investigate the causality of this association and the biological mechanism by which maternal hCG in the late first trimester affects fetal growth.

Supplemental Table 1. The association of maternal hCG cutoffs with SGA.

hCG cut-off	OR	(95%CI)	*P*-value
<5	1.24	(0.88-1.75)	0.22
<10	1.07	(0.82-1.39)	0.61
<15	1.18	(0.95-1.47)	0.14
>85	1.08	(0.86-1.35)	0.52
>90	1.06	(0.81-1.37)	0.69
>95	1.18	(0.84-1.66)	0.34

Table shows the association between different cut-off levels for total hCG (standardized to gestational age; SD) and small for gestational age (defined as birthweight below 10th percentile for gestational age) as odds ratio with 95 percent confidence interval. Analyses were performed after exclusion of women with IVF treatment (N=38), twin pregnancy (N=90) and were adjusted for maternal age, smoking, BMI, parity, placental weight at birth, education level, ethnicity, gestational age at birth, gestational weight gain and fetal sex.

Supplemental Table 2. The association of maternal hCG measured in the 11th or 12th week cutoffs with small for gestational age.

hCG cut-off	OR	(95%CI)	*P*-value
<5	2.21	(1.04-4.70)	0.04
<10	2.00	(1.09-3.67)	0.03
<15	1.80	(1.05-3.10)	0.03
>85	0.81	(0.39-1.66)	0.57
>90	1.02	(0.45-2.32)	0.96
>95	1.52	(0.58-4.01)	0.40

Table shows the association between different cut-off levels for total hCG (standardized to gestational age; SD) and small for gestational age (defined as birthweight below 10th percentile for gestational age) as odds ratio with 95 percent confidence interval. Analyses were performed after exclusion of women with IVF treatment (N=38), twin pregnancy (N=90) and were adjusted for maternal age, smoking, BMI, parity, placental weight at birth, education level, ethnicity, gestational age at birth, gestational weight gain and fetal sex.

Supplemental Table 3. Association of hCG (sd) with birth weight (sd)

	11th week of gestation		12th week of gestation		13th week of gestation
hCG (sd) < 0	0.191 (-0.026, 0.408)	**hCG (sd) < 0**	0.194 (0.063, 0.326)**	**hCG (sd)**	0.006 (-0.043, 0.056)
hCG (sd) ≥ 0	-0.115 (-0.398, 0.167)	**hCG (sd) ≥ 0**	-0.053 (-0.211, 0.105)		

Values are regression coefficients (95% confidence intervals) from the linear regression models for total hCG (standardized according to gestational age at measurement; SD) and birthweight (standardized according to gestational age at birth; SD). Analyses were performed after exclusion of women with IVF treatment (N=38), twin pregnancy (N=90) and were adjusted for maternal age, smoking, BMI, parity, placental weight at birth, education level, ethnicity, gestational weight gain and fetal sex. Non-linear associations are presented with the two estimates for hCG (sd) and the cut-off was based on the shape of the association.
* $P<0.05$
** $P<0.001$

Supplemental Table 4. The association of maternal hCG measured in the 11th or 12th week and SGA stratified by fetal sex.

Amongst male fetuses				Amongst female fetuses			
hCG cutoff	**OR**	**(95%CI)**	***P*-value**	**hCG cutoff**	**OR**	**(95%CI)**	***P*-value**
<5	2.08	(0.73-5.92)	0.17	**<5**	2.39	(0.70-8.07)	0.16
<10	2.77	(1.27-6.02)	0.01	**<10**	1.22	(0.39-3.80)	0.73
<15	2.58	(1.26-5.28)	0.009	**<15**	1.16	(0.47-2.86)	0.76
>85	1.09	(0.99-1.20)	0.07	**>85**	0.85	(0.35-2.04)	0.71
>90	0.33	(0.04-2.96)	0.32	**>90**	1.14	(0.45-2.93)	0.78
>95	0.53	(0.05-5.24)	0.58	**>95**	1.88	(0.59-5.93)	0.28

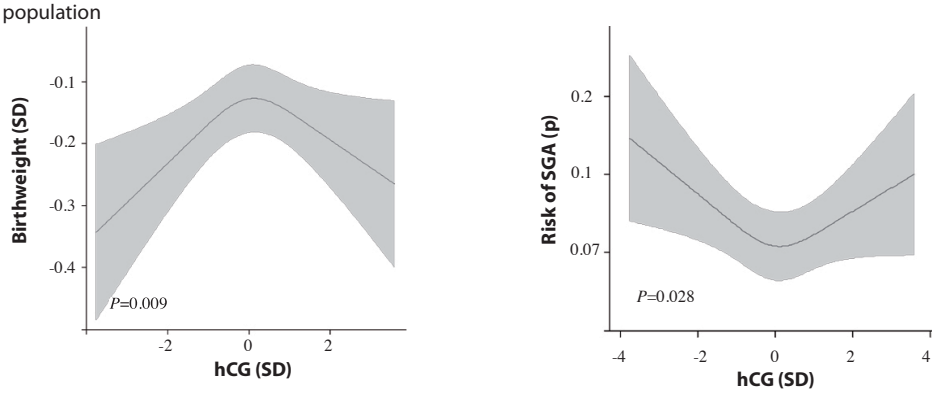
Table shows the association between different cutoff levels for total hCG (standardized to gestational age; SD) and small for gestational age (defined as birthweight below 10th percentile for gestational age) as odds ratio with 95 percent confidence interval. Analyses were performed after exclusion of women with IVF treatment (N=38), twin pregnancy (N=90) and were adjusted for maternal age, smoking, BMI, parity, placental weight at birth, education level, ethnicity, gestational age at birth, gestational weight gain and fetal sex.

Supplemental Table 5. The association of maternal hCG with fetal growth throughout gestation stratified by fetal sex.

Week of hCG measurement	Fetal sex	Beta	±SE	P-value	95% Confidence interval
All weeks	male	0.012	±0.009	0.06	(-0.000, 0.003)
11th and 12th week only	male	0.003	±0.002	0.19	(-0.002, 0.007)
All weeks	female	0.002	±0.001	0.0075	(0.001, 0.004)
11th and 12th week only	female	0.006	±0.002	0.005	(0.002, 0.010)

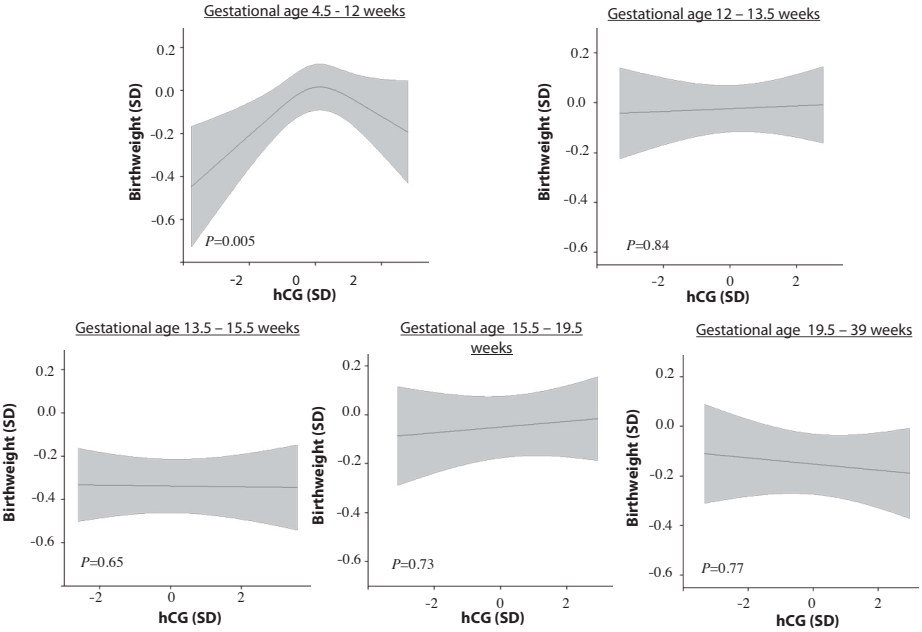
Table shows the repeated measurement model for the association of total hCG (SD), fetal weight (measured twice during pregnancy) and birthweight, as predicted mean with 95 percent confidence interval. Analyses were performed after exclusion of women with IVF treatment (N=38), twin pregnancy (N=90) and were adjusted for maternal age, smoking, BMI, parity, education level, ethnicity, gestational weight gain and placental weight at birth.

Supplemental Figure 1. The association of maternal hCG with birth weight and SGA in the whole study population



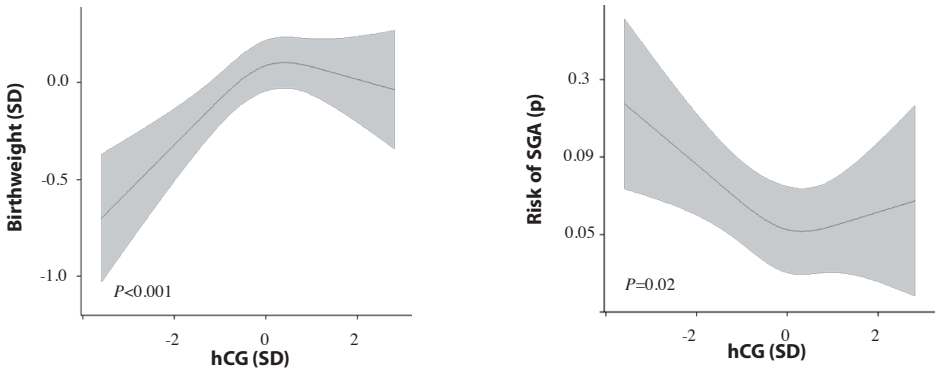
Plots show the linear and logistic regression models for total hCG (standardized to gestational age; SD) and birthweight (standardized to gestational age at birth; SD) as predicted mean with 95 percent confidence interval. Analyses were adjusted for maternal age, smoking, BMI, parity, placental weight at birth, education level, ethnicity, gestational age at birth, gestational weight gain and fetal sex.

Supplemental Figure 2. The association of maternal hCG with birth weight stratified by gestational age at hCG measurement



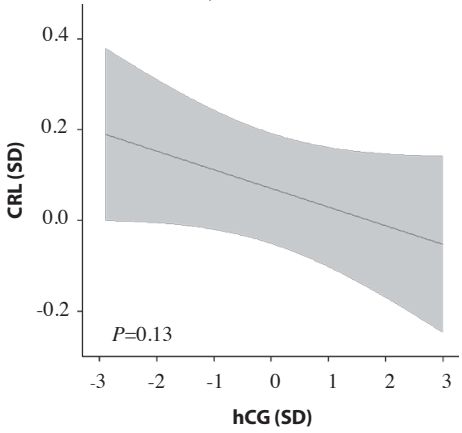
Plots show the linear regression model for the association between total maternal hCG (standardized to gestational age at measurement; SD) and birthweight (standardized to gestational age at birth; SD) as predicted mean with 95 percent confidence interval. Analyses were adjusted for maternal age, smoking, BMI, parity, placental weight at birth, education level, ethnicity, gestational age at birth, gestational weight gain and fetal sex.

Supplemental Figure 3. The association of hCG measured in the 11th or 12th week with birth weight and SGA



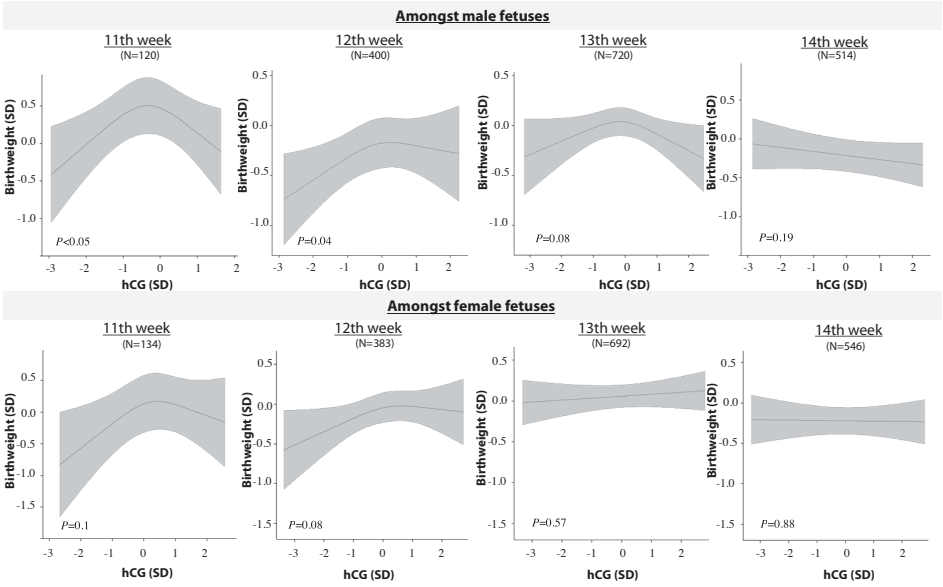
Plots show the linear and logistic regression models for total hCG (standardized to gestational age at measurement; SD) and birthweight (standardized to gestational age at birth; SD) as predicted mean with 95 percent confidence interval. Analyses were adjusted for maternal age, smoking, BMI, parity, placental weight at birth, education level, ethnicity, gestational age at birth, gestational weight gain and fetal sex.

Supplemental Figure 4. The association of maternal hCG with crown-rump length within the subgroup of women with a reliable cycle and known last menstrual period (N=1526)



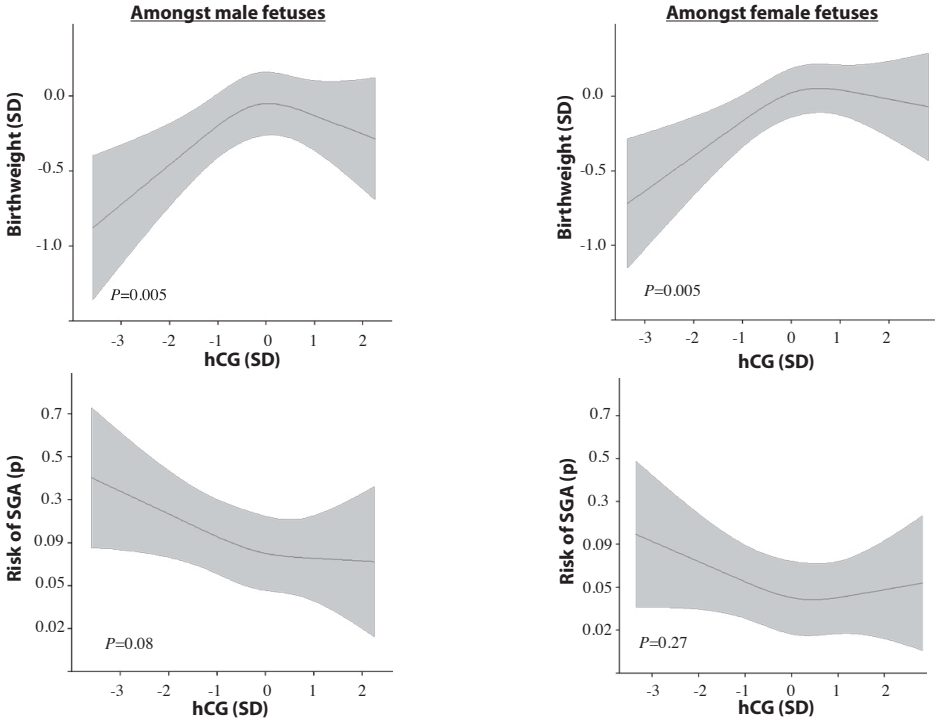
Plot shows the linear regression model for total hCG (SD) and crown-rump length (adjusted according to week of gestation; available in n=1526;SD) as predicted mean with 95 percent confidence interval. Analyses were adjusted for maternal age, smoking, BMI, parity, education level, ethnicity, gestational weight gain and fetal sex.

Supplemental Figure 5. The association of maternal hCG per week of measurement with birth weight stratified by fetal sex



Plots show the logistic and linear regression models for total hCG (standardized according to gestational age at measurement; SD) and birthweight (standardized according to gestational age at birth; SD) or small for gestational age (defined as birthweight below 10th percentile for gestational age) as predicted mean with 95 percent confidence interval. Analyses were adjusted for maternal age, smoking, BMI, parity, placental weight at birth, education level, ethnicity and gestational weight gain.

Supplemental Figure 6. The association of maternal hCG measured in the 11th or 12th week with birth weight stratified by fetal sex



Plots show the linear and logistic regression models for total hCG (standardized to gestational age; SD) and birthweight (standardized to gestational age at birth; SD) or small for gestational age (defined as <10th percentile of gestational age specific birth weight) as predicted mean with 95 percent confidence interval. Analyses were adjusted for maternal age, smoking, BMI, parity, placental weight at birth, education level, ethnicity, gestational age at birth and gestational weight gain.

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CHAPTER 4

Thyroid function in childhood

CHAPTER 4.1

Childhood thyroid function reference ranges and determinants: a literature overview and a prospective cohort study

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ABSTRACT

Background: Reported cutoffs for childhood TSH and FT4 reference ranges vary widely and the knowledge on the determinants of childhood thyroid function is sparse. We aimed to summarize the existing studies on thyroid function reference ranges in children. Furthermore, our objective was to investigate the determinants of childhood TSH and FT4 concentration in a population based-prospective cohort.

Methods: First, to identify studies on childhood thyroid reference ranges, we systematically searched The National Library of Medicine's PubMed, Embase, Ovid Medline, Web of Science and Google Scholar databases. Second, in a non-selected sample of 4273 children (median age 6.0 years, range 4.9 – 9.1 years) from our cohort, we studied the associations of age, sex, anthropometric characteristics, ethnicity, maternal education, time and season at venipuncture with TSH and FT4 concentrations. We also investigated to what extent between-individual variations in the determinants of TSH and FT4 could influence the calculation of reference ranges.

Results: Published reference ranges for TSH and FT4 differ per age range and within age ranges (cutoffs low TSH: 0.13 to >1 mU/L; high TSH: 2.36 to >10 mU/L; low FT4: 7.0 to >10 pmol/L; high FT4: 15.5 to >30 pmol/L). In our cohort, weight, sex and ethnicity were determinants of TSH ($P \leq 0.03$) and FT4 concentrations ($P \leq 0.01$), height and time at venipuncture were determinants of TSH only ($P < 0.0001$). The between-individual variation depending on clinical determinants for TSH ranged between 0.64 and 0.96 mU/L (total population 0.87 mU/L) for the lower limit and 4.30 and 5.62 mU/L (total population 5.20 mU/L) for the upper limit, whereas for FT4, the lower limit ranged between 13.6 and 14.2 pmol/L (total population 13.8 pmol/L) and the upper limit ranged between 20.2 and 23.0 pmol/L (total population 20.8 pmol/L).

Conclusions: Considerable differences exist in the reported reference ranges for childhood TSH and FT4 across and within age ranges and assays. In our cohort, we show only a minimal association between TSH and FT4 suggesting that the hypothalamus-pituitary-thyroid axis remains unaffected by thyroid interfering factors. We identified various determinants of TSH and FT4 in children which accounted for a considerable variation of reference range cutoffs.

INTRODUCTION

Adequate thyroid function is important for proper growth and development in childhood [1,2]. Hypothyroidism in childhood is associated with cognitive deficits, decelerated growth, delayed skeletal maturation and delayed puberty, whereas overt hyperthyroidism is associated with growth acceleration, advanced bone age, tachycardia and mood disorders [2,3]. Furthermore, even mild forms of thyroid dysfunction in childhood are associated with suboptimal outcomes including weight gain, impaired growth velocity, poor school performance, impaired psychomotor skills, disturbed cognitive development and increased cholesterol levels [4-6].

In order to diagnose thyroid disease, adequate reference ranges for TSH and FT4 are essential. The guidelines of the European Thyroid Association for the management of subclinical hypothyroidism in children recommend the use of age-related normative values [7]. However, there is no further consensus on the definition of TSH and FT4 reference ranges during childhood. This complicates the interpretation of thyroid function tests and clinical diagnosis of thyroid dysfunction, as is for example illustrated by the wide range of TSH cutoffs (between 5.0 to 10 mU/L) currently used to define subclinical hypothyroidism during childhood [7].

Multiple studies have been performed to define thyroid function reference ranges in pediatric populations [8-43]. Although some studies adhere to the recommendations by the International Federation of Clinical Chemistry, there is considerable between-study heterogeneity as studies have been conducted across various age ranges, using different assays and in populations comprising different ethnicities and from different geographical conditions. It is currently unknown to what extent between-study variations in methodology and between-population differences in thyroid function determinants add to this heterogeneity.

Although some studies have indicated that TSH and FT4 reference ranges are influenced by child age, Tanner stage, ethnicity, anthropometric characteristics and/or iodine intake, data on determinants of thyroid function during childhood are sparse [22,43,44]. Further knowledge on determinants of TSH and FT4 concentrations during childhood may help to identify specific causes that underlie an abnormal test result. In addition, such knowledge enables physicians to assess the generalizability of described reference ranges to a specific patient population. With regards to the research setting, knowledge on determinants is important to define mediating and/or confounding factors that can influence studies on the effects of thyroid function on clinical outcomes.

The aim of the current study was to systematically assess and summarize the current literature on thyroid function reference ranges during childhood in order to create a general overview of TSH an FT4 reference ranges during childhood. Subsequently, in a large, iodine sufficient pediatric population, we aimed to investigate which clinical

characteristics are determinants of thyroid function and quantify to what extent these determinants affect reference ranges for TSH and FT4.

METHODS

Literature overview

A systematic literature search of The National Library of Medicine's PubMed, Embase, Ovid Medline, Web of Science and Google Scholar databases was performed to identify studies published from inception until November 18th, 2016 (search terms are outlined in the supplemental appendix). Two reviewers (IO, MB) independently screened the obtained titles and abstracts and, subsequently reviewed the full manuscripts for their eligibility for the literature overview. These individual results were cross-checked and upon disagreement a third reviewer (TK) assessed the manuscript to reach consensus. Studies with less than 400 participants, non-representable sample selection for the underlying population and the use of parametric methods (other than those corresponding to a 95% range) used to calculate reference ranges for thyroid function were not included for summary results. [11,15,18,20,21,23-26,28,31-33,38,42]

Original study

Design and participants

This study was embedded in The Generation R Study, a population-based prospective cohort from early fetal life onwards in Rotterdam, the Netherlands. This study has been described in detail elsewhere [45]. In total, all children with consent for follow-up during childhood (N=8305) were invited to visit the research center of which 6674 children visited. After consent by the mother and child, serum samples were obtained for 4593 children and TSH and/or FT4 concentrations were determined in 4286 samples with adequate serum volumes. Children with known thyroid disease, chronic illness (endocrine, inflammatory, autoimmune, cancer or kidney disease) or thyroid (interfering) medication usage (levothyroxine or growth hormone) were excluded (N=13), resulting in a final population of 4273 children (median age 6.0 years, range 4.9 – 9.1 years).

Determinants and covariates

We selected potential determinants based on the literature, biological plausibility and data availability [13,22,46]. These included age, sex, ethnicity, height, weight, maternal education level (as a marker of social economic status), time and season of venipuncture. Information on these determinants was obtained by questionnaires and measurements

during the visit to the research center (on the same day as blood sampling). Medical history was assessed by questionnaires and answers were verified by certified medical doctors. Information on maternal education level was obtained through postal questionnaires. Child ethnicity was determined by the country of origin of the child and/or parents and was defined according to the classification of Statistics Netherlands and categorized according to the major ethnic groups in Rotterdam.[45] These were: Dutch, Turkish, Moroccan, Surinamese, Dutch Antilles, African/Cape Verdian, other Western (European, Oceanian and Caucasian descent Americans /Asians) and other non-Western.

Procedures

Plain tubes were centrifuged and serum was stored at -80°C. Child TSH and FT4 concentrations were determined using an electrochemiluminescence immunoassay on the Cobas e601immunoanalyzer (Roche Diagnostics, Germany). The intra- and interassay coefficients of variation were 1.1 – 3.0 % for TSH at a range of 0.4 – 0.04 mU/l and 1.6 – 5.0 % for FT4 at a range of 1.6 - 24.1 pmol/l.

Statistical analyses

Reference ranges for TSH and FT4 in the Generation R were defined by the 2.5th and 97.5th percentiles. For analyses aimed to identify thyroid function determinants, TSH concentrations were log transformed to adhere to model assumptions (back-transformed values are displayed in graphs to allow for better interpretation). We used multiple linear regression models to investigate the association between the determinants and childhood TSH or FT4. Non-linearity of the association between continuous variables and childhood TSH or FT4 concentrations was investigated by ordinary least squares linear regression models with restricted cubic splines utilizing 3-5 knots. As a sensitivity analysis, we repeated the analyses after exclusion of children with TSH or FT4 concentrations outside of the 95% range to investigate the effect of potential data outliers. To study the effects of highest and lowest values of TSH and FT4 determinants on the TSH and FT4 reference ranges, we calculated the 95% range for both TSH and FT4 at the highest 10% and lowest 10% of each determinant. We used multiple imputation to cope with missing data for determinants/covariates. The multiple imputation model included maternal education level, ethnicity of the child, height, weight, age, sex, time of venipuncture and season (missing data in 15.4%, 2.5%, 0.2%, 0.2% and for the rest, 0%, respectively) and TSH and FT4 concentrations were used as prediction variables only. Five imputed data sets were created and pooled for further analyses. There were no statistically significant differences between the original and imputed datasets. All analyses were performed using Statistical Package of Social Sciences version 20.0 for Windows (SPSS Inc. Chicago, IL, USA) and R statistical software version 3.03 (‘*rms’* package).

RESULTS

Literature overview

The systematic search yielded 4704 studies of potential interest of which 4620 studies were excluded after assessment of the title and the abstract. After further examination, 35 studies were finally included for extraction of the data on TSH and/or FT4 reference ranges (Supplemental Figure 1). An overview of all included studies and reference ranges for various age categories is shown in Supplemental Tables 1-8. In general, the variability of reported upper and lower limits reference ranges for TSH and FT4 was the highest in the first week of life and became lower as the age of the study population becomes older. Similar effects were observed in studies with longitudinal data on TSH and FT4 reference ranges [22-28]. There were considerable differences in the reported lower and upper limits of TSH and FT4, as is shown in Table 1. Taken together, in children aged ≥1 years the lower limit for TSH ranged between 0.32 and 1.30 mU/L while the upper limit for TSH ranged from 2.36 to 6.57 mU/L (Table 1). Furthermore, in children aged ≥1 years, the lower limit for FT4 in these age groups ranged between 7.0 and 18.0 pmol/L, and the upper limit for FT4 ranged between 15.5 and 34.7 pmol/L (Table 1).

Table 1. The ranges for lower and upper limits of TSH and FT4 reference ranges

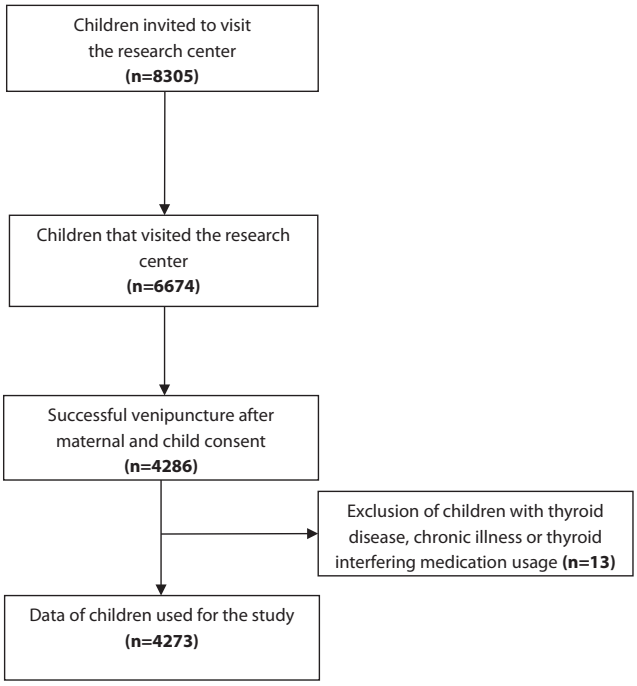
	1 to 7 days	7 days to 3 months	3 months to 1 year	1 year to 5 years	5 years to 10 years	11 years to 20 years
TSH cut-offs (mU/l)						
Lower limit	0.13 – 1.79	0.16 – 1.80	0.30 – 1.80	0.53 – 0.97	0.48 – 1.30	0.32 – 0.88
Upper limit	9.23 – 57.2	4.38 – 12.56	4.23 – 8.14	2.94 – 6.57	3.36 – 5.66	2.36 – 6.45
FT4 cut-offs (pmol/l)						
Lower limit	8.9 – 20.3	8.9 – 19.3	9.2 – 12.3	9.0 – 18.0	8.3 – 14.4	7.0 – 14.2
Upper limit	26.8 – 46.6	21.3 – 33.1	19.5 – 25.3	19.0 – 34.7	16.4 – 24.6	15.5 – 31.5

Reference ranges derived from 2.5th and 97.5th percentiles. Reference ranges that were calculated in populations with overlapping age ranges were counted for the category with most overlap.
Derived from the data extracted from the reviewed studies

Original study

We studied which child characteristics are determinants of thyroid function. Subsequently, we aimed to identify to what extent differences in such determinants between populations underlie the large between-study differences in reference range limits for TSH and FT4. Descriptive characteristics of the study population are shown in Supplemental Table 9. After exclusions, the final study population comprised N=4273 children (Figure 1). There were no considerable differences in characteristics between children with or without data available on TSH or FT4 concentrations (Supplemental Table 10). Child serum samples were obtained at a median age of 6.0 years (95% range 5.7 – 8.0

Figure 1. Flowchart showing selection procedure of the study population

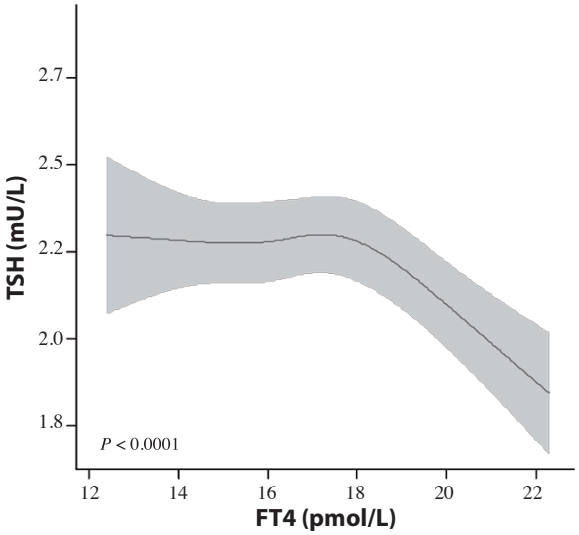


years) and the majority of subjects were of Dutch origin (57.8%; Supplemental Table 9). The number of drawn samples was equally distributed throughout the year and samples were on average taken in the afternoon (median time 14:02h, 95% range: 11.17-5.17h, Supplemental Table 9). The median and reference range (2.5th - 97.5th percentile) for TSH concentrations were 2.30 and 0.87 – 5.20 mU/L, respectively (Table 2). The median and reference range (2.5th - 97.5th percentile) for FT4 were 16.8 and 13.8 – 20.8 pmol/L, respectively (Table 2). There was a negative, non-linear association of FT4 with TSH, exhibiting a stable TSH concentration across FT4 concentrations ranging between roughly 12 and 18 pmol/L (Figure 2).

Assessment of thyroid function determinants

Boys had a higher TSH concentration than girls (Figure 3; P<0.0001). TSH differed according to ethnicity, with the lowest concentration in children of Dutch Antilles origin and the highest concentration in Dutch children (Figure 3; P=0.0003). Height was negatively associated with TSH (P<0.0001) and there was a positive linear association of weight with TSH (Figure 3; P=0.03). There was a U-shaped association of time at venipuncture with TSH, with higher TSH concentrations during the morning and late afternoon (Figure 3; P<0.0001). Age, maternal education and season at venipuncture were not associated with TSH concentrations (Figure 3, P=0.90; Supplemental Figure 2, P=0.20 and P=0.15, respectively).

Figure 2. The association of FT4 with TSH concentrations



Plot shows the association of FT4 with TSH in children (median age 6 years, 95% range 5.7-8.0) with corresponding 95% confidence interval, adjusted for age, sex, ethnicity, height, weight, time at venipuncture, season and maternal education.

Boys had a lower FT4 concentration than girls (Figure 4; $P<0.0001$). FT4 differed according to ethnicity, with the lowest concentration in Dutch children and the highest concentration in children of non-Western or Surinamese origin (Figure 4; $P<0.0001$). There was a negative linear association of weight with FT4 concentrations (Figure 4; $P=0.002$). There was a non-linear association of age with FT4 concentrations in which FT4 was higher at the lower age range (Figure 4; $P=0.01$). Season at venipuncture was associated with FT4, with the highest FT4 concentration during autumn and higher maternal education was associated with lower FT4 (Supplemental Figure 2; $P=0.006$ and $P<0.0001$, respectively). Height and time at venipuncture were not associated with FT4 (Figure 4, $P=0.10$ and $P=0.23$, respectively). All results remained similar after exclusion of children outside of the 95% reference range for TSH and/or FT4 or when age-standardized values for height or weight were studied.

Subsequently, reference ranges were stratified according to the studied thyroid function determinants and their highest and lowest values (10% and 90% cut-offs; Table 2). The lower limit of TSH in our study ranged between 0.64 and 0.96 mU/L (total population 0.87 mU/L) according to between-individual variation in clinical determinants (Table 2). The upper limit ranged between 4.30 and 5.62 mU/L (total population 5.20 mU/L; Table 2). For FT4, the lower limit ranged between 13.6 and 14.2 pmol/L (total population 13.8 pmol/L) and the upper limit ranged between 20.2 and 23.0 pmol/L (total population 20.8 pmol/L) according to between-individual variation in clinical determinants (Table 2).

Table 2. Reference ranges for TSH and FT4 stratified by the 10 and 90% cut-offs of thyroid function determinants in the total population.

	TSH (median: 2.30, 95% range: 0.87 – 5.20 mU/L)				**FT4** (median: 16.8, 95% range 13.8 – 20.8 pmol/L)			
	Low range (<10%)	**High range** (>90%)	**Difference** (mU/L)		**Low range** (<10%)	**High range** (>90%)	**Difference** (pmol/L)	
			Lower limit	Upper limit			Lower limit	Upper limit
Age	0.88 – 5.28	0.67 – 5.07	0.21	0.21	13.8 – 20.7	13.8 – 21.4	0.0	0.7
Height	0.85 – 5.36	0.79 – 4.97	0.06	0.39	13.7 – 21.4	13.8 – 20.8	0.1	0.6
Weight	0.82 – 5.09	0.86 – 5.19	0.04	0.10	13.7 – 21.1	13.7 – 20.5	0.0	0.6
Time at venipuncture*	0.86-5.12	0.75–4.45 0.89-5.38	0.14	0.93	14.0 – 21.2	13.9 – 20.3	0.1	0.9
Gender								
Boys	0.95 – 5.28		0.12	0.18	13.7 – 20.4		0.1	0.7
Girls	0.83 – 5.10				13.8 – 21.1			
Ethnicity								
Dutch	0.90 – 5.35		0.32	1.32	13.7 – 20.5		0.6	2.5
Other non-Western	0.76 – 5.22				13.8 – 21.0			
Cape Verdian/African	0.79 – 5.05				13.9 – 20.5			
Moroccan	0.85 – 4.30				14.2 – 20.5			
Dutch Antillean	0.64 – 4.68				13.7 – 23.0			
Surinamese	0.75 – 5.15				13.7 – 21.7			
Turkish	0.96 – 5.24				14.0 – 21.1			
Other Western	0.95 – 5.62				13.6 – 20.7			
Maternal education level								
Low education	0.87 – 5.17		0.15	0.16	13.9 – 21.8		0.2	1.6
Middle	0.90 – 5.28				13.8 – 21.1			
Higher phase 1	0.85 – 5.12				13.7 – 20.6			
Higher phase 2	1.00 – 5.24				13.7 – 20.2			

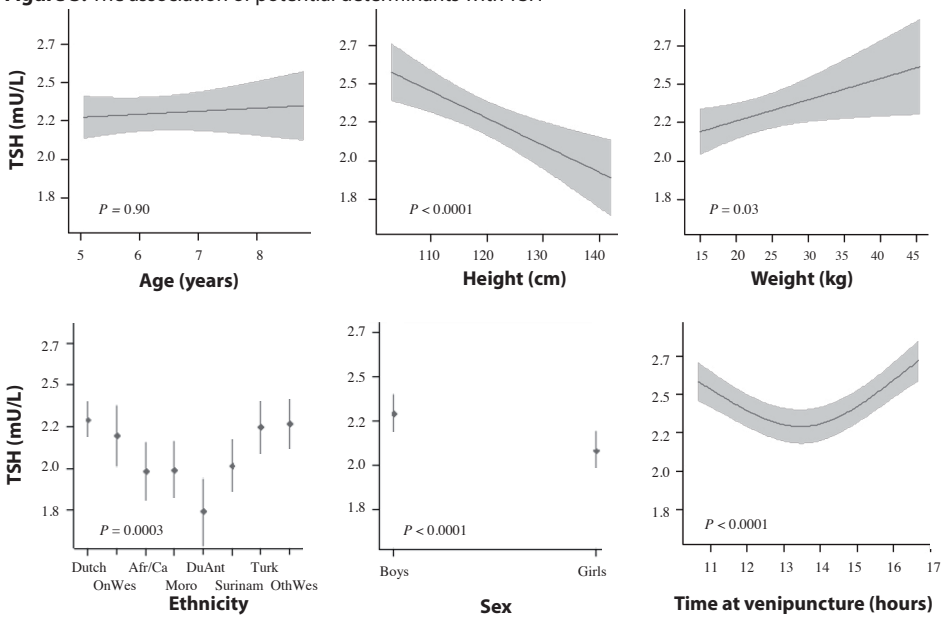
Table 2. (continued)

	TSH (median: 2.30, 95% range: 0.87 – 5.20 mU/L)				**FT4** (median: 16.8, 95% range 13.8 – 20.8 pmol/L)			
	Low range (<10%)	**High range** (>90%)	**Difference** (mU/L)		**Low range** (<10%)	**High range** (>90%)	**Difference** (pmol/L)	
			Lower limit	Upper limit			Lower limit	Upper limit
Season at venipuncture								
Spring	0.86 – 5.30				13.6 – 20.2			
Summer	0.77 – 4.9		0.19	0.49	13.7 – 21.1		0.3	0.9
Autumn	0.90 – 5.35				13.9 – 20.9			
Winter	0.96 – 5.39				13.7 – 20.7			

Table shows the 95% range of TSH and FT4 in the highest and lowest 10% values of the determinants.

* Data shown as median 10% versus highest 10% and lowest 10%, due to a non-linear association of time at venipuncture with TSH

Figure 3. The association of potential determinants with TSH



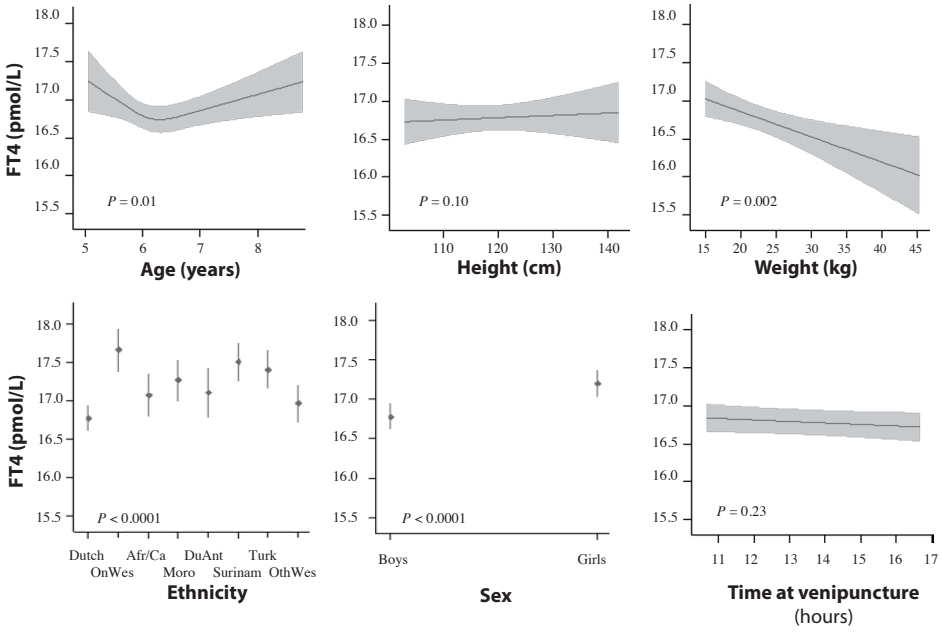
Different biological determinants and their association with TSH with corresponding 95% confidence interval. Every association has been adjusted for the remaining determinants and further adjusted for season of the year and maternal education status (supplement).
OnW: Other non western ethnicities. Afr/Ca: African and Cape Verdian. Moro: Moroccan. DuAnt: Dutch Antilles. Surinam: Surinamese. Turk: Turkish. OthWes: other western ethnicities.

We performed sensitivity analyses to examine whether the association of the various determinants with TSH and FT4 concentrations differs based on ethnicity but we did not identify any relevant effect modification (data not shown).

DISCUSSION

In the current study, we provide a literature overview of published reference ranges for thyroid function in children, demonstrating large differences in the reported reference ranges for TSH and FT4 during childhood. Differences were present across different age-categories, between studies using different assays but also between studies utilizing a similar assay. Subsequently, in our population-based cohort, from an iodine sufficient area, we identified child age, sex, ethnicity and various anthropometric characteristics as thyroid function determinants. Already within this population, between-individual variation in a single clinical determinant accounted for variation in the lower and upper cut-offs of 0.64 to 0.96 mU/L and 4.30 to 5.62 mU/L for TSH, and 13.6 to 14.2 pmol/L and 20.2-23.0 pmol/L for FT4.

Figure 4. The association of potential determinants with FT4



Different biological determinants and their association with FT4 with corresponding 95% confidence interval. Every association has been adjusted for the remaining determinants and further adjusted for season of the year and maternal education status (supplement). OnW: Other non western ethnicities. Afr/Ca: African and Cape Verdian. Moro: Moroccan. DuAnt: Dutch Antilles. Surinam: Surinamese. Turk: Turkish. OthWes: other western ethnicities.

In both the clinical as well as the research setting, there is very little consensus on how to define an abnormal thyroid function in children and reference ranges [7]. For example, some studies from our literature overview defined pediatric reference ranges for thyroid function using a non-parametric approach utilizing the 2.5th-97.5th range or the 5th-95th range to define a normal TSH or FT4, while others used a (semi-) parametric approach defining normality based on +/- 1.96 or 2 standard deviations from the mean [25]. Such methodological differences are the most likely cause of the large differences in pediatric reference ranges for TSH and FT4 in the literature, as shown in our literature overview. These differences hamper translation of research findings to the clinical setting and also affect the accuracy of the literature summary. Our findings demonstrate the need for standardization of reference range methodology in this field and suggest that further studies are required to optimize clinical diagnosis of thyroid disease in children.

Apart from differences in the methodology of calculating reference ranges, also the study size, study population selection and exclusion of individuals with major disease known to affect thyroid function may play an important role [47]. Many studies identified through our literature overview lack a sufficiently sized population to generate appro-

priate reference ranges for different age intervals. Although a minimum of 120 subjects is often proposed to define reference ranges, this is only recommended as an absolute minimum for the calculation of non-parametric 90% coverage intervals (e.g. 5th and 95th percentile reference ranges) [48-51]. However, because of the high inter-individual variability and skewness of TSH and to some extent also FT4, a minimum of approximately 400 individual measurements per partition is required for these measurements [48-51]. As shown in our literature overview, 14 studies presented data derived from less than 100 measurements [11,15,18,20,21,23,24,26,28,31-33,38,42].

Another important determinant of the large differences in reference ranges for TSH and FT4 concentrations is the assay that is used. While most studies used an immunoassay, some studies used equilibrium dialysis and/or LCMS [8,20]. However, even when similar assays were used, large between-study differences were present. For comparison, for the 5 to 8-year-old children from our cohort study, the reference range was 0.87 – 5.20 mU/L for TSH. Studies that used a similar assay report a TSH reference range that lies anywhere between 0.48 and 5.66 mU/L [8,12,22]. This variation may suggest that also differences in population characteristics can account for some of the between-study differences in thyroid function reference ranges. Although characteristics such as child age and anthropometry have previously been identified as determinants of thyroid function, it is unknown to what extent these may affect reference ranges [22,43,44]. In our population-based cohort study, we show that already within a population representing children from a small geographical area, lower and upper cut-offs for TSH may vary up to 0.21 mU/l and 0.93 mU/L (up to 21% and 31%), respectively, according to a single thyroid function determinant. For lower or upper FT4 cut-offs, this variation was much lower (0-2.5 pmol/L, or up to 4.3% and 12.0%, respectively). This larger variation according to determinants in TSH than in FT4 may reflect only mild alterations in the hypothalamic-pituitary-thyroid axis (HPTa). In the vast majority of young children, it is likely that the HPTa is not yet subjected to pathophysiological processes such as development of toxic nodules or thyroid autoimmunity. This is supported by the results from our cohort study, showing a stable association of FT4 with TSH. Therefore, we speculate that the majority of the between-individual differences in TSH and FT4 presented in this study are more likely caused by differences in the HPTa set point, for example based on genetic variation [52]. In order to further clarify the explained variability in thyroid function, genetic studies in children could thus prove to be valuable. Higher TSH values in boys compared to girls that we identified differ from study results in adults in which women tend to have higher TSH values compared to men [53]. These differences could perhaps be explained by genetic differences which cause more prominent TSH differences at the age where less pathophysiology is present, or alternatively, slight sex differences could be present in the maturation of the HPTa. Another relevant difference in determinants of TSH and FT4 as identified in the current paper is that height was associated with TSH but not

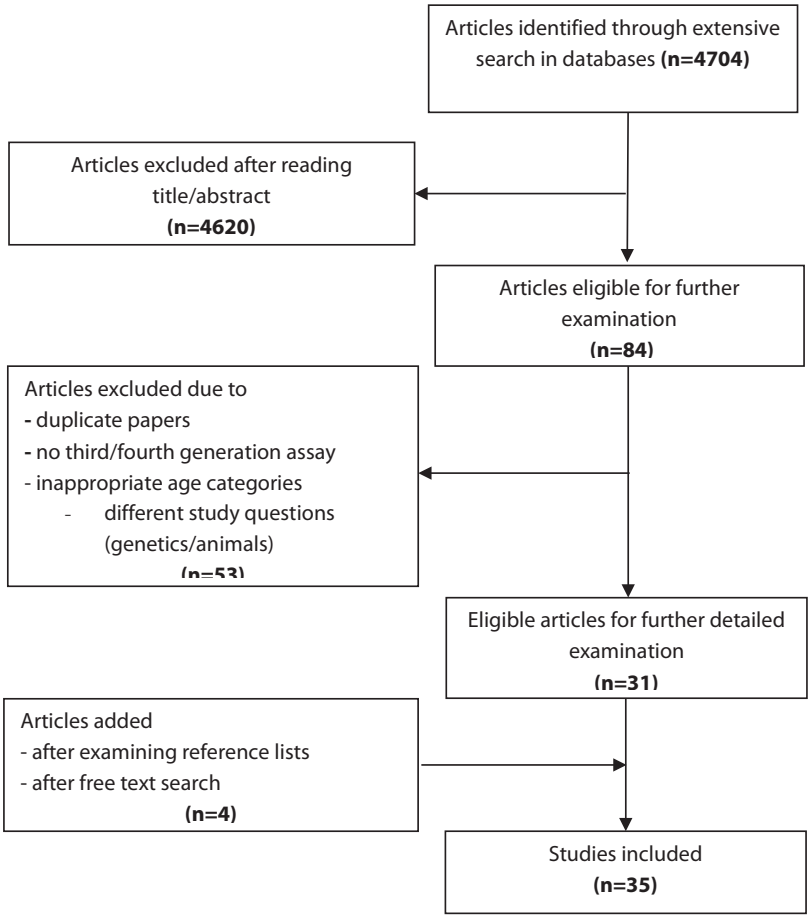
FT4 while weight was associated with both TSH and FT4. This may indicate that the association of height with TSH is perhaps caused by genetic pleiotropy, implicating the existence of genes affecting both the HPTa set-point as well as height, while weight is more likely to interfere with the HPTa [54]. Although the fat component of weight could potentially increase TSH and decrease FT4 via higher leptin concentrations and higher TBG concentrations [54], respectively, we demonstrated that the lean body mass but not fat mass is associated with FT4 concentrations in a previous study [55]. Since it is likely that the association of body composition with thyroid function is at least partly subject to reverse causation [56], further studies are needed to clarify the underlying mechanisms of our results.

In the current literature overview, we provide a detailed summary of the existing studies on the thyroid function reference ranges during childhood. Furthermore, we were able to study clinical determinants of thyroid function in a large prospective population-based cohort of children living in an iodine sufficient area. A limitation of this study is the fact that our population comprised a relatively narrow age range. It is therefore not possible to extrapolate our results to other age categories. These data should be collected in the future. In addition, venipuncture in our study was mostly done in the afternoon (median time 14:02 h, 95% range 11:20 – 17:20 h) in a non-fasting state. In clinical practice, determining thyroid function usually occurs in the morning, in a fasting state. Current literature about childhood reference ranges does not often consider the time of venipuncture or the fasting state [9,14,19,22-28,31,36,41,42], which makes the comparison between studies challenging. Furthermore, several studies have determined reference ranges in different fasting and non-fasting states [13,21,29,33], which could potentially complicate the interpretation of reference ranges even further. The lower concentrations of TSH in our study when the time of venipuncture was in the early afternoon could be partially mediated by food intake, as FT3 concentrations rise and TSH concentrations decrease postprandially [57]. However, the design of this study was not adequate for investigating postprandial thyroid function changes as breakfast or lunch were not consumed at set times and snacks were provided to the children during the visit. In addition, opposite to some studies [13,21,29,33], the majority of studies on childhood thyroid function reference ranges do not consider the time of venipuncture or the fasting state [9,14,19,22-28,31,36,41,42]. Based on our results regarding the time of venipuncture, this makes the comparison of thyroid function tests between studies and between individuals in a clinical setting more challenging. Another potential limitation of our cohort study is that we did not have available data on TPO antibodies (Ab). However, it is unlikely that the relatively short exposure to thyroid autoimmunity in children with a median age of 6 years already affects thyroid function. This is illustrated by the fact that lower FT4 concentrations were not associated with higher TSH concentrations in our cohort study. Moreover, it has been shown that thyroid function in children with positive serum TPO Abs is not lower than in

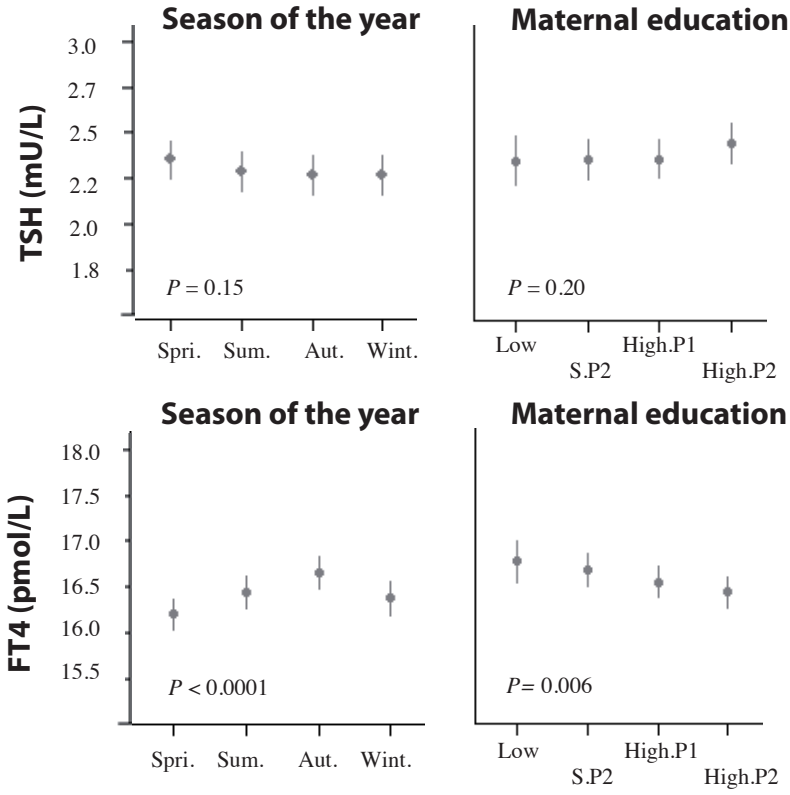
TPOAbs negative children [58]. Finally, the observational nature of the population-based cohort study leaves the possibility of residual confounding and the uncertainty about causality within studied associations.

In conclusion, in the current literature overview we demonstrate a large heterogeneity in pediatric thyroid function reference ranges in the existing literature. In our population-based cohort study, we demonstrate a minimal association of TSH and FT4, suggesting that the HPTa in children is still unaffected by thyroidal pathological processes and we show that child age, sex, ethnicity, anthropometry and time of venipuncture are determinants of TSH and/or FT4 concentrations and that between-individual variations in these determinants can influence the calculation of reference ranges. The identification of these determinants and quantification of their effects can help with the interpretation of thyroid function tests. Future efforts should focus on generating evidence based recommendations to define abnormal thyroid function in children, in order to tackle the large heterogeneity in the current literature.

Supplemental Figure 1. Flowchart of the literature search strategy



Supplemental Figure 2. Association of the season of blood sampling and maternal education with thyroid function with 95 percent confidence interval



Supplemental Table 1. Reference ranges of TSH for children from 1 day to 1 year old in literature. *5th and 95th percentile , **18% of samples measured by Vitros 5600, † reference ranges based on -2 to 2 standard deviation, **NC** = size of study population is not reported**, NR** – median not reported.

Assay	N	Females (F) Males (M)	TSH as 2.5th – 50th – 97.5th percentile					
			1 – 7 days	8 – 15 days	15 days – 1 month	1 – 3 months	3 – 6 months	6 – 12 months
Abbott Architect								
Bailey et al., 2013	**278**	**F: 139 M: 139**			0.73 – NR – 4.77			
Chaler Aldrimer et al., 2012 (Abbott AxSYM)		**694**			0.92 – 2.30 – 4.38		0.79 – 2.24 – 4.23	0.84 – 2.37 – 4.31
Soldin et al., 2010	**290**	**F: 152 M: 138**					F: 1.12– NR – 4.47 M: 0.96 – NR – 4.90	
Chan et al., 2009		**71**			0.88 – NR – 5.42			
Access								
Romero et al., 2014	**175**	**F: 78 M: 97**		F: 0.31 – 2.70 – 6.68 M: 0.99 – 2.31 – 6.82		F: 0.85 – 1.82 – 7.06 M: 0.79 – 2.06 – 6.17		F: 0.72 – 1.84 – 5.17 M: 0.77 – 1.74 – 6.11
Djemli et al., 2004* (Access 2)	**24**	**F: 12 M: 12**		F: 1.5 – 3.3 – 6.5 M: 0.7 – 2.4 – 9.8				
Advia Centaur								
Loh et al., 2015** (Advia Centaur Vitros 5600)		**NC**	0.82 – NR – 12.08	0.91 – NR – 10.63	1.12– NR – 8.77			
Strich et al., 2012		**425**		1.08 – NR – 11.80		0.68– NR – 12.56 (to 2 months)	0.62 – NR – 7.3 (from 2 months)	
Kapelari et al., 2008		**64**		0.7 – 3.5 – 18.10			1.12 – 2.85 – 8.21	
Hubner et al., 2002		**460**	0.13– NR – 9.23 (to 3 days)	0.16 – NR – 8.48 (from 4 days)		0.3 – NR – 5.88 (from 2 months)		

Supplemental Table 1. (continued)

Assay	N	Females (F) Males (M)	TSH as 2.5th – 50th – 97.5th percentile					
			1 – 7 days	8 – 15 days	15 days – 1 month	1 – 3 months	3 – 6 months	6 – 12 months
Delfia								
Zurakowski et al., 1999	**289**	**F: 131** **M: 158**				F: 0.8 – NR – 6.3 (to 11 months) M: 0.8 – NR – 6.3		
Immulite								
Verburg et al., 2011		**308**	0.32 – 3.11 – 12.27	0.34 – 3.01 – 11.44	0.36 – 2.80 – 9.75	0.32 – 3.25 – 11.21		0.38 – 2.62 – 8.14
Fideleff et al., 2010		**334**	1.10 – 4.70 – 12.70 (2-3 days)	1.50 – 4.10 – 7.70		1.20 – 3.90 – 6.90 (28-40 days)		
Najam et al. 2003		**1104**	1.24 – 8.00 – 27.50 (0-4 days) 0.4 – 5.00 – 13.95 (5-7 days)			0.7 – 9.00 – 17.56 (1 month)		0.31 – 2.5 – 14.51 (1 year)
Elmlinger et al., 2001		**85**	1.79 – 4.63 – 9.69	1.80 – 3.71 – 7.97				
Roche								
Omuse et al., 2016 (Roche e601)	**1639**	**F: 865** **M: 774**	F: 0.56 – 2.74 – 11.00 M: 0.59 – 3.31 – 12.84		0.90 – 2.71 – 7.46			
Kratzsch et al., 2008 (Roche Elecsys)		**273**	0.71 – 6.88 –57.2		0.99 – 3.89 –10.9		0.61 – 3.42 – 10.7	
Vitros								
Lem et al., 2012† (Vitros Eci Technology)		**512**	1.90 – 5.54 – 17.58 (1 day) 1.40 – 4.64 – 13.10 (2 days) 0.94 – 3.75 – 9.65 (3 days) 0.60 – 2.85 – 6.82 (4 days) 0.58 – 2.14 – 5.58 (7 days)	0.58 – 2.14 – 5.57 (1 month)			0.58 – 2.14 – 5.57 (3 months) 0.58 – 2.14 – 5.56 (6 months)	0.57 – 2.13 – 5.54 (1 year)

Supplemental Table 2. Reference ranges of TSH for children from 1 year to 5 years old in literature. *5th and 95th percentile, **18% of samples measured by Vitros 5600, † reference ranges based on -2 to 2 standard deviation , **NC** = size of study population is not reported, **NR** – median not reported.

Assay	N	Females (F) Males (M)	TSH as 2.5th – 50th – 97.5th percentile			
			1 – 2 years	2 – 3 years	3 – 4 years	4 – 5 years
Abbott Architect						
Chaler et al., 2012 (Abbott AxSYM)		**1826**	0.97 – 2.46 – 4.35		0.97 – 2.49 – 4.38 (to 6 years)	
Soldin et al., 2010	**961**	**F: 467** **M: 494**	F: 1.00 – NR – 4.37 M: 0.93 – NR – 4.79		F: 0.85 – NR – 4.07 M: 0.83 – NR – 4.37	
Chan et al., 2009	**307**	**F: 155** **M: 152**		F: 0.66 – NR – 4.75 M: 0.67 – NR – 4.50		
Access						
Romero et al., 2014	**230**	**F: 118** **M: 112**	(13-18 months) F: 0.51 – 2.03 – 4.85 M: 0.50 – 2.22 – 4.56 (19-23 months) F: 0.51 – 1.79 – 4.98 M: 0.87 – 2.34 – 5.33	F: 0.57 – 1.71 – 4.23 M: 0.70 – 1.89 – 4.45	(to 6 years) F: 0.79 – 2.00 – 5.32 M: 1.07 – 1.84 – 4.20	
Djemli et al., 2004* (Access 2)	**73**	**F: 27** **M: 46**	(from 1 month) F: 1.0 – 2.4 – 5.7 M: 0.7 – 2.1 – 5.9			
Advia Centaur						
Loh et al., 2015** (Advia Centaur Vitros 5600)		**NC**	0.74 – NR – 5.68 (from 2 months)			
Strich et al., 2012		**2782**		0.75 – 6.57		
Kahapola et al., 2012	**215**	**F: 91** **M: 124**		0.69 – 1.85 – 3.91 F: 0.65 – 1.94 - 3.82 M: 0.81 – 1.85 – 3.92		
Kapelari et al., 2008		**218**		0.80 – 2.70 – 6.26		
Hubner et al., 2002		**460**		0.42 – 1.98 – 4.79		

Supplemental Table 2. (continued)

Assay	N	Females (F) Males (M)	TSH as 2.5th – 50th – 97.5th percentile			
			1 – 2 years	2 – 3 years	3 – 4 years	4 – 5 years
Delfia						
Cioffi et al., 2001* (AutoDelfia)		**778**	0.3 – NR – 5.9 (2 years)	1.2 – NR – 5.8 (3 years)	1.0 – NR – 6.1 (4 years)	0.8 – NR – 4.5 (5 years)
Zurakowski et al., 1999	**1182**	**F: 523 M: 659**	F: 0.7 – 5.9 M: 0.7 – 6.0			
Immulite						
Verburg et al., 2011		**83**			0.66 – 2.18 – 5.15	
Elmlinger et al., 2001		**<86**	0.63 – 2.04 – 4.12 (from 1 month to 3 years)			0.53 – 1.60 – 2.94 (to 6 years)
Roche						
La'ulu et al., 2016 (Roche E170)	**594**	**F: 281 M: 313**	(from 6 months) F: 0.85– NR – 5.78 M: 1.07 – NR – 7.57		F: 0.80 – NR – 6.90 M: 1.10 – NR – 6.56	
Henderson et al., 2011 (Roche E170)		**45**	(to 6 years) Non-parametric: 1.3 – NR – 5.5 (to 6 years) Robust: 0.7 – NR – 6.1			
Kulasingam et al., 2010 (Roche cobas 6000)		**189**	0.84 – NR – 6.22 (from birth - 5 years)			
Kratzsch et al., 2008 (Roche Elecsys)		**247**	0.60 – 2.60 –5.80		0.63 – 2.57 –5.63	
Vitros						
Lem et al., 2012† (Vitros Eci Technology)		**512**	0.57 – 2.13 – 5.54 (1 year) 0.57 – 2.12 – 5.51 (2 years)			0.56 – 2.08 – 5.41 (5 years)

Supplemental Table 3. Reference ranges of TSH stratified on age (5-10 years) and assay, *5th and 95th percentile, **NA** = not available, **18% of samples measured by Vitros 5600, *** 3rd – 97th percentile, †reference ranges based on -2 to 2 standard deviation, **NC** = size of study population is not reported, **NR** – median not reported.

Assay	N	Females (F) Males (M)	TSH as 2.5th – 50th – 97.5th percentile		
			5 – 6 years	7 – 8 years	9 – 10 years
Abbott Architect					
Radicioni et al., 2013		**72**	Pre-pubertal (median age 8.9 years, range: 6.2-12.1 years) 0.87 – 1.95 – 5.19		
Bailey et al., 2013	**1280**	**F: 640 M: 640**	0.7 – NR – 4.17 (6 months – <14 years)		
Aldrimer et al., 2012		**457**	0.89 – NR – 4.97 (6 months – 12 years)		
Chmaler et al., 2012 (Abbott AxSYM)		**1266**		0.82 – 2.36 – 4.74	
Soldin et al., 2010	**1234**	**F: 697 M: 537**	F: 0.89 – NR – 4.07 M: 0.81 – NR – 4.07		
Southcott et al., 2010b(Abott Ci8200)	**196**	**F: 242 M: 254**		F: 0.80 – 1.85 – 3.47 M: 0.88 – 1.85 – 3.66	
Access					
Djemli et al., 2004* (Access 2)	**207**	**F: 101 M: 106**			F: 0.9 – 2.0 – 4.0 M: 1.0 – 1.9 – 3.7
Advia Centaur					
Loh et al., 2015**		**NC**	NA – NR – 5.11 (from 4 years)	0.62 – NR – 4.52	
Strich et al., 2012		**3531**	0.79 – NR – 6.0 (from 6 years)		
Kahapola et al., 2012	**605**	**F: 334 M: 271**	0.75 – 1.91 – 3.97 F: 0.79 – 1.90 – 3.95 M: 0.74 – 1.94 – 4.02		
Kapelari et al., 2008		**315**	0.80 – 2.30 – 5.40 (from 6 years)		
Hubner et al., 2002		**460**	0.48 – 1.87 – 4.67 (from 6 years to 10 years)		

Supplemental Table 3. (continued)

Assay	N	Females (F) Males (M)	TSH as 2.5th – 50th – 97.5th percentile		
			5 – 6 years	7 – 8 years	9 – 10 years
Delfia					
Cioffi et al., 2001* (AutoDelfia)		**1368**	0.8 – NR – 4.5 (5years) 0.9 – NR – 3.9 (6 years)	1.3 – NR – 3.6 (7 years) 1.0 – NR – 4.4 (8 years)	0.7 – NR – 3.8 (9 years) 0.1 – NR – 3.6 (10 years)
Stichel et al., 2000***		**280**	(median age 10 years, range 3.0 – NR - 15.5) 0.54 – 1.69 – 3.36		
Zurakowski et al., 1999	**1260**	**F: 562** **M: 698**	F: 0.6– NR – 5.1 M: 0.7 – NR – 5.4		
Immulite					
Verburg et al., 2011		**91**		0.80 – 2.35 – 5.24 (7 years)	
Najam et al., 2003		**38**	0.39 – NR – 19.86		
Elmlinger et al., 2001		**121**		0.80 – 1.86 – 3.48	0.85 – 2.00 – 3.50
Roche					
La'ulu et al., 2016 (Roche E170)	**137**	**F: 252** **M: 259**	F: 0.85– NR – 5.83 M:1.00 – NR – 6.51	1.12– NR – 5.66 (7 years)	F: 0.94– NR – 5.40 M: 1.14 – NR – 6.41 (from 8 to 9 years)
Iwaku et al., 2013 (Roche ECLIA)		**134**	0.62 – NR – 4.90 (from 4 years)	0.53– NR – 5.16	0.67– NR – 4.52
Kulasingam et al., 2010 (Roche cobas 6000)	**108**	**F:61** **M: 47**		F: 0.48 – NR – 4.81 M: 1.18 – NR – 5.33	
Kratzsch et al., 2008 (Roche Elecsys)		**241**	0.76 – 2.38 – 5.35 (from 6 years)		1.04 – 2.54 – 5.61 (to 11 years)
Vitros					
Lem et al., 2012† (Vitros Eci Technology)		**512**	0.56 – 2.08 – 5.41 (5 years) 0.55 – 2.04 – 5.31 (8 years)		

Supplemental Table 4. Reference ranges of TSH for children from 11 years to 21 years old in literature. *5th and 95th percentile, **18% of samples measured by Vitros 5600,† reference ranges based on -2 to 2 standard deviation; **NC** = size of study population is not reported, **NR** – median not reported.

Assay	N	Females (F) Males (M)	TSH as 2.5th – 50th – 97.5th percentile										
			11 years	12 years	13 years	14 years	15 years	16 years	17 years	18 years	19 years	20 years	21 years
Abbott Architect													
Ehrenkranz et al., 2015		**52765**	0.53 – NR – 6.45 (1 to 20 years)										
Radicioni et al., 2013		**368**	0.76 – 1.75 – 4.51 Pubertal (range: 9.6 – 17.9 y)										
Bailey et al., 2013	**518**	**F: 259 M: 259**					0.47 – NR – 3.41						
Aldrimer et al., 2012	**214**	**F: 119 M: 95**					F: 0.43 – NR – 3.35 M: 0.81 – NR – 3.61						
Chaler et al., 2012 (Abbott AxSYM)		**3830**	0.88 – 2.53 – 4.76 (from 9 years)			0.88 – 2.28 – 4.65				0.71 – 1.86 – 4.88			
Soldin et al., 2010	**3233**	**F: 1940 M:1293**		F: 0.67– NR – 3.72		M: 0.79 – NR – 3.98				F: 0.47 – NR – 3.63 M: 0.55 – NR – 3.55			
Chan et al., 2009	**294**	**F: 201 M: 93**		F: 0.47– NR – 4.13 M: 0.58 – NR – 3.59									
Access													
Djemli et al., 2004* (Access 2)	**402**	**F: 202 M: 200**		F: 0.7 – 1.7 -3.4 M: 0.8 – 1.8 – 3.9				F: 0.6 – 1.5 – 3.7 M: 0.7 – 1.4 – 2.8					
Advia Centaur													
Loh et al., 2015** (Vitros 5600)		**NC**					0.47 – NR – 3.74						
Strich et al., 2012		**4573**		0.72 – NR – 5.77				0.63 – 6.28					
Kahapola et al., 2012	**7069**	**F: 5121 M: 1948**		All 0.62 – 1.71 – 3.88 F: 0.57 – 1.63 – 3.88 M: 0.84 – 1.82 – 3.79						All 0.51 – 1.50 – 3.59 F: 0.51 – 1.46 – 3.56 M: 0.52 – 1.63 – 3.72			
Kapelari et al., 2008		**588**		0.70 – 2.10 – 4.61				0.50 – 1.70 – 4.33					
Hubner et al., 2002		**460**		0.53 – 1.78 – 4.58				0.56 – 2.00 – 4.53					

Supplemental Table 4. (continued)

Assay	N	Females (F) Males (M)	TSH as 2.5th – 50th – 97.5th percentile									
			11 years	12 years	13 years	14 years	15 years	16 years	17 years	18 years	19 years	20 years	21 years
Delfia													
Cioffi et al., 2001* (AutoDelfia)		**1410**	1.0 – 4.4	0.6 – 4.7	0.6 – 4.8	0.9 – 3.0	0.1 – 4.6	0.2 – 3.2					
Zurakowski et al., 1999	**2827**	**F: 1866 M: 961**		F: 0.5 – NR – 4.4 M: 0.6 – NR – 4.9						F: 0.5 – NR – 3.9 M: 0.5 – NR – 4.4			
Diagnostic products corp.													
Li et al., 2011	**370**	**F: 184 M: 186**		F: 0.86 – NR – 1.97 M: 1.03 – NR – 7.41				F: 0.69 – NR – 6.03 M: 0.72 – NR – 6.32					
Guan et al., 2008		**250**	0.39 – NR – 5.66 (aged <19 years)										
Immulite													
Verburg et al., 2011		**83**		0.66 – 2.11 – 4.88						0.49 – 1.79 – 3.38			
Elmlinger et al., 2001		**419**	0.85 – NR – 3.33	0.86 – NR – 3.21	0.80 – NR – 3.08	0.76 – NR – 2.83	0.70 – NR – 2.55	0.64 – NR – 2.51	0.62 – NR – 2.42	0.52 – NR – 2.36			
Nichols Institute Diag.													
Boucai et al., 2011		**NC**			0.41 – 1.30 – 3.78								
Roche													
La'ulu et al., 2016 (Roche E170)	**1042**	**F: 521 M: 521**	F: 0.94 – NR – 4.71 M: 0.78 – NR – 6.11		F:0.88–NR–4.71 M:0.77– N –4.32	F: 0.47 – NR – 4.56 M: 0.65 – NR – 4.16		F: 0.56 – NR – 4.62 M: 0.63 – NR – 4.58					
Iwaku et al., 2013 (ECLIA)		**190**	0.62 – NR – 3.36		0.54 – NR – 2.78		0.32 – NR – 3.00						
Mosso et al., 2013		**NC**					F: 0.76 – 2.24 – 5.43, M: 0.95 – 2.66 – 6.09						

Supplemental Table 4. (continued)

Assay	N	Females (F) Males (M)	TSH as 2.5th – 50th – 97.5th percentile										
			11 years	12 years	13 years	14 years	15 years	16 years	17 years	18 years	19 years	20 years	21 years
Henderson et al., 2011 (Roche E170)		**245**		(from 7 years) Non-parametric: 1.0 – NR – 6.2 (from 7 years) Robust: 0.7 – NR – 5.4									
Kulasingam et al., 2010 (Roche cobas 6000)		**179**		0.76 – NR – 4.20					F: 0.38 – NR – 2.82		M: 0.64 – NR – 5.37		
Kratzsch et al., 2008 (Elecsys)		**230**		0.51 – 2.14 –4.60						0.38 – 1.66 –3.47			
Vitros													
Lem et al., 2012† (Vitros Eci)		**512**		0.53 – NR – 5.16			0.52 – NR – 5.05			0.51 – NR – 4.93			

Supplemental Table 5. Reference ranges of fT4 for children from 1 day to 1 years old in literature rounded to 1 decimal place. *5th and 95th percentile, **18% of samples measured by Vitros 5600, †only data on T4, ***Liquid chromatography tandem mass spectrometry, †† reference ranges based on -2 to 2 standard deviation, **NC** = size of study population is not reported, **NR** – median not reported.

Assay	N	Females (F) Males (M)	FT4 as 2.5th – 50th – 97.5th percentile					
			1 – 7 days	8 – 15 days	15 day – 1 month	1 – 3 months	3 – 6 months	6 – 12 months
Abbott Architect								
Bailey et al., 2013 *	**782**	**F:391 M: 391**	13.5– NR – 41.3 (5 to <15 days)		8.8 – NR – 32.6		11.5 – NR – 21.9	
Chaler et al., 2012 (Abbott AxSYM)		**659**			13.0 – 17.2 – 26.8		11.6 – 16.3 – 23.3	11.1 – 16.2 – 24.4
Soldin et al., 2010	**612**	**F: 298 M: 314**	F: 8.9 – NR – 23.9 (to 2 months); M: 10.1 – NR – 23.6				F: 8.9 – NR – 19.2 (from 2 months); M: 9.2 – NR – 20.1	
Chan et al., 2009	**79**	**F: 43 M: 36**	F: 11.0 – NR – 20.6; M: 11.9 – NR – 23.6					
Access								
Romero et al., 2014	**175**	**F: 78 M: 97**	F: 0.88 – 1.25 – 2.75 (ng/ml) M: 0.65 – 1.24 – 2.38 (ng/ml)			F: 0.67 – 0.86 – 1.34 (ng/ml) M: 0.61 – 0.86 – 1.20 (ng/ml)		F: 0.66 – 0.79 – 1.14 (ng/ml) M: 0.63 – 0.80 – 0.97 (ng/ml)
Djemli et al., 2004* (Access 2)	**24**	**F: 12 M:12**	F: 11.0 – 13.6 – 22.3 M: 9.8 – 12.2 – 23.2					
Advia Centaur								
Loh et al., 2015** (Centaur Vitros 5600)		**NC**	19.9 – NR – 46.6	17.2 – NR – 33.1	13.2 – NR – 21.8	11.3 – NR – 21.3 (to 2 months)		
Strich et al., 2012		**422**		12.4 – NR – 27.4		12.4 – NR – 21.8 (to 2 months)	10.8 – NR – 19.5 (from 2 months)	
Kapelari et al., 2008		**68**		8.50 – 20.10 – 30.50			9.17 – 15.50 – 25.28	
Hubner et al., 2002		**460**	10.8 – NR – 26.8 (0-3 days)		10.9 – NR – 25.5 (from 4 days)		11.4 – NR – 20.9 (from 2 months)	

Supplemental Table 5. (continued)

Assay	N	Females (F) Males (M)	FT4 as 2.5th – 50th – 97.5th percentile					
			1 – 7 days	8 – 15 days	15 day – 1 month	1 – 3 months	3 – 6 months	6 – 12 months
Delfia								
Zurakowski et al., 1999		**47**					9.5 – NR – 39.5 (to 11 months)	
Immulite								
Verburg et al., 2011		**308**	8.9 – 18.0 – 33.6	8.9 – 17.9 – 32.9	9.0 – 17.7 – 31.8	10.2 – 17.7 – 25.2		12.0 – 17.4 – 23.1
Fideleff et al., 2010		**334**	20.3 – 29.3 – 41.0 (2-3 days)	19.3 – 24.4 – 34.5		14.8 – 19.4 – 26.9 (28-40 days)		
Najam et al., 2003†		**1109**	8.98 – 17.0 – 26.0 (0 – 4 days) 5.79 – 12.6 – 20.7 (5 – 7 days)			7.1 – 11.4 – 19.1 (1 month)		4 – 10.2 – 18.6 (1 year)
Elmlinger et al., 2001		**<171**	29.6 – 62.4 – 79.2	18.0 – 42.3 – 63.6			11.1 – 19.7 – 27.3 (to 3 years)	
LCMS* **								
Soldin et al., 2009 Ultrafiltration at 37°C/25°C		**140**					at 37 °C: 1.3 – 2.8 at 25 °C: 0.9 – 1.9	
Roche								
Omuse et al., 2016 (Roche e601)	**1329**	**F: 695 M: 634**	13.6 – 25.1 – 34.8	13.5 – 22.3 – 30.2	14.2 – 19.6 – 24.8 (15-20 days) 13.2 – 18.2 – 23.4 (23 to 30 days)			
Kratzsch et al., 2008 (Roche Elecsys)		**258**	10.9 – 17.6 –34.5		12.70 – 17.8 – 30.0		12.30 – 16.9 –23.5	
Vitros								
Lem et al., 2012†† (Eci Technology)		**512**	12.3 – 21.6 – 52.5 (1 week)	12.8 – 21.1 – 44.3 (1 month)			13.4 – 20.3 – 36.8 (3 months)	13.8 – 19.7 – 31.4 (6 months) 14.1 – 19.2 – 28.2 (1 year)

Supplemental Table 6. Reference ranges of fT4 for children from 1 year to 5 years old in literature rounded to 1 decimal place. *5th and 95th percentile, † reference ranges based on -2 to 2 standard deviation, **NR** – median not reported.

Assay	N	Females (F) Males (M)	FT4 as 2.5th – 50th – 97.5th percentile			
			1 – 2 years	2 – 3 years	3 – 4 years	4 – 5 years
Abbott Architect						
Chaler et al., 2012 (Abbott AxSYM)		**1826**	11.4 – 16.3 – 24.8		12.0 – 16.6 – 25.0 (to 6 years)	
Soldin et al., 2010	**931**	**F: 455** **M: 476**	F: 9.4 – NR – 18.2 M: 10.6 – NR – 17.0		F: 10.2 – NR – 17.8 M: 10.3 – NR – 17.0	
Chan et al., 2009	**194**	**F: 93** **M: 101**	F: 12.0 – NR – 18.6 M: 11.0 – NR – 20.8			
Access						
Romero et al., 2014	**230**	**F: 118** **M: 112**	(13-18 months) F: 0.7 – 0.8 – 1.0 (ng/ml) M: 0.7 – 0.8 – 1.2 (ng/ml) (19-23 months) F: 0.6 – 0.8 – 1.1 (ng/ml) M: 0.6 – 0.9 – 1.5 (ng/ml)	F: 0.6 – 0.8 – 1.2 (ng/ml) M: 0.7 – 0.8 – 1.0 (ng/ml)	(to 6 years) F: 0.7 – 0.9 – 1.1 M: 0.6 – 0.8 – 1.0	
Djemli et al., 2004* (Access 2)	**75**	**F: 28** **M: 47**	F: 9.0 – 11.3 – 16.1 (from 1 month) M: 8.7 – 11.7 – 16.2			
Advia Centaur						
Strich et al., 2012		**2722**	11.7 – NR – 19.0			
Kapelari et al., 2008		**229**	10.5 – 15.7 – 22.4			
Hubner et al., 2002		**460**	11.4 – 14.7 – 19.0			
Delfia						
Cioffi et al., 2001* (AutoDelfia)		**778**	9.9 – NR – 17.3 (2 years)	11.5 – NR – 19.7 (3 years)	8.9 – NR – 22.5 (4 years)	12.2 – NR– 23.6 (5 years)
Zurakowski et al., 1999		**91**	9.0 – 37.2			

Supplemental Table 6. (continued)

Assay	N	Females (F) Males (M)	FT4 as 2.5th – 50th – 97.5th percentile			
			1 – 2 years	2 – 3 years	3 – 4 years	4 – 5 years
ED and LCMS						
La'ulu et al., 2016	**840**		18.0 – NR – 34.7 (from 6 months to 6 years)			
Immulite						
Verburg et al., 2011	**83**				13.4 – 17.7 – 22.1	
Elmlinger et al.,2001	**<51**					12.9 – 17.3 – 23.9 (to 6 years)
Liquid chromatography tandem mass spectometry						
Soldin et al., 2009 LCMS, Ultrafiltration at 37°C/25C	**274**		16.7 – NR – 30.9 at 37 °C 11.6 – NR – 20.6 at 25 °C		(From 3 to 8 years) 16.7 – NR – 36.6 at 37 °C 11.6 – NR – 20.6 at 25 °C	
Roche						
Henderson et al., 2011 (Roche E170)	**46**		Non-parametric: 14.5 – 19.8 Robust: 14.2 – NR – 20.3 (both until 6 years)			
Kulasingam et al., 2010 (Roche cobas 6000)	**208**		10.9 – NR – 36.3 (from birth – 5 years)			
Kratzsch et al., 2008 (Roche Elecsys)	**247**		13.9 – 17.0 –21.4		13.3 – 17.1 –20.3	
Vitros						
Lem et al., 2012† (Vitros Eci Technology)	**512**		14.1 – 19.2 – 28.2 (1 year) 14.3 – 18.8 – 26.3 (2 years)			13.9 – 18.1 – 24.8 (5 years)

Supplemental Table 7. Reference ranges of FT4 stratified on age (5-10 years) and assay rounded to 1 decimal place. *5th and 95th percentile, **18% of samples measured by Vitros 5600, *** 3rd – 97th percentile, †only data on T4, †† reference ranges based on -2 to 2 standard deviation, **NC** = size of study population is not reported **, NR** – median not reported.

Assay	N	Females (F) Males (M)	FT4 as 2.5th – 50th – 97.5th percentile		
			5 – 6 years	7 – 8 years	9 – 10 years
Abbott Architect					
Radicioni et al., 2013		**72**	Pre-pubertal (median age 8.9 years, range: 6.2-12.1 years) 13.1 – 16.4 – 20.6		
Aldrimer et al., 2012		**471**	10.8 – 16.40 (6 months to 12 years)		
Chmaler et al., 2012 (Abbott AxSYM)		**1266**		11.7 – 16.2 – 24.6	
Soldin et al., 2010	**1171**	**F: 655 M: 516**	F: 9.9 – NR – 17.0 M: 10.1 – NR – 16.6		
Southcott et al., 2010 (Abott Ci8200)	**508**	**F: 249 M: 259**	(median age 8,1 years, 2.5 - 97.5 percentile range: 7.5 – 8.8 y) F: 12.6 – 14.8 – 17.3 M: 12.2 – 14.7 – 16.7		
Chan et al., 2009		**139**	10.9 – 19.0 (from 6 years)		
Access					
Djemli et al., 2004* (Access 2)	**205**	**F: 103 M: 102**			F: 9.6 – 11.6 – 14.5 M: 9.7 – 11.7 – 14.2
Advia Centaur					
Loh et al., 2015 ** (Advia Centaur Vitros 5600)		**NC**		10.91 – 20.58	
Strich et al., 2012		**3452**	11.3 – NR – 18.7 (from 6 years)		
Kapelari., 2008		**327**	10.6 – 15.9 – 20.9 (from 6 years)		
Hubner et al., 2002**		**460**	11.0 – 14.2 – 18.8 (from 6 years to 10 years)		

Supplemental Table 7. (continued)

Assay	N	Females (F) Males (M)	FT4 as 2.5th – 50th – 97.5th percentile		
			5 – 6 years	7 – 8 years	9 – 10 years
Delfia					
Cioffi et al., 2001* (AutoDelfia)		**1368**	12.2 – NR – 23.6 (5 years) 5.8 – NR – 29.5 (6 years)	11.3 – NR – 22.8 (7 years) 11.6 – NR – 44.8 (8 years)	11.3 – NR – 18.7 (9 years) 10.8 – NR – 21.8 (10 years)
Stichel et al., 2000***		**280**		(median age 10 years, range 3.0 - 15.5) 6.0 – 9.0 – 13.1 (ug/dL)	
Zurakowski et al., 1999		**57**		8.3 – 34.1	
Immulite					
Verburg et al., 2011		**91**		13.2 – 17.4 – 21.6 (7 years)	
Najam et al., 2003†		**39**	6.6 – 9.3 – 17.5 (5 years)		
Elmlinger et al.,2001		**121**		12.9 – 17.3 – 23.9	10.3 – 17.0 – 23.8
Liquid chromatography tandem mass spectometry					
Soldin et al., 2009 LCMS, Ultrafiltration at 37^{0}C/25^{0}C		**129**			(From 3 years to 8 years) at 37 ^{0}C 16.7 – NR – 30.9 at 25 ^{0}C 11.6 – NR – 20.6
Roche					
Iwaku et al., 2013 (ECLIA)		**134**	14.4 – NR – 21.5 (from 4 years)	13.8 – NR – 20.7	12.4 – NR – 20.6
Kulasingam et al., 2010 (Roche cobas 6000)		**238**		10.4 – NR – 27.1 (to 15 years)	
Kratzsch et al., 2008 (Roche Elecsys)		**241**	13.7 – 17.0 – 21.7 (from 6 years)		13.2 – 16.9 – 21.6 (to 11 years)

Supplemental Table 7. (continued)

Assay	N	Females (F) Males (M)	FT4 as 2.5th – 50th – 97.5th percentile		
			5 – 6 years	7 – 8 years	9 – 10 years
Vitros					
Lem et al., 2012†† (Vitros Eci Technology)		**512**	13.9 – 18.1 – 24.8 (5 years) 13.4 – 17.5 – 24.1 (8 years)		

Supplemental Table 8. Reference ranges of fT4 for children from 11 year to 20 years old in literature rounded to 1 decimal place. *5th and 95th percentile, **18% of samples measured by Vitros 5600, † reference ranges based on -2 to 2 standard deviation, †† Equilibrium dialysis-liquid chromatography tandem mass spectrometry, **NC** = size of study population is not reported, **NR** – median not reported.

Assay	N	Females (F) Males (M)	FT4 as 2.5th – 50th – 97.5th percentile									
			11 years	12 years	13 years	14 years	15 years	16 years	17 years	18 years	19 years	20 years
Abbott Architect												
Ehrenkranz et al., 2015	**18344**		10.0 – NR – 18.7 (1 to 20 years)									
Radicioni et al., 2013	**368**		10.9 – 13.9 – 19.1 (range: 9.6 – 17.9 years)									
Bailey et al., 2013	**1904**	**F: 952 M: 952**	11.5 – NR – 17.6 (1 to <19 years)									
Aldrimer et al., 2012	**215**				10.2 – NR – 15.50							
Chaler et al., 2012 (Abbott AxSYM)	**3830**		10.9 – 16.0 – 25.2 (from 9 years)			10.4 – 14.9 – 24.7			9.6 – 14.8 – 25.0			
Soldin et al., 2010	**3034**	**F: 1805 M: 1229**	F: 8.5 – NR – 15.7 M: 8.9 – NR – 15.9						F: 8.6 – NR – 15.7 M: 8.6 – NR – 15.7			
Chan et al., 2009		**324**	10.0 – 16.9						10.2 – 17.3			
Access												
Djemli et al., 2004* (Access 2)	**406**	**F: 204 M: 202**	F: 8.8 – 10.7 – 13.5 M: 8.4 – 10.8 – 13.0					F: 8.7 – 10.7 – 13.6 M: 9.5 – 11.8 – 15.0				

Supplemental Table 8. (continued)

Assay	N	Females (F) Males (M)	FT4 as 2.5th – 50th – 97.5th percentile									
			11 years	12 years	13 years	14 years	15 years	16 years	17 years	18 years	19 years	20 years
Advia Centaur												
Loh et al., 2015** (Vitros 5600)		**NC**	10.2 – NR – 20.1 (from 10 years)									
Strich et al., 2012		**4448**		10.5 – NR – 17.9				10.4 – NR – 18.0				
Kapelari et al., 2008		**597**		10.4 – 15.2 – 21.4				10.6 – 15.2 – 22.6				
Hubner et al., 2002		**460**		10.8 – 13.6 – 18.7				10.7 – 14.4 – 18.7				
Amerlite												
Christofides et al., 1995 (Amerlite – MAB)		**29**				11.8 – NR – 25.2 (age range: 0 – 20 year)						
Delfia												
Cioffi et al., 2001* (AutoDelfia)*		**1410**	10.6–NR– 20.5	9.4–NR– 20.7	10.0 –NR– 17.1	9.8–NR– 16.7	9.8–NR–18.9	8.9–NR–21.2				
Zurakowski et al., 1999		**158**			7.6 – NR – 31.5				7.0 – NR – 28.7			
ED-LCMS††												
La'ulu et al., 2016		**<1373**				14.2 – NR – 25.7 (from 7 years)						
Immulite												
Verburg et al., 2011		**83**		12.7–NR– 21.2						12.3–NR –20.9		
Elmlinger et al.,2001		**419**	11.8 – 22.7	10.4–NR– 22.9	8.5–NR–22.5	12.2–NR–23.3	9.1–NR–23.4	12.9–NR– 23.3	11.8–NR– 22.5	9.3–NR– 20.5		

Supplemental Table 8. (continued)

Assay	N	Females (F) Males (M)	FT4 as 2.5th – 50th – 97.5th percentile									
			11 years	12 years	13 years	14 years	15 years	16 years	17 years	18 years	19 years	20 years
LCMS												
Soldin et al*., 2009 Ultrafiltration at 37/25C	**632**	**F:376 M: 256**		16.7–NR– 30.9 at 37 °C 11.6 –NR– 20.6 at 25 °C			16.7–NR– 30.9 at 37°C 11.6–NR– 20.6 at 25°C		16.7–NR– 30.9 at 37°C 11.6 –NR–20.6 at 25°C			
Roche												
Iwaku et al., 2013 (Roche ECLIA)		**190**		13.1– NR – 19.6		12.4 – NR – 19.6		12.2 – NR – 19.7				
Henderson et al., 2011 (Roche E170)		**250**		Non-parametric: 13.0 – NR – 20.3 Robust: 12.3 – NR – 20.0 (from 7 years)			Non-parametric: 12.8 – NR – 20.6 Robust: 12.1 – NR – 20.2					
Kulasingam et al., 2010 (Roche cobas 6000)	**64**	**F: 36 M: 28**							F: 10.0 – NR – 18.7 M: 9.9 – NR – 36.2			
Kratzsch et al., 2008 (Roche Elecsys)		**230**				12.0 – 15.4 –22.0				12.2 – 17.0 –22.2		
Vitros												
Lem et al., 2012† (Vitros Eci Technology)		**512**		12.7 – NR – 23.3			12.3 – NR – 22.8			12.0 – NR –22.3		

Supplemental Table 9. Descriptive statistics of the study population. *European, Oceania, American western, Asian western, **Indonesian, American non - western, Asian non - western

	Median or N per group	(95% range or %)
Age (years)	6.0	(5.7 – 8.0)
Child sex (boys %)	2202	(51.5)
Length (cm)	119	(109.1 – 132.9)
Weight (kg)	22.6	(17.6 – 34.7)
Ethnicity child (N%)		
Dutch	2402	(57.8)
Moroccan	253	(6.1)
Dutch Antilles	132	(3.2)
Surinamese	296	(7.1)
Turkish	296	(7.1)
Cape Verdian/African	218	(5.2)
Other Western *	351	(8.4)
Education of mother (N%)		
No education finished/primary school/secondary phase 1	457	(12.6)
Secondary phase 2	1110	(30.5)
Higher phase 1	1002	(27.5)
Higher phase 2	1072	(29.4)
Season (N%)		
Spring	1159	(27.1)
Summer	1074	(25.1)
Autumn	1111	(26.0)
Winter	929	(21.7)
Average time of venipuncture (h)	14:02	(11.17 – 5.17)

Supplemental Table 10. Statistically significant differences found in non-response analysis. Estimates shown are derived from linear regression analyses with the clinical characteristics as the dependent variable and missing TSH/FT4 (binary yes/no) as the independent variable. Other tested variables such as maternal education level, season, BMI and ethnicity did not differ.

	Dependent determinants					
	Age (years)		Height (cm)		Weight (kg)	
	β	***P***	**β**	***P***	**β**	***P***
Independent						
Missing TSH	-0.057	*P*<0.001	-0.864	*P*<0.001	-0.369	*P*=0.001
Missing FT4	-0.056	*P*<0.001	-0.838	*P*<0.001	-0.351	*P*=0.001

Supplemental appendix. *Search queries for all databases*

Database	Predetermined search terms
PubMed	(("Thyroid Hormones"[Mesh]) AND ("Reference Values"[Mesh] OR "Reference Standards"[Mesh])) AND "Child"[Mesh]
Embase	('thyroid hormone'/de OR 'thyroxine'/de OR 'thyrotropin'/de OR 'thyroid hormone blood level'/exp OR 'thyrotropin blood level'/de OR 'thyroid function'/exp OR 'thyroid disease'/de OR hypothyroidism/exp OR 'hyperthyroidism'/exp OR 'thyroid function test'/de OR 'hypophysis hormone'/de OR (((thyroid* OR hypophysis OR pituitar*) NEAR/6 (hormone* OR function* OR dysfunction*)) OR thyroxin* OR thyrotropin* OR tsh OR free-T4 OR euthyroid* OR hypothyroid* OR hyperthyroid*):ab,ti) AND ('reference value'/de OR 'normal value'/de OR (((reference* OR standard* OR normal) NEAR/6 (value* OR interval* OR range* OR limit* OR data OR level* OR mean OR median OR concentrat*)) OR (reference* NEAR/3 standard*)):ab,ti) AND (child/exp OR adolescent/exp OR adolescence/exp OR 'child behavior'/de OR 'child parent relation'/de OR pediatrics/exp OR childhood/exp OR 'child nutrition'/de OR 'infant nutrition'/exp OR 'child welfare'/de OR 'child abuse'/de OR 'child advocacy'/de OR 'child development'/de OR 'child growth'/de OR 'child health'/de OR 'child health care'/exp OR 'child care'/exp OR 'childhood disease'/exp OR 'child death'/de OR 'child psychiatry'/de OR 'child psychology'/de OR 'pediatric ward'/de OR 'pediatric hospital'/de OR 'pediatric anesthesia'/de OR (adolescen* OR preadolescen* OR infan* OR newborn* OR (new NEXT/1 born*) OR baby OR babies OR neonat* OR child* OR kid OR kids OR toddler* OR teen* OR boy* OR girl* OR minors OR underag* OR (under NEXT/1 (age* OR aging)) OR juvenil* OR youth* OR kindergar* OR puber* OR pubescen* OR prepubescen* OR prepubert* OR pediatric* OR paediatric* OR school* OR preschool* OR highschool*):ab,ti) AND ('observational study'/exp OR 'cohort analysis'/exp OR 'longitudinal study'/exp OR 'retrospective study'/exp OR 'prospective study'/exp OR 'health survey'/de OR 'health care survey'/de OR 'epidemiological data'/de OR 'case control study'/de OR 'cross-sectional study'/de OR 'population research'/de OR 'family study'/de OR 'major clinical study'/de OR 'multicenter study'/de OR 'comparative study'/de OR 'follow up'/de OR 'clinical study'/de OR 'clinical article'/de OR 'community trial'/de OR 'review'/exp OR 'systematic review'/exp OR 'meta analysis'/de OR (((observation* OR epidemiolog* OR communit*) NEAR/6 (stud* OR data OR research)) OR cohort* OR longitudinal* OR retrospectiv* OR prospectiv* OR population* OR (national* NEAR/3 (stud* OR survey)) OR (health* NEAR/3 survey*) OR ((case OR cases OR match*) NEAR/3 control*) OR (cross NEXT/1 section*) OR multicenter* OR multi-center* OR follow-up* OR followup* OR clinical* OR review* OR meta-analy*):ab,ti) NOT ([Conference Abstract]/lim OR [Letter]/lim OR [Note]/lim OR [Editorial]/lim) NOT ([animals]/lim NOT [humans]/lim)

Supplemental appendix. *(continued)*

Database	Predetermined search terms
Ovid Medline	("Thyroid Hormones"/ OR "thyroxine"/ OR "thyrotropin"/ OR "Thyroid Function Tests"/ OR "Thyroid Diseases"/ OR exp Hypothyroidism/ OR exp "Hyperthyroidism"/ OR "Pituitary Hormones"/ OR (((thyroid* OR hypophysis OR pituitar*) ADJ6 (hormone* OR function* OR dysfunction*)) OR thyroxin* OR thyrotropin* OR tsh OR free-T4 OR euthyroid* OR hypothyroid* OR hyperthyroid*).ab,ti.) AND ("Reference Values"/ OR (((reference* OR standard* OR normal*) ADJ6 (value* OR interval* OR range* OR limit* OR data OR level* OR mean OR median OR concentrat*)) OR (reference* ADJ3 standard*)).ab,ti.) AND (exp Child/ OR exp Infant/ OR exp Adolescent/ OR exp "Child Behavior"/ OR exp "Parent Child Relations"/ OR exp "Pediatrics"/ OR "Child Nutrition Sciences"/ OR "Infant nutritional physiological phenomena"/ OR exp "Child Welfare"/ OR "Child Development"/ OR exp "Child Health Services"/ OR exp "Child Care"/ OR "Child Rearing"/ OR exp "Child development Disorders, Pervasive"/ OR "Child Psychiatry"/ OR "Child Psychology"/ OR "Hospitals, Pediatric"/ OR (adolescen* OR infan* OR newborn* OR (new ADJ born*) OR baby OR babies OR neonat* OR child* OR kid OR kids OR toddler* OR teen* OR boy* OR girl* OR minors OR underag* OR (under ADJ age*) OR juvenil* OR youth* OR kindergar* OR puber* OR pubescen* OR prepubescen* OR prepubert* OR pediatric* OR paediatric* OR school* OR preschool* OR highschool*).ab,ti.) AND ("Observational Study"/ OR exp "Cohort Studies"/ OR "Health Surveys"/ OR exp "Epidemiologic Studies"/ OR "Case-Control Studies"/ OR "Cross-Sectional Studies"/ OR "Multicenter Study"/ OR "Comparative Study"/ OR "Clinical Study"/ OR "Review"/ OR "Meta-Analysis"/ OR (((observation* OR epidemiolog* OR famil* OR comparativ* OR communit*) ADJ6 (stud* OR data OR research)) OR cohort* OR longitudinal* OR retrospectiv* OR prospectiv* OR population* OR (national* ADJ3 (stud* OR survey)) OR (health* ADJ3 survey*) OR ((case OR cases OR match*) ADJ3 control*) OR (cross ADJ section*) OR correlation* OR multicenter* OR multi-center* OR follow-up* OR followup* OR clinical* OR review* OR meta-analy*).ab,ti.) NOT (letter OR news OR comment OR editorial OR congresses OR abstracts).pt. NOT (exp animals/ NOT humans/)
Web of Science	TS=(((((thyroid* OR hypophysis OR pituitar*) NEAR/5 (hormone* OR function* OR dysfunction*)) OR thyroxin* OR thyrotropin* OR tsh OR free-T4 OR euthyroid* OR hypothyroid* OR hyperthyroid*)) AND ((((reference* OR standard* OR normal) NEAR/5 (value* OR interval* OR range* OR limit* OR data OR level* OR mean OR median OR concentrat*)) OR (reference* NEAR/2 standard*))) AND ((adolescen* OR preadolescen* OR infan* OR newborn* OR (new NEAR/1 born*) OR baby OR babies OR neonat* OR child* OR kid OR kids OR toddler* OR teen* OR boy* OR girl* OR minors OR underag* OR (under NEAR/1 (age* OR aging)) OR juvenil* OR youth* OR kindergar* OR puber* OR pubescen* OR prepubescen* OR prepubert* OR pediatric* OR paediatric* OR school* OR preschool* OR highschool*)) AND ((((observation* OR epidemiolog* OR communit*) NEAR/5 (stud* OR data OR research)) OR cohort* OR longitudinal* OR retrospectiv* OR prospectiv* OR population* OR (national* NEAR/2 (stud* OR survey)) OR (health* NEAR/2 survey*) OR ((case OR cases OR match*) NEAR/2 control*) OR (cross NEAR/1 section*) OR multicenter* OR multi-center* OR follow-up* OR followup* OR clinical* OR review* OR meta-analy*)))
Google Scholar	"thyroid|hypophysis|pituitary hormones|function"|thyroxin|thyrotropin "reference|standard|normal values|intervals|ranges|limits|data|levels"|"reference standard" adolescents|children|infants|newborns|child

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CHAPTER 4.2

Childhood Thyroid Function, Body Composition and Cardiovascular Function

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ABSTRACT

Objective: The cardiovascular system is a known target for thyroid hormone. Early-life cardiovascular alterations may lead to a higher risk of cardiovascular disease in adulthood. Little is known about the effects of thyroid hormone on cardiovascular function during childhood, including the role of body composition in this association.

Design: Population-based prospective cohort of children (N=4251, median age 6 years, 95% range 5.7-8.0 years).

Methods: Thyroid stimulating hormone (TSH) and free thyroxine (FT4) concentrations were measured to assess thyroid function. Left ventricular (LV) mass was assessed with echocardiography. Arterial stiffness was assessed with carotid-femoral pulse wave velocity (CFPWV). Systolic and diastolic blood pressure (BP) was measured. Body composition was assessed by dual-energy X-ray absorptiometry scan.

Results: FT4 was inversely associated with LV mass (P=0.002), and with lean body mass (P<0.0001). The association of FT4 with LV mass was partially mediated through variability in lean body mass (55% mediated effect). TSH was inversely associated with LV mass (P=0.010), predominantly in boys. TSH was positively associated with systolic and diastolic BP (both P<0.001). FT4 was positively associated with CFPWV and diastolic BP (P<0.0001, P=0.008, respectively), and the latter association attenuated after adjustment for CFPWV.

Conclusions: At the age of 6, higher FT4 is associated with lower LV mass (partially through effects on lean body mass) and with higher arterial stiffness which may lead to higher BP. Our data also suggest different mechanisms via which TSH and FT4 are associated with cardiovascular function during early childhood.

INTRODUCTION

Cardiovascular disease is the leading cause of morbidity and mortality worldwide [1]. Structural and functional cardiac parameters, as well as blood pressure, track from childhood to adulthood [2-6], suggesting that early-life metabolic and physiological alterations may lead to an adaptive response of the cardiovascular system. Consequently, the risk of developing cardiovascular disease in adult life may increase [7] and the determinants of childhood cardiac structure and function could have predictive value for clinical events in adulthood. Childhood cardiovascular structure and function are mainly determined by changes in the body size that occur due to somatic growth [8], however, little is known about other determinants.

The cardiovascular system is a well-known target for thyroid hormone (TH), as is exhibited by profound effects of TH on systemic vascular resistance, contractility, heart rate, blood volume and cardiac mass [9]. Different types of evidence also support a role for TH in the regulation of early cardiac function and growth [10,11]. During fetal and postnatal life, cardiomyocytes express TH receptors (TRα and TRβ) [12] and the transcription of genes encoding contractile proteins of myosin heavy chains is TH-dependent [13].

Although the role of thyroid function in the regulation of cardiovascular system has been extensively studied in adults, data about the effects of variation in thyroid function on cardiovascular development and function during childhood are scarce. Furthermore, there is a lack of data on the effects of thyroid function on body composition and its potential role in the association of TH with cardiovascular system in the pediatric population. Therefore, we aimed to study the association of childhood thyroid function with cardiovascular structure and function and body composition measurements.

SUBJECTS AND METHODS

Study population

This study was embedded in Generation R, a population-based prospective cohort from early fetal life onwards in Rotterdam, the Netherlands [14]. The study was designed to identify early environmental and genetic causes leading to normal and abnormal growth, development and health during fetal life and childhood [14]. All children were born between 2002 and 2006 [14]. In total, 6690 children (median age 6 years, 95% range 5.7-8.0 years) attended the research center for the follow-up measurements. Successful serum sampling was performed in 4593 children and thyroid-stimulating hormone (TSH) and free thyroxine (FT4) concentrations were determined in 4306 serum samples. Children with thyroid/chronic disease and thyroid (interfering) medication, cardiac abnormalities or with missing data on cardiac ultrasound (N=23, N=32, N=182, respectively) were excluded from the analysis (Supplemental figure 1).

Thyroid measurements

Serum samples were obtained at the time of the visit to the research center. Plain tubes were centrifuged and serum was stored at -80°C. TSH and FT4 concentrations were determined using an electrochemiluminescence immunoassay on the Cobas e601 immunoanalyzer (Roche Diagnostics). The intra- and interassay coefficients of variation were 1.1-3.0% for TSH at a range 0.04-0.4 mU/L and 1.6-5.0% for FT4 at a range of 1.6-24.1 pmol/L.

Cardiovascular measurements

M-mode echocardiographic measurements were performed using the ATL-Philips Model HDI 5000 (Seattle, WA, USA) or the Logiq E9 (GE Medical Systems, Wauwatosa, WI, USA) device [15,16]. Experienced sonographers performed the measurements and were supervised by a pediatric cardiologist. To minimize the inter-observer differences, quality checks were frequently carried out and feedback was provided regularly. To assess reproducibility of echocardiographic measurements, the intraobserver intraclass correlation coefficient were calculated for LAD, AOD, interventricular end-diastolic septum thickness (IVSD), left ventricular diastolic diameter (LVDD) and left ventricular posterior wall thickness (LVPWD) in 28 subjects (median age 7.5 years, interquartile range 3.0 – 11.0) and varied between 0.91 to 0.99 and 0.78 and 0.96, respectively [17]. Missing echocardiograms were random, and mainly due to participant circumstances or unavailability of equipment or sonographer. Aortic root diameter (AOD), LVDD, LVPWD and IVSD were measured and fractional shortening (FS) and left ventricular (LV) mass were calculated; LV mass was computed using the formula by Devereux *et al*: LV mass = $0.80 \times 1.04((\text{IVSTD} + \text{LVEDD} + \text{LVPWTD})3 - (\text{LVEDD})3) + 0.6$ [18].

Carotid-femoral pulse wave velocity (CFPWV), the reference method to assess arterial stiffness [19], was measured in a supine position using the automatic Complior SP device (Complior; Artech Medical, Pantin, France). CFPWV was calculated as a ratio of the distance traveled by the pulse wave and the time delay between the upstroke of carotid and femoral waveforms. The mean of at least 10 consecutive pressure waveforms was used in order to cover a complete respiratory cycle. CFPWV can be measured reliably, with good reproducibility, in large pediatric population-based cohorts [20].

Systolic blood pressure (BP) and diastolic BP was measured at the right brachial artery in a supine position, four times with one-minute intervals. The automatic sphygmomanometer Datascope Accutorr Plus (Paramus, New Jersey, USA) was used [21]. The mean value was calculated by using the last three blood pressure measurements. Pulse pressure was calculated as the difference between mean systolic and mean diastolic pressure. In this study high BP was defined as the highest 5 percentiles of the study population [22].

Anthropometric measurements

Height and weight were measured without shoes and heavy clothing and were used to determine body surface area (BSA), calculated according to the Haycock formula [23,24]. Dual-energy X-ray absorptiometry scan (iDXA, General Electrics – Lunar, 2008, Madison, WI, USA) was performed to assess lean and fat mass of the body composition; subsequently, lean mass and fat mass indices were determined according to formula: lean mass index = lean mass / height ^{2} and fat mass index = fat mass / height ^{2} [25].

Covariates

Information on ethnicity was obtained through questionnaires and was classified by the countries of birth of the parents, according to the classification of Statistics Netherlands [14]. Information on the educational level of the mother was used as a proxy for socioeconomic status and was obtained through questionnaires, as well as information about maternal smoking during pregnancy [14].

Statistical analysis

We investigated the associations of TSH and FT4 with mean LV mass, AOD, FS, lean mass, fat mass, CFPWV, systolic and diastolic BP by using multiple linear regression analyses, utilizing restricted cubic splines with three knots to account for possible non-linear associations. Multiple logistic regression models were used to assess the association of TSH with the risk of high BP. All model covariates were selected based on biological plausibility, change of the effect estimate of interest or residual variability of the model. The analyses were adjusted for sex, age, ethnicity and maternal educational level. Maternal smoking during pregnancy did not affect the estimates and was not included in the model. For the models of cardiac ultrasound measurements, sensitivity analyses were performed to examine potential effect modification by BP level. For the BP models, analyses were performed to examine the potential mediation effect of arterial stiffness, as well as to test effect modification by BMI. Multivariable associations were graphically depicted by plots (main manuscript) and β estimates with 95% confidence intervals are shown in Supplemental Table 1. We tested for effect modification with sex by introducing a product interaction term of TSH or FT4 with sex to the model. A *P*-value cut-off of <0.15 was considered for quantification of the effect difference by subsequent stratification of the association.

We accounted for the high number of statistical tests (39 in total) by controlling the false discovery rate using the fdrtool package [26]. This method allows for tailored identification of the expected proportion of false positive results among all rejected null hypothesis. We identified that a q-value of 0.055 (i.e. the cut-off for a 5.5% chance of a type I error) was similar to a *P*-value of 0.05. Therefore, a *P*-value threshold of <0.05 was considered for statistical significance.

As body surface area (BSA) explains a large percentage of the variability in cardiac size [8], BSA-adjusted SDS for AOD and LV mass were constructed for analyses. Height-adjusted SDS for BP and CFPWV were constructed for analyses. All SDS were constructed using Generalized Additive Models for Location, Size and Shape (GAMLSS).

Since TH regulates body composition, which is also an important determinant of LV mass, the association of thyroid function with cardiac ultrasound measurements could be mediated via changes in body composition [27 28]. To examine the mediating role of BSA in the association of FT4 with LV mass, we analyzed the direct and indirect mediation effects of BSA by performing mediation analysis, using the approach described previously by Imai et al. [29]. To further examine the mediating effects of specific body composition components in the association of FT4 with LV mass, we performed similar analyses with lean mass index as a potential mediator.

For covariates with missing data, multiple imputation according to Markov Chain Monte Carlo method was used [30]. The percentage of missing data was 2.7% for ethnicity and 14.8% for maternal educational level variables. Five imputed data sets were created and pooled for analysis. Child ethnicity and maternal educational level were then added to the model. We added age, mean systolic and diastolic BP, TSH and FT4 concentrations and cardiac ultrasound measurements as prediction variables only. No statistically significant differences in descriptive statistics were found between the original and imputed datasets. Statistical analyses were performed using Statistical Package of Social Sciences version 21.0 for Windows (SPSS Inc. Armonk, NY) and R statistical software version 3.2.0 (package *rms, mediation, GAMLSS and fdrtool*).

RESULTS

After exclusions, the final study population consisted of 4251 children (Supplemental figure 1), descriptives of which are shown in Table 1. There were no differences between the participants with or without (N=182) available cardiac ultrasound data (Supplemental Table 2) with the exception of FT4 concentrations (median 16.8 vs 16.4, *P*=0.033). There was no difference between participants with or without available data on CFPVW (Supplemental Table 3) or BP (data not shown).

The association of thyroid function with cardiac structure and function

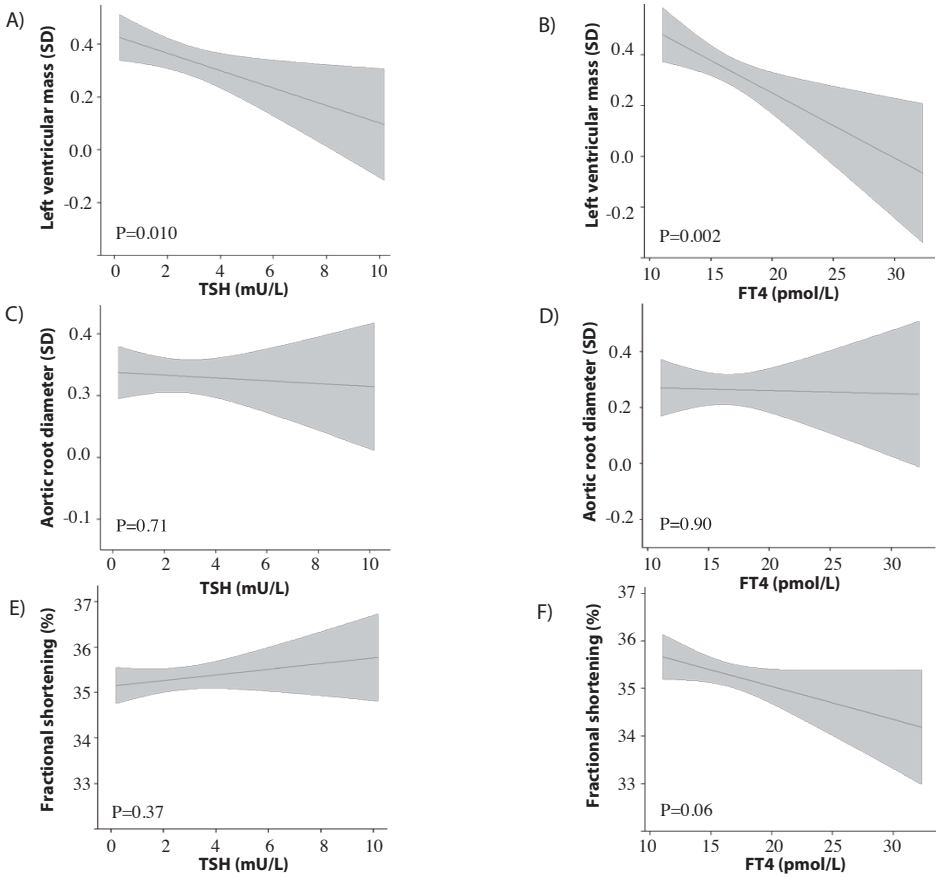
There was an inverse linear association of both TSH and FT4 concentrations with LV mass (*P*=0.010 and *P*=0.002, respectively; Figure 1A,B) which remained same after additional adjustment for blood pressure. TSH and FT4 concentrations were not associated with AOD or FS (Figure 1C-F). After addition of a product interaction term to the model (TSH*sex, *P*=0.09), we stratified the association of TSH with LV mass by sex (Supplemen-

Table 1. Descriptives of the Study Population

Characteristic	Value
TSH, median (95%range), mU/l,	2.29 (0.87-5.20)
FT4, median (95%range), pmol/l	16.8 (13.8-20.7)
Age, median (95%range), years	6.0 (5.7-8.0)
BMI, mean, (sd), kg/m2	16.2 (1.7)
BSA (sd), m^2	0.9 (0.1)
Lean mass index, median (95%range), kg/m^2	1.14 (0.98-1.33)
Fat mass index, median (95%range), kg/m^2	0.37 (0.24-0.79)
Blood pressure, median (95%range), mmHg	
Systolic	102 (88-120)
Diastolic	60 (48-74)
Pulse pressure	42 (30-56)
Carotid-femoral pulse wave velocity, median, (95%range), m/s	5.39 (4.08-7.53)
Aortic root diameter, mean, (sd), mm	19.3 (1.8)
Left ventricular mass, mean, (sd), g	53.8 (11.7)
Fractional shortening, mean (sd), %	35.3 (4.5)
Ethnicity, n(%)	
Dutch	2439 (57.6)
Moroccan	266 (6.3)
Turkish	305 (7.2)
Surinamese	308 (7.3)
Other-Western	339 (8.0)
Other-Non-Western	581 (13.7)
Child sex, n(%)	
Male	2199 (51.5)
Female	2071 (48.5)
Maternal education, n(%)	
No education/primary	196 (4.6)
Secondary	1692 (39.9)
Higher	2350 (55.5)

tal Figure 2). The association of TSH with LV mass was present in boys and not in girls (*P*=0.004 and *P*=0.46, respectively; Supplemental figure 2). There were no sex-specific differences in the association of FT4 concentration with LV mass or in the associations of TSH and FT4 with AOD or FS.

Figure 1. The association of thyroid function with cardiac ultrasound measurements



Plots show the linear regression models for TSH, FT4 and cardiac ultrasound measurements, as predicted mean with 95 percent confidence interval. Analyses were adjusted for ethnicity, age, sex and maternal educational level.

The role of body composition in the association of thyroid function with left ventricular mass

We observed an inverse linear association of FT4 with BSA (beta coefficient = -0.002, 95% confidence interval: (-0.004, -0.001), *P* value < 0.001, data not shown), and we subsequently investigated if the association of FT4 with LV mass could be mediated by BSA. The mediation analysis showed that 26% of the effect of FT4 on LV mass was mediated via changes in BSA (Table 2). As BSA roughly reflects the variability in lean mass and fat mass, and TH may differentially affect these body composition segments [31], we further investigated the association of thyroid function with lean mass and fat mass. There was an inverse linear association of FT4 with lean mass (Figure 2B, *P*<0.0001), whereas FT4

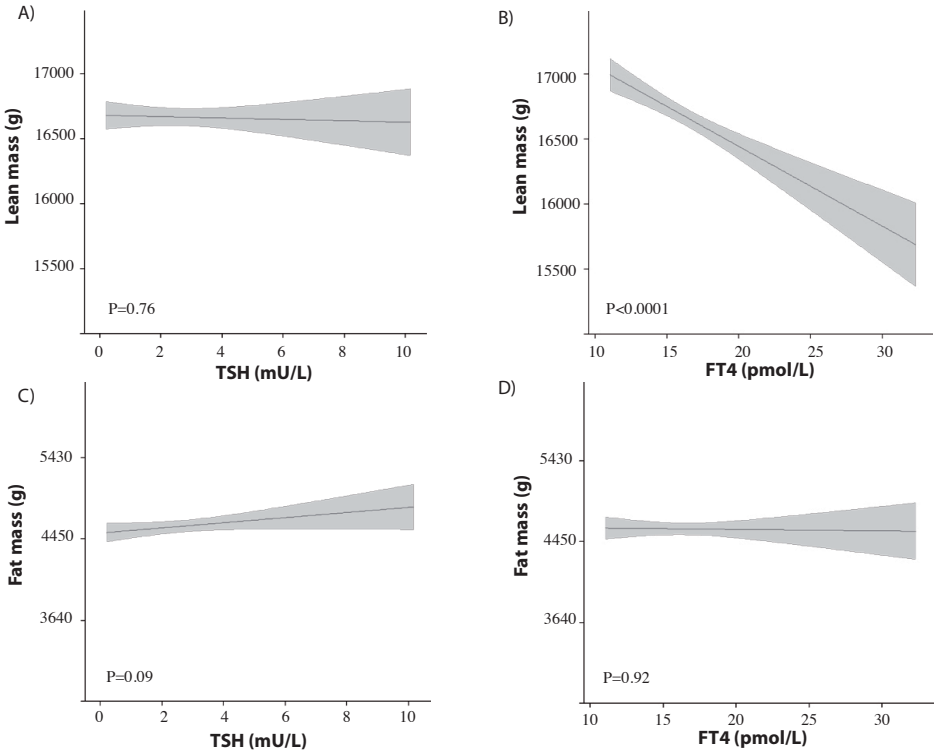
was not associated with fat mass (Figure 2D, *P*=0.92). TSH was not associated with lean mass or fat mass (Figure 2A,C, *P*=0.76, *P*=0.09, respectively).

To specifically study the mediating role of the particular body composition components in the association of FT4 with LV mass, we performed a mediation analysis with the lean mass index as a potential mediator. This analysis showed that 55% of the association was mediated through changes in lean mass (Table 3).

Table 2. BSA as a mediator in the association of FT4 with LV mass

Mediator: BSA	estimate	95% confidence interval	*P* value
Mediated effect	-0.164	-0.275, -0.064	<0.001
Direct effect	-0.465	-0.611, -0.306	<0.001
Total effect	-0.629	-0.830, -0.453	<0.001
Percentage of mediated effect	26%		

Figure 2. The association of thyroid function with body composition components



Plots show the linear regression models for TSH and FT4 with lean mass and fat mass, as predicted mean with 95 percent confidence interval. Analyses were adjusted for sex, ethnicity, age, height and maternal educational level.

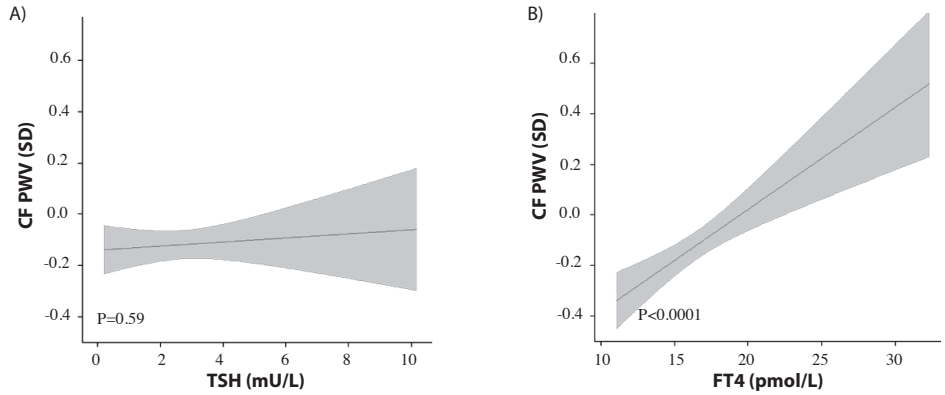
Table 3. Lean body mass as a mediator in the association of of FT4 with LV mass

Mediator: lean body mass index	estimate	95% confidence interval	*P* value
Mediated effect	-0.339	-0.423, -0.249	<0.001
Direct effect	-0.282	-0.465, -0.098	<0.001
Total effect	-0.620	-0.810, -0.421	<0.001
Percentage of mediated effect	55%		

The association of thyroid function with CFPWV and blood pressure

TSH was not associated with CFPWV (*P*=0.59, Figure 3A) whereas there was a positive linear association of FT4 with CFPWV (*P*<0.0001, Figure 3B).

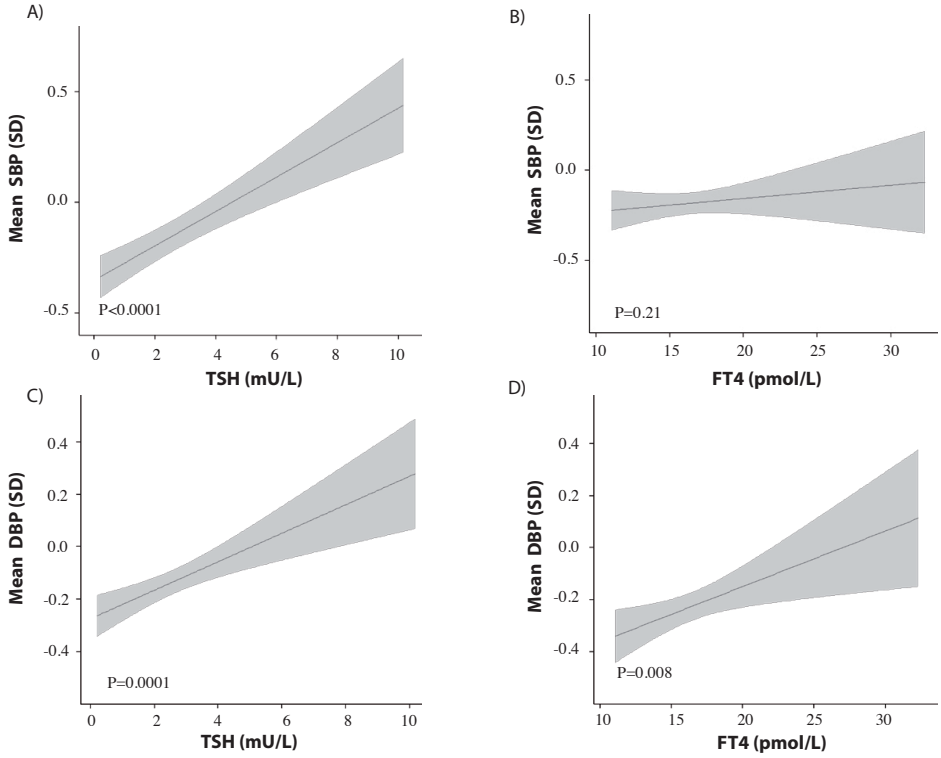
Figure 3. The association of thyroid function with carotid-femoral pulse wave velocity



Plots show the linear regression models for TSH/FT4 and carotid femoral pulse wave velocity, as predicted mean with 95 percent confidence interval. Analyses were adjusted for ethnicity, age, child sex and BMI.

There was a positive linear association of TSH with systolic and diastolic BP (*P*<0.0001 and *P*=0.0001 respectively; Figure 4A,C). The association of TSH with systolic BP was more prominent in girls, and girls with higher TSH concentration had a higher risk of high BP (OR 1.92 – 2.12 depending on the TSH cutoff; Supplemental Figure 3). FT4 was not associated with systolic BP whereas there was a positive linear association of FT4 with diastolic BP (*P*=0.21 and *P*=0.008, respectively; Figure 4B,D). There was a positive linear association of CFPWV with BP (beta coefficient 0.15±0.02, *P*<0.001 and 0.18±0.02, *P*<0.001 for systolic and diastolic BP, respectively, data not shown). After adding CFPWV to the model, the association of FT4 with diastolic BP attenuated (beta coefficient decreased 30% (from 0.022 ±0.009 to 0.016 ±0.009, *P*=0.07, data not shown.) There was no association of TSH or FT4 with pulse pressure (data not shown).

Figure 4. The association of thyroid function with mean arterial blood pressure.



Plots show the linear regression models for TSH, FT4 and systolic/diastolic blood pressure, as predicted mean with 95 percent confidence interval. Analyses were adjusted for ethnicity, age, sex, maternal education level and BMI.

DISCUSSION

Early-life determinants may shape the development of the cardiovascular system and influence subsequent predisposition for cardiovascular disease in adult life. In the current study, we show that thyroid function is a determinant of LV mass and this effect is partially mediated by the effects of FT4 on lean body mass. We also demonstrate a positive association of TSH with BP and a positive association of FT4 with diastolic BP that was dependent on the differences in arterial stiffness, suggesting that TSH and FT4 are associated with BP via different mechanisms.

Little is known about the effects of thyroid hormones on cardiac structure and function at a young age, and to our knowledge, no study has investigated the association of thyroid function with LV mass in children. In adults, population-based studies do not observe an association of TSH concentrations with LV mass [32-34], whereas a higher LV mass

has been described in individuals with subclinical and/or overt hyperthyroidism [35-37], although a longitudinal study showed no effect of subclinical hyperthyroidism on the progression of cardiac hypertrophy in adults [38]. One study in hypertensive individuals reports an inverse linear association of TSH concentration with LV mass [39].

In the current study, there was an inverse linear association of TSH concentration with LV mass in children. Importantly, various differences between adult and childhood factors need to be taken into account when interpreting these associations. In children, the changes in LV mass are predominantly determined by changes in body size [8], and the influence of pathological conditions on the cardiovascular system is much lower than in adults. Factors contributing to a chronically high cardiac load likely underlie the association of thyroid function with LV mass in hyperthyroid and hypertensive state during adulthood [35,36,39], whereas during childhood, LV mass is essentially determined by growth [8]. This is in line with our results showing no effect of additional adjustment for blood pressure. An alternative explanation for the effects of TSH could potentially be the underling effects of thyrotropin-releasing hormone (TRH) on the cardiovascular system, as studies in animals demonstrate a positive association of TRH concentration on LV mass [40,41]. Furthermore, in our study, higher TSH was associated with lower LV mass only in boys. Interestingly, studies in adults report no sex-specific differences in the association of thyroid function with LV mass [32-34,39]. Hence, these differences are more likely to reflect a sex-specific diversity in the rate of both somatic growth as well as heart growth, as LV mass is higher in boys than in girls already in prepubertal period [42]. This is in line with the higher TSH concentrations in boys in The Generation R cohort (unpublished data), suggesting a potential for stronger effects of TSH in boys.

Surprisingly, not only higher TSH, but also higher FT4 was associated with lower LV mass. Most of the previous studies on thyroid function and cardiac structure did not examine the association of FT4 with LV mass [32-35] and only focused on TSH as a measurement of thyroid function. A positive association of FT4 with LV mass has been reported in euthyroid hypertensive adults [39], most likely due to cardiovascular adaptations induced by chronic hypertension and relatively high BMI of the study participants. In our study, the association of FT4 with LV mass was partially mediated via effects of FT4 on the lean body mass but not fat mass. Similar to a previous study, higher FT4 was associated with lower lean mass [31], and we subsequently identified that this effect may mediate approximately 55% of the association of FT4 with LV mass. This is in line with the notion that lean mass is a much stronger determinant of LV mass than fat mass in children [27,43]. Taken all together, our results suggest that the association of thyroid function with LV mass is a reflection of different biological mechanisms through which TSH and FT4 are associated with LV mass, which could explain a similar direction of these associations. The inverse association of FT4 with LV mass in our study suggests that children with lower FT4 are more likely to have higher LV mass at this age, and it remains to be

determined if these individuals have a higher tendency towards LV hypertrophy later in life [2]. The underlying mechanism of this association remains unclear, but it is likely that in the healthy population of a young age, we examined the developmental influence of TH on cardiac structure and function.

We did not observe an association of thyroid function with fractional shortening or aortic root diameter, which is in line with previous reports in adults [33,34]. This lack of association suggests that, at the age of 6 years, the effects of thyroid function are predominant on the cardiac structure and/or growth, as represented by changes in the LV mass. Large differences in thyroid function that could be caused by underlying thyroid disease are practically absent in this young population. As a consequence, any association of thyroid function with cardiac functional parameters will probably be less pronounced. As LV mass tracks from childhood to adulthood [2,3], the observed associations might have implications for cardiac function in the future. However, future studies are needed to further study such long-term effects.

In line with previous reports [44,45], we show that higher TSH is associated with higher BP. A positive association of TSH and BP was reported earlier in obese children as well, but here the association of TSH with BP may have been amplified because obesity has been shown to affect both TSH and BP levels [46,47]. However, we did not find any effect modification by BMI. It has been speculated that this association might be occurring through common genetic factors that are associated with both thyroid function and BP [48].

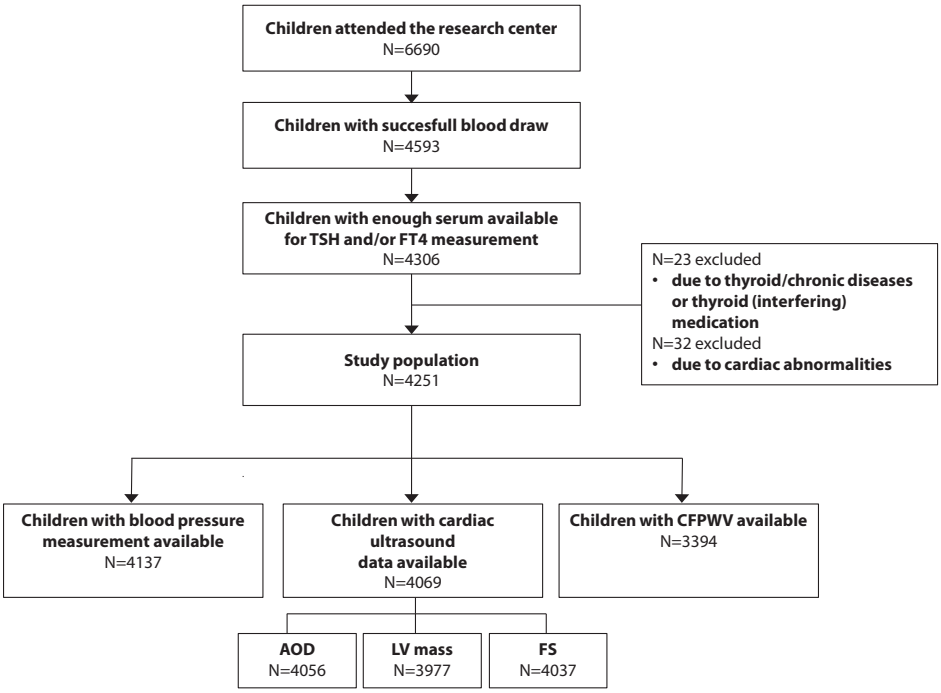
A novel finding of this study is that FT4 is associated with diastolic BP via differences in arterial stiffness. The mechanism underlying this association is likely to be different from the association of TSH with BP, as in our study, CFPWV (a measure of arterial stiffness), was strongly associated with FT4 but not with TSH. Previous studies have also reported a positive association of FT4 with arterial stiffness [49,50] and speculated that FT4 modifies arterial elasticity [50]. Thyroid hormone can initiate endothelial dysfunction [51,52], which is an early sign of atherosclerosis [53] that underlies arterial stiffness. Taken together, this suggests that direct effects of thyroid hormone on arterial tissue may create structural changes that could have consequences for BP levels already during childhood.

To our knowledge, this is the first population-based study that examined the association of thyroid function with cardiovascular structural and functional parameters during childhood. We had detailed data available on thyroid function, cardiac ultrasound measurements, serial BP measurements, arterial stiffness, body composition assessment and potential confounding variables. The main limitation of this study is the cross-sectional study design, which does not allow for studying casual inference and does not impede residual confounding. Another limitation is the narrow age range of children (5.7 - 8.0 years), which limits the generalization of the results to older ages in childhood. Furthermore, in this study, we identified lean mass, and not fat mass, as a potential mediator of

the effects of thyroid function on LV mass. The association of thyroid hormone with fat mass is complex, potentially bidirectional and/or confounded by food intake/fasting [54]. In adults, a positive association of FT4 with fat body mass has been described [31]. In our study, however, FT4 concentration was not associated with fat mass, rendering the possibility of mediation pathway through fat mass implausible.

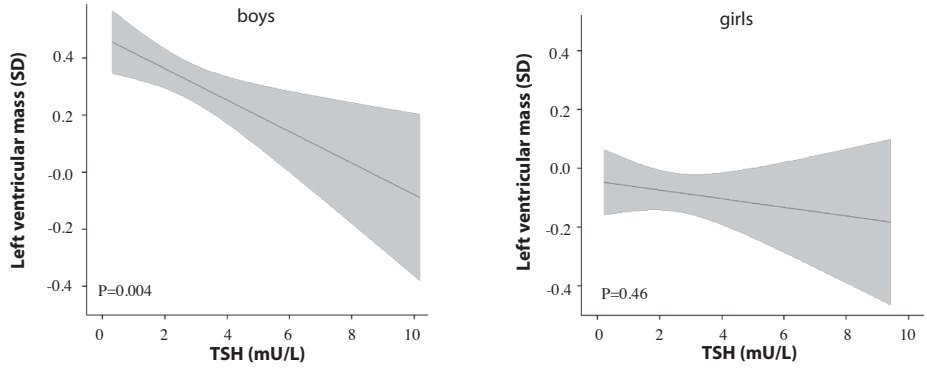
In the current study, we translate the findings of the effects of thyroid function on cardiovascular growth and function from experimental studies to humans in the young age. We observed that thyroid function is associated with the measurement of cardiac growth (LV mass), in part via effects on lean body mass. We also observed that TSH and FT4 might have different underlying mechanisms in the association with BP, with a role of arterial stiffness in the latter case. Although these findings have relatively limited clinical implications, the observed associations designate the importance of thyroid function as an important cardiovascular determinant, as it might affect cardiovascular health during the lifespan. These results need further research to confirm the observations and investigate the association of thyroid function with longitudinal cardiac structure and function measurements during childhood, as well as the extent of arterial stiffness effect in the association of FT4 with BP.

Supplemental Figure 1. Flowchart showing selection procedure of the study population.



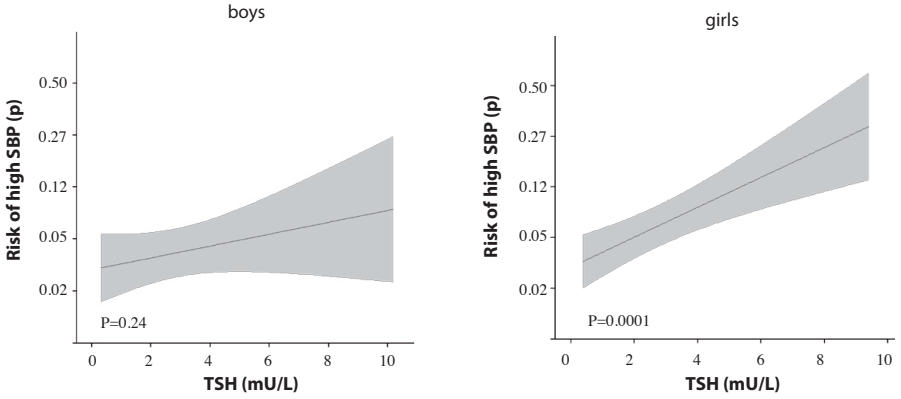
- AOD – aortic root diameter
- LV mass – left ventricular mass
- FS – fractional shortening
- CFPWV – carotid femoral pulse wave velocity

Supplemental Figure 2. The association of TSH with left ventricular mass stratified for sex



Plots show the linear regression models for TSH and left ventricular mass, as predicted mean with 95 percent confidence interval. Analyses were adjusted for ethnicity, age and maternal educational level.

Supplemental Figure 3. The association of thyroid function with the risk of high arterial blood pressure stratified for sex



TSH cutoff	OR	(95%CI)	*P*-value
>95	1.89	(0.88-4.02)	0.10
>90	1.41	(0.75-2.64)	0.29
>85	1.56	(0.92-2.64)	0.10

TSH cutoff	OR	(95%CI)	*P*-value
>95	2.12	(1.06-4.23)	0.033
>90	2.29	(1.35-3.85)	0.002
>85	1.92	(1.21-3.06)	0.006

Plots show the logistic regression models for TSH, FT4 and systolic/diastolic blood pressure, as predicted mean with 95 percent confidence interval. Analyses were adjusted for ethnicity, age, child sex, maternal education level and BMI.

Supplemental Table 1. Regression coefficients (95% confidence intervals) from the multivariate linear regression analysis

Association of thyroid function with cardiac ultrasound measurements				
	Left ventricular mass (SD)	Aortic root diameter (SD)	Fractional shortening	
TSH	-0.035 (-0.063, -0.008)*	-0.005 (-0.032, 0.022)	-0.012 (-0.027, 0.002)	
FT4	-0.027 (-0.044, -0.010) *	-0.001, (-0.017, 0.015)	-0.071 (-0.147, 0.003)	
Sex-specific differences in the association of thyroid function with left ventricular mass				
	boys		**girls**	
TSH	-0.055 (-0.093, -0.017) *		-0.013 (-0.053, 0.026)	
FT4	-0.024 (-0.049, 0.002)		-0.028 (-0.052, -0.004)*	
Association of thyroid function with blood pressure and CFPWV				
	Systolic blood pressure (SD)	Diastolic blood pressure (SD)	Pulse pressure (SD)	CFPWV
TSH	0.079 (0.052, 0.106) **	0.060 (0.034, 0.088) **	0.018 (-0.005, 0.042)	0.009 (-0.022, 0.034)
FT4	0.01 (-0.006, 0.0026)	0.022 (0.006, 0.039) **	-0.012 (-0.027, 0.002)	0.040 (0.022, 0.057) **
Sex-specific differences in the association of TSH with systolic blood pressure				
	Systolic blood pressure (SD)		**Systolic blood pressure (SD)**	
	boys		**girls**	
TSH	0.047 (0.011, 0.083)*		0.117 (0.078, 0.156) **	

Values are regression coefficients (95% confidence intervals) from the multivariate linear regression analyses. Models were adjusted for ethnicity, age, sex, maternal educational level.

* $P<0.05$

** $P<0.001$

Supplemental Table 2. Descriptives of the Study Population– Non response analysis

Characteristic	Value (response)	Value (non response)	*P* value
TSH, median (95%range), mU/l,	2.29 (0.87-5.18)	2.25 (0.89-5.55)	0.36
FT4, median (95%range), pmol/l	16.8 (13.8-20.8)	16.4 (13.6-20.4)	0.033
Age, median (95%range), years	6.0 (5.7-8.0)	6.0 (5.7-7.0)	0.38
BSA (sd), m^2	0.9 (0.1)	0.88 (0.08)	0.62
Lean body mass index, mean (95%range), kg/m^2	1.14 (0.98-1.33)	1.14 (0.98-1.33)	0.76
Fat body mass index, median (95%range), kg/m^2	0.37 (0.24-0.79)	0.36 (0.22-0.81)	0.12
Ethnicity, n(%)			0.81
Dutch	2236 (57.6)	103 (56.6)	
Moroccan	254 (6.3)	12 (6.6)	
Turkish	289 (7.1)	16 (8.8)	
Surinamese	298 (7.3)	10 (5.5)	
Other-Western	321 (7.9)	18 (9.9)	
Other-Non-Western	558 (13.8)	23 (12.6)	
Child sex, n(%)			0.65
Male	2085 (51.4)	97 (53.3)	
Female	1971 (48.6)	85 (46.7)	
Maternal education, n(%)			0.84
No education/primary	189 (4.7)	7 (3.8)	
Secondary	1617 (39.9)	75 (41.2)	
Higher	2250 (55.5)	100 (54.9)	

Supplemental Table 3. Descriptives of the Study Population – Non Response for CFPWV

Characteristic	Value	*P* value – difference between the participants with and without available data
TSH, median (95%range), mU/l,	2.30 (0.78-5.50)	0.17
FT4, median (95%range), pmol/l	16.6 (13.6-20.7)	0.05
Age, median (95%range), years	6.0 (5.6-7.6)	0.40
BMI, mean, (sd), kg/m2	16.3 (1.96)	0.32
Blood pressure, median (95%range), mmHg		
Systolic	102 (87-120)	0.44
Diastolic	60 (48-76)	0.19
Pulse pressure	42 (30-56)	0.69
Ethnicity, n(%)		0.02
Dutch	477 (58.4)	
Moroccan	36 (4.4)	
Turkish	51 (6.2)	
Surinamese	72 (8.8)	
Other-Western	55 (6.7)	
Other-Non-Western	126 (15.4)	
Child sex, n(%)		0.92
Male	422 (51.7)	
Female	395 (48.3)	
Maternal education, n(%)		0.89
No education/primary	37 (4.5)	
Secondary	321 (39.3)	
Higher	459 (56.2)	

Supplemental Table 4. Difference in participants' characteristics based on availability of thyroid function measurement

Characteristic	Value (available thyroid function)	Value (unavailable thyroid function)	*P* value
Age, mean (sd), years	6.2 (0.5)	6.2 (0.5)	0.83
Ethnicity, n(%)			0.03
Dutch	2098 (58.5)	1709 (55.0)	
Moroccan	216 (6.0)	253 (8.1)	
Turkish	278 (7.8)	221 (7.1)	
Surinamese	260 (7.3)	211 (6.8)	
Dutch Antillean	181 (5.0)	279 (9.0)	
Other-Western	281 (7.8)	269 (8.7)	
Other-Non-Western	271 (7.5)	250 (8.1)	
Child sex, n(%)			0.65
Male	1819 (50.7	1533 (49.4)	
Female	1766 (49.3)	1572 (50.6)	
Maternal education, n(%)			0.001
No education/primary	140 (3.9)	177 (5.7)	
Secondary	1452 (40.5)	1300 (41.9	
Higher	1993 (55.6)	1628 (52.4)	
Lean body mass, mean (sd), kg	16.4 (2.3)	16.3 (2.4)	0.05
Fat body mass, mean (sd), kg	5.9 (2.4)	5.9 (2.4)	0.82
Blood pressure, mean (sd)			
Systolic	102 (8)	102 (8)	0.71
Diastolic	60 (7)	60 (7)	0.59
Carotid-femoral pulse wave velocity, mean, (sd), (m/s)	5.5 (0.9)	5.5 (0.9)	0.17
Aortic root diameter, mean, (sd), mm	19.3 (1.9)	19.1 (1.8)	0.01
Left ventricular mass, mean, (sd), g	53.8 (11.8)	52.9 (11.7)	0.01
Fractional shortening, mean, (sd), %	35.3 (4.5)	35.2 (4.7)	0.14

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CHAPTER 4.3

The association of thyroid function with bone density during childhood

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Submitted

ABSTRACT

Background: The skeleton is a well-known target organ for thyroid hormone. Little data are available on the association of thyroid function and bone development during childhood. The aim of this study was to prospectively examine and quantify the effects of thyroid function on bone density in a population-based cohort of children.

Methods: Serum TSH and FT4 were measured in samples collected at median age of 6 years (95% range 5.6-7.9 years). For 4204 participants of The Generation R Study, information on thyroid function and bone density at the age of 6 was available; for 3404 participants information was available for thyroid function and bone density at the age of 10. A total body dual-energy x-ray absorptiometry (DXA) (used for none mineral density (BMD) and bone mineral content (BMC)) scan was performed at median age of 6 and 10 years (95% range 5.6-7.9 years and 9.0-10.9 years, respectively).

Results: At the age of 6, TSH and FT4 were inversely associated with both BMD and BMC (TSH: *P*=0.003 and *P*=0.038, respectively, and FT4: *P*=0.047 and *P*=0.003, respectively). TSH was not associated with BMD and BMC at the age of 10 (*P*=0.15 and *P*=0.52, respectively), whereas FT4 was inversely associated with BMD and BMC at the age of 10 (*P*=0.048 and *P*=0.018, respectively). In the European subgroup TSH was not associated with BMD and BMC at the age of 6 (*P*=0.11 and *P*=0.21, respectively) or at the age of 10 (*P*=0.79 and *P*=0.79, respectively) and FT4 was not associated with BMD (*P*=0.10), whereas FT4 was inversely associated with BMC at 6 *(P*=0.014) and BMC and BMD at 10 years (*P*=0.031 and *P*=0.037, respectively).

Conclusion: In this large population-based cohort, we observed an inverse association of FT4 with bone density at the age of 6 and at the age of 10 years. We did not detect consistent associations of TSH with bone density measurements.

INTRODUCTION

Thyroid hormone regulates metabolism in practically all tissues of the human body [1] and plays an important role during childhood growth and development [2], in neurodevelopment and neuropsychological function [3,4] and in the cardiovascular system [5]. Bone is a well-known target organ for thyroid hormone, which is especially important for proper linear growth and development during childhood [2]. In line with this, congenital hypothyroidism is associated with delayed skeletal development and impaired bone maturation, leading to short stature [2] and thyroid hormone replacement can induce 'catch-up' growth and acceleration of skeletal maturation [6], leading to achievement of normal height and normal bone mass [7,8]. On the other hand, juvenile hyperthyroidism is associated with accelerated skeletal development and rapid growth [9,10]. In adults, hyperthyroidism increases the risk of osteoporosis, which is reflected by low bone mineral density (BMD) and the risk of fracture [11]. Studies in healthy adults suggest that even high-normal thyroid hormone and low-normal thyroid stimulating hormone (TSH) concentrations are associated with high bone turnover state, leading to a net increased bone resorption and thereby a decreased BMD and consequently a higher risk of fracture [2,12,13].

In vitro and animal studies suggest that besides thyroid hormone, TSH might play a role in the skeletal development and maintenance by inhibiting osteoclastogenesis and function [2,14] and possibly by exerting effects on osteoblasts as well [2,14]. It was also shown that deiodinases are likely to regulate thyroid hormone activity in bone [15-17], although the exact mechanisms remain unclear. Despite the known regulating role of thyroid hormone in the skeleton in experimental setting, little epidemiological data are available that have translated these effects to human data focusing on childhood growth and development.

The aim of this study was to investigate the association of thyroid function with BMD and bone mineral content (BMC) during childhood in a population-based prospective cohort. We set out to investigate the potential effects of TSH and FT4 on longitudinally measured bone density in children.

METHODS

Study design

This study was embedded in Generation R, a large prospective population-based cohort from early fetal life onwards, in Rotterdam, the Netherlands [18]. The study was designed to identify early environmental and genetic determinants of growth, development and health during fetal life, childhood and young adulthood [18]. Eligible participants were

6690 live-born children born between 2002 and 2006 that visited the research center at roughly 6 years of age[18].

Thyroid measurements

TSH and FT4 were measured in cord blood samples that were obtained directly after birth (median gestational age at birth 40.1 weeks, 95% range 35.8-42.3 weeks) and in serum samples obtained at the age of 6. Plain tubes were centrifuged and serum was stored at -80°C. TSH and free thyroxine (FT4) concentrations were determined in cord blood samples using chemiluminescence assays (Vitros ECI; Ortho Clinical Diagnostics, Rochester, NY). The intra- and interassay variation coefficients were <4.1% for TSH at a range of 3.97–22.7 mU/l and <5.4% for FT4 at range of 14.3 – 25.0 pmol/l. At 6 years of age, TSH and FT4 were measured using an electrochemiluminescence immunoassay on the Cobas e601 immunoanalyzer (Roche Diagnostics). The intra- and interassay coefficients of variation were 1.1-3.0% for TSH at a range 0.04-0.4 mU/L and 1.6-5.0% for FT4 at a range of 1.6-24.1 pmol/L.

Bone density measurements

At the age of 6 (95% range 5.6-7.9 years) and at the age of 10 years (median 9.7 years, 95% range 9.0-10.9 years) BMD was measured using a dual-energy X-ray absorptiometry (DXA) scan (Idxa; General Electrics – Lunar, 2008) using procedures described previously [19]. BMD measured by DXA was expressed as BMC (in g) per projected bone area (in cm^2). As recommended by the International Society for Clinical Densitometry, BMC and BMD values of the total body without the head was used, so that bone density of the skeleton is best represented [20]. Due to size effects, all models were adjusted for height and soft tissue mass [21]. For the same reason, BMC values were standardized for bone area [21].

Covariates

Weight and height were measured without shoes and heavy clothing. DXA scans were used to assess the lean and fat mass of the body composition; subsequently soft tissue mass was determined by adding lean mass to fat mass. Information on ethnicity was obtained through questionnaires and was defined by the country of origin of the parents, according to the classification of Statistics Netherlands [18]. Information on educational level of the mother was used as a proxy for socioeconomic status and was obtained through questionnaires. Information about participants' sex, birth weight and gestational age at birth was obtained using medical records and hospital registries.

Statistical Analysis

The association of TSH and FT4 with BMD and BMC was examined using multiple linear regression models. We used restricted cubic splines utilizing three knots to account for

possible non-linearity. Multivariable associations were graphically depicted by plots and standardized beta estimates with 95% confidence intervals for the regression models are presented in Supplemental Table 2. All model covariates were selected based on biological plausibility, a 10% change in the effect estimate or residual variability of the model. All models were adjusted for age, sex, soft tissue mass, height and educational level of the mother. Cord blood models were additionally adjusted for the SD score of birthweight. As already in childhood a substantial variation is present in bone density depending on the ethnic origin [22], the associations were additionally examined in a subgroup of European participants. Multiple imputation according to Markov Chain Monte Carlo method was used to cope with the missing values of the covariates [23]. The percentage of missing data was: 16.6% for educational level of the mother at child's age 6, 9.2% for education level of the mother at enrolment, 2.5% for ethnicity, 2.1% for birth weight, 2.0 % for child's sex, 1.6% for gestational age at birth and <1% for other covariates. Data on thyroid function and bone density measurements were not imputed and were used as predictor variables only. Five imputed datasets were created and pooled for analysis. There were no statistically significant differences between the imputed and the original dataset. All analyses were performed using Statistical Package of Social Sciences version 21 for Windows (SPSS Inc. Chicago, IL, USA) and R statistical software version 3.3.3 ('lm. beta', 'rms'and'foreign' package).

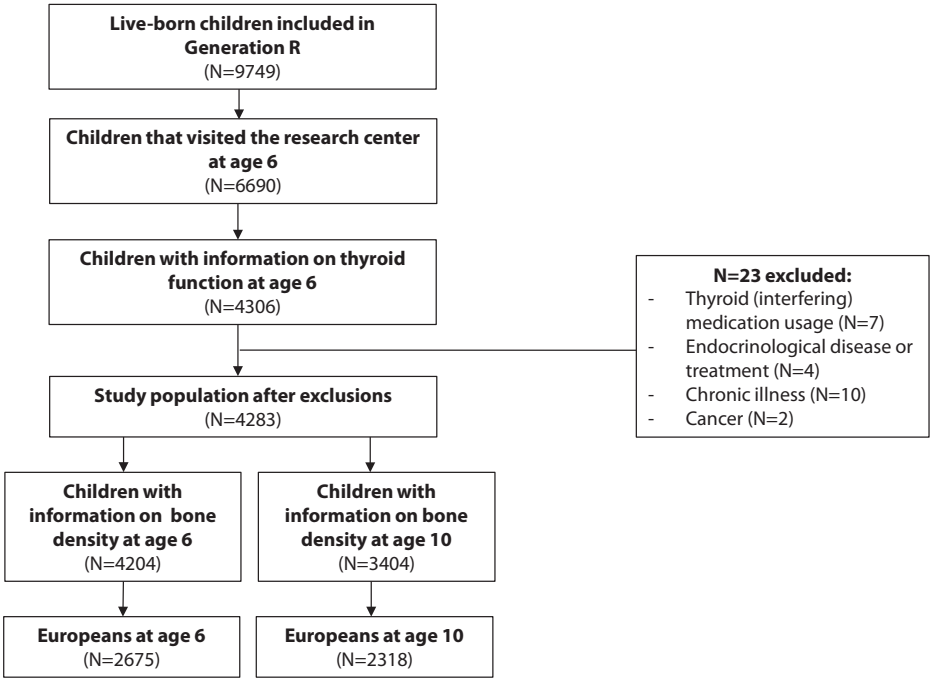
RESULTS

At median age of 6 years 6690 participants visited the research center (Figure 1). For 4306 children information on thyroid function was available (Figure 1). Participants that used medication that could interfere with thyroid function (n=7) or that suffered from endocrinological disease (n=4), chronic illness (n=10) or cancer (n=2) were excluded (Figure 1). After exclusions, the total study population comprised of 4204 participants at median age of 6 years and 3404 participants at median age of 10 years, (Figure 1); descriptives of the study population are shown in Table 1. Of the total group, 2675 and 2318 participants at the median age of 6 and 10 years, respectively, were of European origin. Descriptives of the European subgroup are presented in Supplemental Table 1.

Thyroid function and bone density in the total population

In the total population, at median age of 6 years, TSH was inversely associated with both BMD and BMC (P=0.003 and P=0.038, respectively; Figure 2) and FT4 was inversely associated with BMD and BMC (P=0.047 and P=0.003, respectively; Figure 2). TSH was not associated with BMD and BMC at median age of 10 years (P=0.15 and P=0.52, respectively; Figure 3), whereas FT4 was inversely associated with both BMD and BMC at median age

Figure 1. Flowchart showing selection procedure of the study population



of 10 years (*P*=0.048 and *P*=0.018, respectively; Figure 3). These effects attenuated when the models were adjusted for bone density at median age of 6 years (*P*=0.80 and *P*=0.76, respectively; Supplemental Table 2).

Thyroid function and bone density in the subgroup of European participants

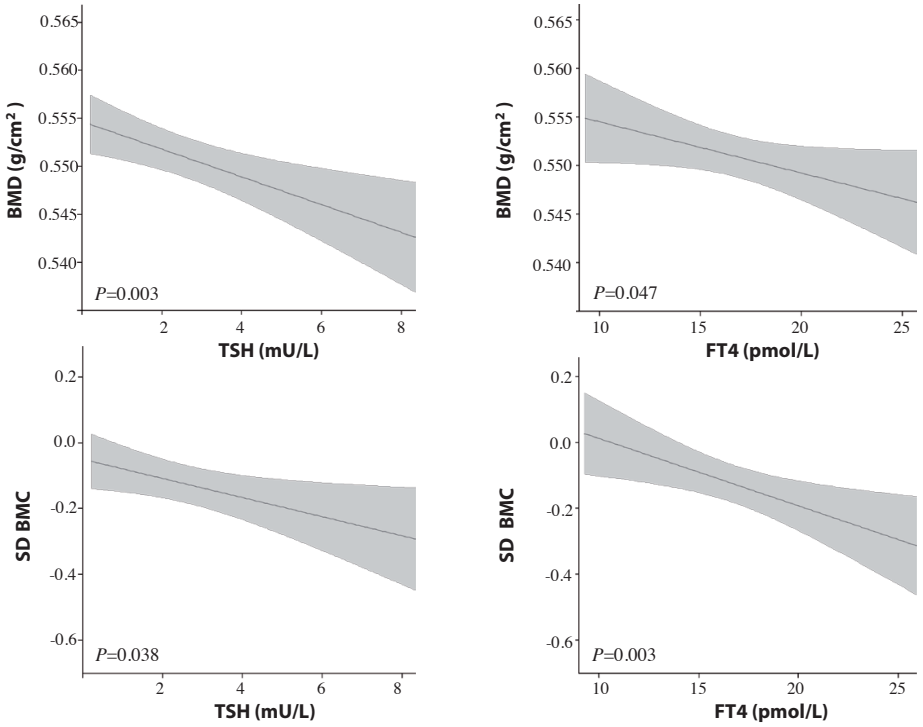
As Generation R is a multi-ethnic cohort and ethnicity-related differences in bone density are already present in childhood [22], we performed a sensitivity analysis by investigating the associations in a subgroup of participants of European descent. TSH was not associated with BMD or BMC assessed at median age of 6 years (*P*=0.11 and *P*=0.21, respectively; Figure 4). FT4 was not associated with BMD, but there was an inverse association of FT4 with BMC (*P*=0.10 and *P*=0.014, respectively; Figure 4).

TSH was not associated with BMD or BMC at the median age of 10 years (*P*=0.79 and *P*=0. 79, respectively; Figure 5). FT4 was inversely associated with both BMD and BMC at median age 10 (*P*=0.031 and *P*=0.037, respectively; Figure 5). These effects attenuated when the models were adjusted for bone density at the age of 6 (*P*=0.85 and *P*=0.87, respectively; Supplemental Table 2).

Table 1. Descriptives of the total study population

Characteristic	Value	
At birth		
TSH cord blood, median (95%range), mU/L	9.35	(3.35-33.5)
FT4 cord blood, median (95%range), pmol/L	20.5	(15.4 - 28.8)
Birth weight, median (95%range), g	3450	(2306 - 4475)
Gestational age at birth, median (95%range), weeks	40.1	(35.8 - 42.3)
Educational level of the mother, n(%)		
No education or primary education	670	(11.8%)
Secondary education	2374	(41.7%)
Higher education	2647	(46.5%)
At median 6 years		
BMD without head, median (95%range), g/cm^2	0.55	(0.47 - 0.70)
BMC without head, median (95%range), g	514	(366 - 792)
TSH, median (95%range), mU/L	2.29	(0.87 - 5.18)
FT4, median (95%range), pmol/L	16.8	(13.8 - 20.8)
Age, median (95%range), years	6.0	(5.6 - 7.9)
Weight, median (95%range), kg	22.6	(17.6 - 34.2)
Height, median (95%range), cm	119	(109 - 133)
Educational level of the mother, n(%)		
No education or primary education	522	(9.2%)
Secondary education	2205	(38.7%)
Higher education	2964	(52.1%)
At median 10 years		
BMD without head, median (95%range), g/cm^2	0.68	(0.57 - 0.82)
BMC without head, median (95%range), g	921	(661 - 1320)
Age, median (95%range), years	9.7	(9.0 - 10.9)
Weight, median (95%range), kg	34.8	(25.6 - 52.0)
Height, median (95%range), cm	141	(130 - 155)
General characteristics		
Child's sex, n(%)		
Male	2891	(50.8%)
Female	2800	(49.2%)
Ethnicity child, n(%)		
Dutch	3204	(56.3%)
Turkish	416	(7.3%)
Surinamese	423	(7.4%)
North-African	351	(6.2%)
Asian	206	(3.6%)
Other western	509	(8.9%)
Other non-western	582	(10.2%)

Figure 2. The association of thyroid function with bone density at 6 years in the total study population



Plots show the linear regression models of TSH and FT4 with bone mineral density (BMD) and bone mineral content (BMC) standardized for bone area at age 6, as predicted mean with 95% confidence interval. Analyses were adjusted for age , sex, soft tissue mass, height, ethnicity and educational level of the mother .

Cord blood thyroid function and bone density

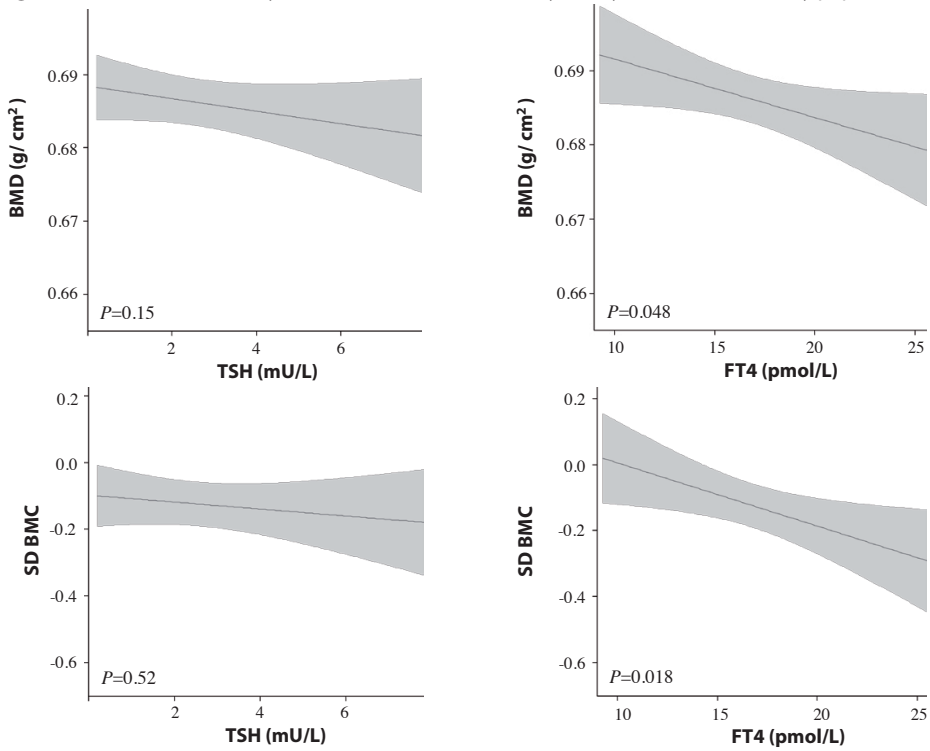
Cord blood thyroid function was not associated with bone density measurements at any age, apart from an inverse association of FT4 with BMD at the age of 6 in the total population (Supplemental Figure 1-4).

DISCUSSION

In the current study, we show that thyroid function is associated with bone density measurements during childhood. We demonstrate that higher FT4 is associated with lower bone density at median age of 6 and 10 years. We did not detect consistent associations of TSH with bone density during childhood.

Population studies in both euthyroid and hyperthyroid adults show that higher thyroid hormone concentrations and/or lower TSH concentrations are associated with lower BMD [2,12]. Children with untreated hyperthyroidism are reported to have a lower

Figure 3. The association of thyroid function with bone density at 10 years in the total study population

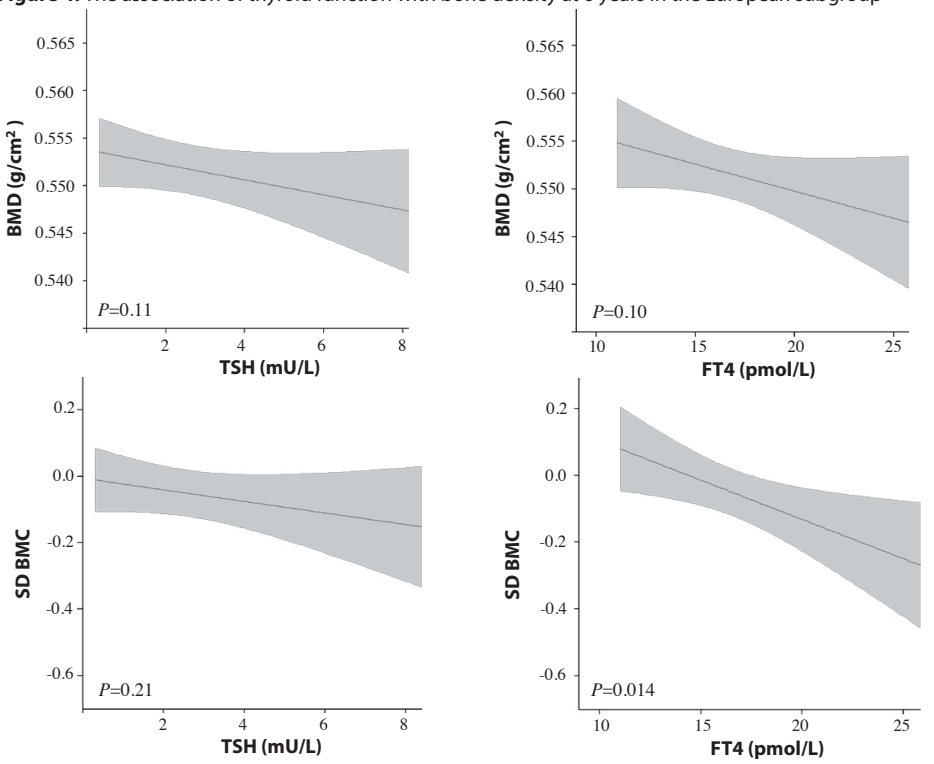


Plots show the linear regression models of TSH and FT4 with bone mineral density (BMD) and bone mineral content (BMC) standardized for bone area at age 10, as predicted mean with 95% confidence interval. Analyses were adjusted for age , sex, soft tissue mass, height, ethnicity and educational level of the mother .

BMD compared to healthy controls [24,25], but these findings are not generalizable to a healthy pediatric population. Little epidemiological data are available on the influence of thyroid function on the bone development, and to our knowledge, this is the first study to investigate the association of thyroid function with bone density in healthy children. Optimizing peak bone mass in children and adolescents is considered as an important strategy to prevent osteoporosis in later life [26]. Understanding the physiology of bone mass accrual will help to identify potential risk factors and targets for intervention.

In the current study, we identified an inverse association of FT4 with BMD and BMC in early childhood, suggesting that high thyroid hormone levels might lead to lower bone density already during childhood. These results are in line with data from experimental studies which show that thyroid hormone stimulates osteoblast proliferation, bone matrix synthesis and mineralization [2,12,27]. Moreover, it has also been shown that thyroid hormone regulates bone mass [28] and that it stimulates osteoclast activity and bone resorption directly, or indirectly via osteoblasts or other pathways [2,12,27].

Figure 4. The association of thyroid function with bone density at 6 years in the European subgroup

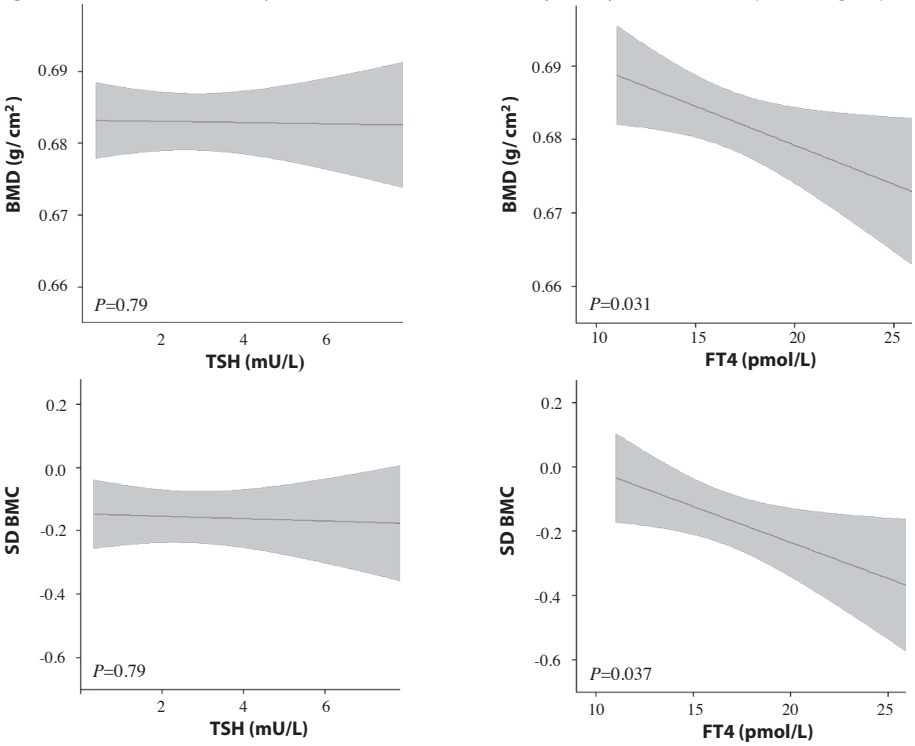


Plots show the linear regression models of TSH and FT4 with bone mineral density (BMD) and bone mineral content (BMC) standardized for bone area , as predicted mean with 95% confidence interval. Analyses were adjusted for age , sex, soft tissue mass, height and educational level of the mother .

In the European population we detected stronger effects of FT4 on BMC than on BMD at the age of 6. A potential explanation is the method of assessing bone density, because using BMD measurement can overestimate true BMD when measuring large bones, whereas on small bones BMD can be underestimated, suggesting that, during childhood growth, BMD might be a less reliable measurement for bone density [21]. BMC adjusted for bone area, height and soft tissue mass, reflects true bone density better and is therefore more reliable and may provide a more precise measurement [29].

Our results suggest that the inverse association of FT4 with bone density at the age of 10 is driven by the effects of FT4 earlier in childhood, in this case at 6 years. Furthermore, the 'Bone Mineral Density in Childhood Study' showed that bone density tracks in time, which means that if adjusted bone density scores are low in childhood, they will remain low later in childhood and adolescence as well [30]. This suggests that early influences during childhood may be important for bone density in later age. Hence, early childhood thyroid function might influence the quality of bone accrual throughout early

Figure 5. The association of thyroid function with bone density at 10 years in the European subgroup



Plots show the linear regression models of TSH and FT4 with bone mineral density (BMD) and bone mineral content (BMC) standardized for bone area , as predicted mean with 95% confidence interval. Analyses were adjusted for age , sex, soft tissue mass, height and educational level of the mother .

childhood, and might therefore be important for bone density at a later age as well. Further studies are needed to investigate the effects of early life thyroid function on bone density in adulthood.

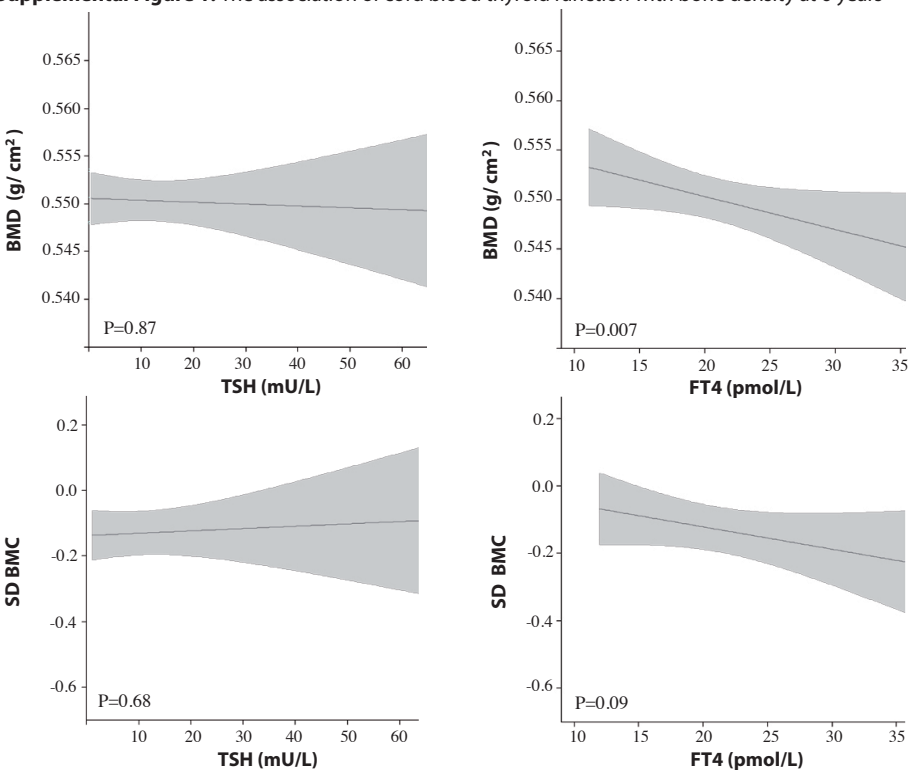
Experimental studies suggest that TSH might directly exert effects on bone by inhibiting osteoclastogenesis and function [2,14] and possibly by exerting effects on osteoblasts as well [2,14]. However, the results are not consistent and this remains a controversial hypothesis [2,28]. Some studies in adults suggest that high TSH might protect against bone loss [31-33], although it is postulated that FT4 has a greater impact on bone turnover than any direct effect of TSH [33]. In the longitudinal analysis within the total population and in the European subgroup overall, we did not detect associations of TSH with bone density in childhood. The association of TSH with bone density we detected in the total population cross-sectionally might therefore be subject to residual or unmeasured confounding. Apart from the ethnicity related differences in bone density [22,34,35], TSH levels are also known to differ between ethnic groups in the Generation R cohort (unpublished data). Moreover, genetics are known to have great impact on both bone density [36] and thyroid

function [37]. Further research is needed to examine the possible ethnic and/or genetic differences in the association of TSH with bone density.

For the current study, we were able to study the association of childhood thyroid function with longitudinally measured bone density in a population-based setting using detailed information on covariates for a large number of participants. The main limitation of this study is that only one thyroid function measurement at median age of 6 years was available, which limits generalization of the results to older ages in childhood. Moreover, the observational design of this study does not allow for inference of causality and does not preclude the existence of residual confounding even though we were able to adjust for a large number of covariates.

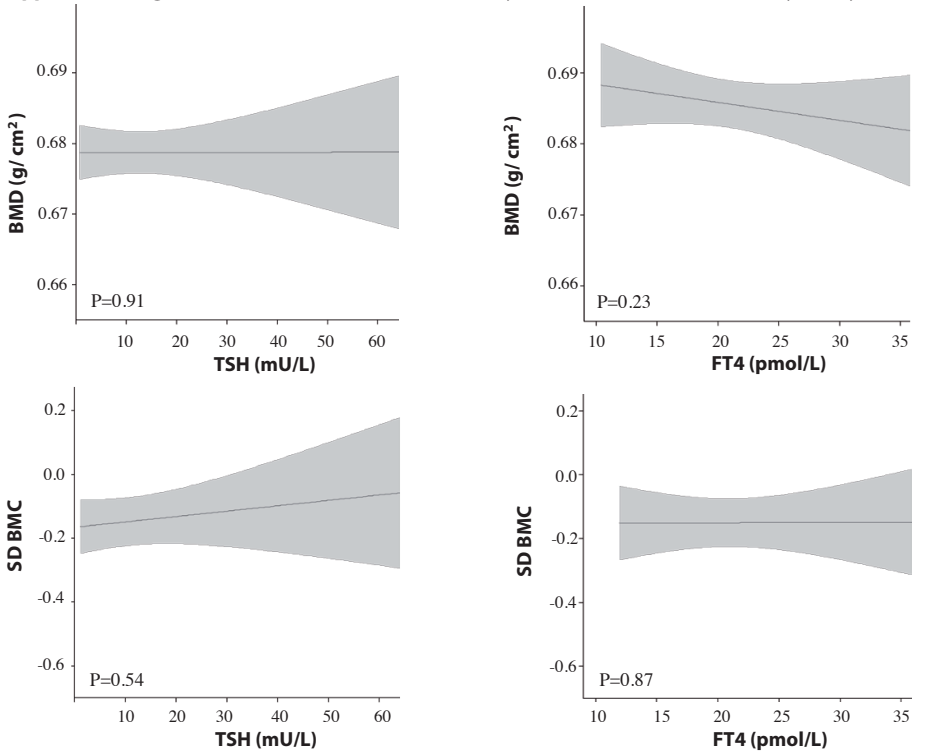
In conclusion, this study provides new insights into the influence of thyroid function on bone density during childhood. We show an inverse association of FT4 with bone density and our data suggest that early childhood thyroid function could be a determinant of the quality of bone mineral accrual throughout childhood. We did not detect consistent evidence for an effect of TSH on bone density. Future studies are needed to investigate whether these associations persists later in childhood and adulthood and to investigate whether these effects are genetically determined.

Supplemental Figure 1. The association of cord blood thyroid function with bone density at 6 years



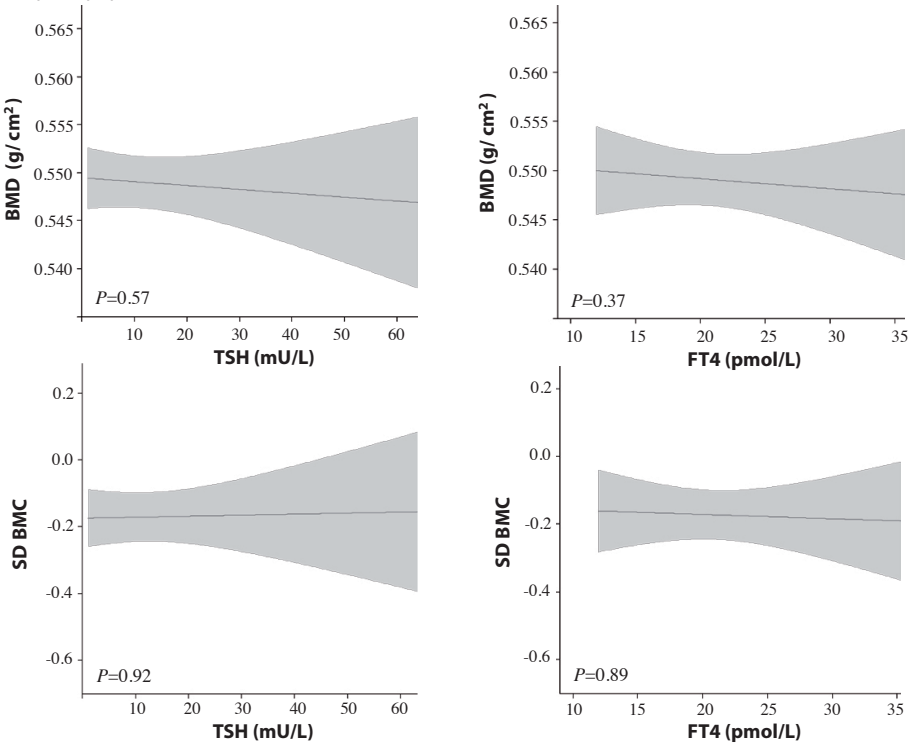
Plots show the linear regression models of cord blood TSH and FT4 with bone mineral density (BMD) and bone mineral content (BMC) regressed for bone area at age 6, as predicted mean with 95% confidence interval. Analyses were adjusted for age, sex, soft tissue, height, SDS birth weight, ethnicity and educational level of the mother.

Supplemental Figure 2. The association of cord blood thyroid function with bone density at 10 years



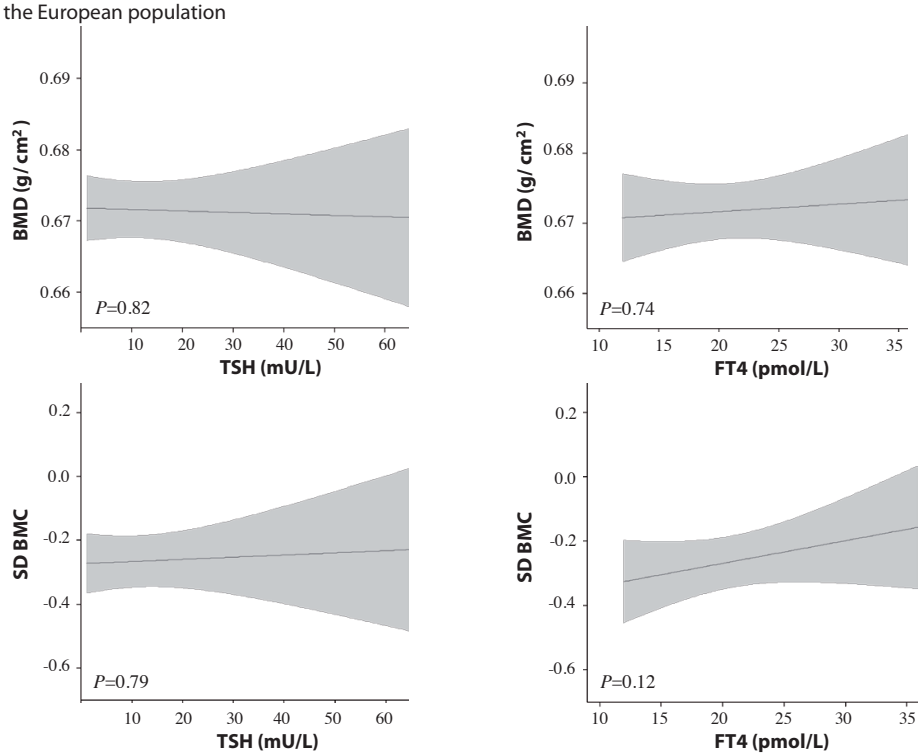
Plots show the linear regression models of cord blood TSH and FT4 with bone mineral density (BMD) and bone mineral content (BMC) regressed for bone area at age 10, as predicted mean with 95% confidence interval. Analyses were adjusted for age , sex, soft tissue, height, SDS birth weight, ethnicity and educational level of the mother .

Supplemental Figure 3. The association of cord blood thyroid function with bone density at 6 years in the European population



Plots show the linear regression models of cord blood TSH and FT4 with bone mineral density (BMD) and bone mineral content (BMC) standardized for bone area at age 6, as predicted mean with 95% confidence interval. Analyses were adjusted for age , soft tissue mass, height , SDS birth weight, and educational level of the mother .

Supplemental Figure 4. The association of cord blood thyroid function with bone density at 10 years in the European population



Plots show the linear regression models of cord blood TSH and FT4 with bone mineral density (BMD) and bone mineral content (BMC) standardized for bone area at age 10, as predicted mean with 95% confidence interval. Analyses were adjusted for age , sex, soft tissue mass, height , SDS birthweight and educational level of the mother at birth.

Supplemental Table 1. Descriptives of the European study population

Characteristic	Value	
At birth		
TSH cord blood, median (95%range), mU/L	9.79	(6.54, 35.1)
FT4 cord blood, median (95%range), pmol/L	20.7	(15.6, 28.9)
Birth weight, median (95%range), g	3500	(2360, 4520)
Gestational age at birth, median (95%range), weeks	40.1	(35.7, 42.3)
Educational level of the mother, n (%)		
No education or primary education	152	(4.2%)
Secondary education	1360	(37.5%)
Higher education	2110	(58.3%)
At median 6 years		
BMD without head, median (95%range), g/cm^2	0.54	(0.47, 0.65)
BMC without head, median (95%range), g	508	(366, 749)
TSH, median (95%range), mU/L	2.38	(0.91, 5.38)
FT4, median (95%range), pmol/L	16.5	(13.7, 20.5)
Age, median (95%range), years	6.0	(5.6, 7.6)
Weight, median (95%range), kg	22.4	(17.7, 31.4)
Height, median (95%range), cm	119	(109, 132)
Educational level of the mother, n(%)		
No education or primary education	69	(1.9%)
Secondary education	1214	(33.5%)
Higher education	2340	(64.6%)
At median 10 years		
BMD without head, median (95%range), g/cm^2	0.68	(0.57, 0.80)
BMC without head , median (95%range), g	915	(665, 1275)
Age, median (95%range), years	9.7	(9.1, 10.7)
Weight, median (95%range), kg	34.4	(25.6, 49)
Height, median (95%range), cm	142	(130, 154)
General characteristics		
Child's sex, n(%)		
Male	1823	(50.3%)
Female	1800	(49.7%)
Ethnicity child, n(%)		
Dutch	3204	(88.4%)
Other European	419	(11.6%)

Supplemental Table 2. Estimates for the association of childhood thyroid function with bone density

		Total study population			European study population		
Model	Outcome	Standardized β	95% CI	*P* value	Standardized β	95% CI	*P* value
Childhood thyroid function with bone density at age 6							
TSH	BMD	-0.028	-0.046, -0.010	0.003	-0.017	-0.039, 0.004	0.11
TSH	BMC	-0.027	-0.052, -0.001	0.038	-0.018	-0.048, 0.011	0.21
FT4	BMD	-0.011	-0.022, -0.000	0.047	-0.012	-0.026, 0.002	0.10
FT4	BMC	-0.023	-0.038, -0.008	0.003	-0.024	-0.043, -0.005	0.014
Childhood thyroid function with bone density at age 10							
TSH	BMD	-0.015	-0.035, 0.005	0.15	-0.003	-0.027, 0.020	0.79
TSH	BMC	-0.009	-0.036, 0.018	0.52	-0.004	-0.035, 0.027	0.79
FT4	BMD	-0.012	-0.024, -0.000	0.048	-0.017	-0.032, -0.002	0.031
FT4	BMC	-0.019	-0.035, -0.003	0.018	-0.021	-0.042, -0.001	0.037
Childhood thyroid function with bone density at age 10 adjusted for bone density at 6							
TSH	BMD	-0.003	-0.017, 0.011	0.67	0.000	-0.015, 0.017	0.95
TSH	BMC	0.009	-0.018, 0.020	0.93	0.008	-0.022, 0.023	0.94
FT4	BMD	-0.001	-0.009, 0.007	0.80	0.001	-0.009, 0.011	0.85
FT4	BMC	-0.002	-0.013, 0.010	0.76	0.001	-0.013, 0.016	0.87

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CHAPTER 5

General discussion

This thesis examines the associations of thyroid function and hCG with various outcomes in early life. The association of thyroid function with pregnancy outcomes is investigated with a specific interest in potential underlying mechanisms such as the effects on the placental function and metabolic effects in terms of homocysteine concentrations. Also, the associations of the pregnancy hormone hCG (which is known to stimulate the thyroid) with pre-eclampsia and fetal growth were investigated, and we studied if thyroid function could be a pathway or an effect modifier of these associations. Furthermore, the studies presented in this thesis investigate major determinants of childhood thyroid function, as well as the associations of childhood thyroid function with relevant thyroid hormone target organs including the cardiovascular system and bone. This chapter provides a general discussion of the main findings presented in previous chapters, methodological considerations, clinical implications and future perspectives for the field.

REFLECTION OF MAIN FINDINGS

Thyroid function in pregnancy

Thus far, a great effort has been put into investigation of the adverse effects of gestational thyroid dysfunction and the optimization of thyroid hormone availability in pregnancy [1,2]. The necessity of such studies is substantiated by the clinical associations of thyroid (dys) function with pregnancy complications of major public health concern. These include premature delivery, pre-eclampsia and low birth weight [3-8] which altogether underlie a large portion of maternal and fetal morbidity and mortality worldwide. However, little is known on the factors that could mediate the effects of gestational thyroid function on these relevant outcomes. Elucidating such mechanisms could provide novel insights into clinical follow-up, identification of high-risk groups and could eventually allow for early and targeted interventions. One of the first potential mechanisms that we investigated is the placental function. Experimental studies have shown that thyroid hormone exerts effects on both endometrium and trophoblast function [9-13] and it has been postulated that an optimal thyroid hormone concentration is crucial for a successful placentation [13,14]. However, these associations are largely unexplored in epidemiological studies, which is particularly important for verification of the results on a population level and extraction of potential clinical implications. Chapter 2.1 of this thesis aims to translate the experimental data into a population setting and investigates the association of gestational thyroid function with placental function, as assessed by placental vascular resistance. Moreover, we investigate how relevant this potential association is for the effects of thyroid function on pregnancy complications that represent a major public health burden.

In Chapter 2.1, we demonstrate that a higher maternal FT4 concentration in early pregnancy is associated with higher placental vascular resistance in mid- and late pregnancy and that high thyroid function may influence placental function, which could be occurring potentially through impaired placentation. The association of high FT4 with a higher vascular resistance in both the fetal and maternal compartment of the placenta (umbilical and uterine arteries) suggests that thyroid hormone affects the formation of the placenta as a whole, i.e. feto-placental and utero-placental circulation. The association of high FT4 (measured in early pregnancy) with a higher placental vascular resistance measured repeatedly in the second and third trimester suggests that the effects of thyroid hormone on placental development may have a persistent impact throughout pregnancy. These associations were similar in a subgroup of euthyroid women, suggesting that even 'high-normal' thyroid hormone availability may lead to a suboptimal placentation. In general, placentation is a complex process and impairment at any stage may result in pregnancy complications [15]. We hypothesized that the detected associations of thyroid hormone with placental vascular resistance could be explained via regulating effects of thyroid hormone on trophoblast function as well as on angiogenic factors and cytokines that play a role in the process of placentation [10,16] because this could result in impaired placental function and manifest as high placental vascular resistance later in pregnancy. The findings presented in Chapter 2.1 provide a basis for future studies that may want to assess the exact mechanisms and particularly investigate the effects of thyroid hormone variation on early placentation. Future studies should aim to explore the crucial time point when trophoblast is vulnerable to variation in thyroid hormone concentration and quantify to what extent the availability of thyroid hormone plays a role in the functionality of placental tissue.

When it comes to metabolic effects of thyroid function, there is abundant evidence about the regulating effects of thyroid hormone on metabolism throughout the whole body, from early development onwards [17]. It is particularly interesting that thyroid function may have a role in the specific pathways of homocysteine metabolism. Homocysteine is a non-protein amino acid, known for its detrimental effects when present in high concentrations, especially for cardiovascular system and cerebrovascular health [18]. It has been suggested that high homocysteine has a role in female reproductive health, in pathophysiology of pregnancy complications and in adverse outcomes in neonates [19-25]. Experimental studies in animals have shown that thyroid hormone regulates the activity of methylenetetrahydrofolate reductase (MTHFR) and methionine synthase, enzymes required for re-methylation of homocysteine [26]. Furthermore, thyroid hormone may stimulate processes that have depleting effects on folate and vitamin B12, while these vitamins are important co-factors for the metabolism of homocysteine [27-33]. The population-based data on the effects of thyroid function on homocysteine are scarce, especially during pregnancy, and therefore we cannot be sure if relevant findings from

animal studies affect human physiology and pathophysiology that may be relevant for the outcomes of pregnancy and in early life. In Chapter 2.2 we aim to translate the experimental data to human physiology by investigating the effects of thyroid function on homocysteine concentrations and relevant metabolic factors in pregnancy and in the neonatal period. This chapter adds a new perspective to the knowledge on the specific role of thyroid hormone availability in the homocysteine metabolism. In part, because this is the first population-based study investigating these associations, but also because the focus was on both pregnancy and neonatal period – crucial time points that most likely reflect different thyroid hormone related effects with an impact on child development and health. We investigated which of the two hypothesized mechanisms may underlie the effects of thyroid hormone on homocysteine metabolism and show that in pregnant women, higher FT4 concentrations are associated with higher homocysteine whereas, in neonates, higher FT4 and lower TSH are associated with higher homocysteine. In the latter case, the associations were mediated by the effects of thyroid function on folate and vitamin B12 concentrations. These results suggest that in case of neonates, the effects of high thyroid hormone on high homocysteine concentration may be mediated by depleting effects of thyroid hormone on folate and vitamin B12. This was not the case in pregnant women. We also investigated whether the underlying mechanism of the detected associations might involve effects on the enzymes of the re-methylation cycle and did not detect any difference based on the genetic polymorphism of MTHFR, which is one of the enzymes of the re-methylation cycle. These findings may serve as a starting point for future studies that are required to replicate our results and further investigate the underlying mechanism for the detected associations in pregnant women, for which we may have been statistically underpowered. Of particular interest would also be to investigate the role of vitamin B6 on the effects of thyroid function on homocysteine metabolism, given that besides the re-methylation pathway, a large proportion of homocysteine is actually metabolized to cystathionine via vitamin B6-dependent pathway [34]. Such studies could be performed in a population-based setting to investigate the potential mediating effects of vitamin B6 in the association of thyroid hormone with homocysteine. Of interest would also be to investigate potential differences in metabolic effects between women that were taking multivitamin supplementation versus folic acid supplements only during pregnancy.

One of the aims in an epidemiological study is to investigate and unravel potential mediating pathways in the association of an exposure and an outcome [35]. Based on the associations in Chapters 2.1 and 2.2 and based on previous studies of thyroid function on pregnancy outcomes, we investigated the potential mediating role of placental function and homocysteine in the associations of thyroid function with pregnancy complications. Results of these analyses suggest that up to 10.4% and 12.5% of the previously shown associations of thyroid function with pre-eclampsia and birth weight, respectively,

could be mediated via changes in placental hemodynamics, whereas up to 13.3% of the association of thyroid hormone with birth weight could be mediated via effects on homocysteine concentration. These analyses are important in unraveling the physiology and potential underlying mechanisms for the associations studied. The mediating effects detected in these chapters were not consistent across pregnancy outcomes and ultrasound measurements in case of placental function, suggesting that the associations of thyroid hormone with pregnancy outcomes still remain largely explained either by direct effects of thyroid hormone, or via other mediators. Future studies are needed to confirm our findings, investigate the extent of mediation via placenta and metabolic effects on homocysteine and explore other potential pathways in the effects of thyroid function with pregnancy outcomes. Of particular interest would be experimental investigation of the effects of early thyroid hormone variation on the placental formation.

Chapter 2 investigates functional and metabolic effects of thyroid function during pregnancy. These results suggest that higher thyroid hormone concentrations are associated with higher placental vascular resistance and with higher homocysteine concentrations and these associations might have a small contributory role in the previously studied effects of gestational thyroid function on pregnancy outcomes, such as pre-eclampsia and low birth weight. This suggests that early pregnancy thyroid hormone concentrations are relevant for various aspects of pregnancy, including early effects, such as placentation, but also metabolic effects related to homocysteine concentrations, which may be implicated in pregnancy complications.

hCG and pregnancy outcomes

Multiple studies have suggested that hCG metabolism is associated with pregnancy outcomes including pre-eclampsia and fetal growth [36-45]. The complicating factor in drawing a conclusion from these results are considerable between-study differences in terms of gestational age of hCG measurement, specific isoform of hCG that was assessed and regarding the outcome assessment such as for example specific phenotype of pre-eclampsia. Moreover, because of the exceptional ability of hCG to stimulate the thyroid during pregnancy, it is not known whether the associations of hCG with major pregnancy complications could in part be explained by the effects of hCG on thyroid function and we examined this hypothesis in Chapters 3.1 and 3.2. In addition, due to the co-regulatory role of hCG in angiogenesis, we investigated whether the effects on vascular endothelial growth factors in pregnancy could partially explain the associations of interest.

In Chapter 3.1 we show an association of high total hCG concentration in combination with high sFlt-1/PlGF ratio and a high risk of pre-eclampsia. The results suggest that up to 40% of the association of hCG with pre-eclampsia may be mediated via effects of hCG on the ratio of angiogenic factors - sFlt-1 and PlGF. Thus far, it has been accepted that

a hyperinflammatory response plays a role in the pathophysiology of pre-eclampsia, although the exact pathophysiological mechanism is unclear [46-48]. Because it is known that sFlt-1/PlGF ratio is higher in the circulation of pre-eclamptic women compared to healthy women [49,50], we aimed to examine and quantify the potential effects of hCG on the balance between pro- and anti-angiogenic growth factors and its role in the association of hCG with pre-eclampsia. These findings add to the current understanding of the pathophysiology of pre-eclampsia, which underlies a great portion of maternal and fetal morbidity and mortality worldwide [46,51-53]. Future studies should replicate our results regarding the mediating role of sFlt-1/PlGF in the pathophysiology involved and further elucidate underlying mechanisms of the association of hCG with pre-eclampsia for example by repeatedly measuring hCG and studying effects on pro- and antiangiogenic factors concentration and their ratio from very early pregnancy onwards to detect the crucial time-point when the unfavorable sequence of events predisposing to pre-eclampsia occur.

Furthermore, in Chapter 3.2, we demonstrate that low early-pregnancy hCG is associated with a lower birth weight and with a risk of being born small for gestational age (SGA). These associations seem to arise due to a decrease in fetal growth in the second half of pregnancy. Sex-specific differences were detected in the pattern of longitudinally examined association of hCG with fetal growth, mainly in higher concentrations of hCG, whereas the associations of low hCG with low birth weight and a decrease in fetal growth were independent of fetal sex. The novel findings shown in this chapter suggest a specific role of hCG in fetal growth during the transitional period from first to second trimester, as the association of low hCG with low birth weight and SGA was limited to hCG concentrations measured in 11th and 12th week of gestation. This period is important as it reflects the start of maternal blood supply to the intervillous space and thereby a shift from hypoxic to an oxygenated fetal environment [54]. The underlying mechanisms might involve effects of hCG on trophoblast shell and arterial plugs [55]. These results suggest that SGA in general may be pre-determined potentially by a sequence of events related to low hCG concentrations at a specific time-point in early pregnancy, which is different from cases of for example low birth weight occurring in pre-eclampsia, as we show that high hCG concentrations are associated with pre-eclampsia partially via effects on the balance between pro- and antiangiogenic factors. Future studies that would replicate our findings may want to investigate additional mechanisms underlying detected associations.

We did not detect a mediating or modifying role of thyroid function in the associations of hCG with pre-eclampsia or fetal growth. This suggests that although the physiology of hCG and thyroid function in pregnancy is interrelated, these two hormones have a separate role in same pregnancy complications. For example, we hypothesized that the association of hCG with pre-eclampsia is partly due to the contributing effects of high

hCG on the unfavorable balance between pro- and anti-angiogenic factors in pre-eclampsia, or that it is a compensatory response of trophoblast to feto-placental hypoxia, whereas the association of thyroid hormone with pre-eclampsia is hypothesized to be due to the effects of thyroid hormone on endothelial cell dysfunction [5]. However, in this thesis, we investigated the associations of hCG concentration with fetal growth and pre-eclampsia, whereas future studies may want to assess the possibility of the mediating or modifying effects of thyroid function in the association of hCG with different pregnancy complications.

The results of Chapter 3 show the associations of hCG concentration with the risk of pre-eclampsia as well with the risk of fetal growth restriction and being born SGA. These results suggest that the two extremes of the hCG range have different mechanisms in contributing to pregnancy complications, as we show an association of low hCG with decreased fetal growth and risk of SGA in general and an association of high hCG with the risk of pre-eclampsia which practically may also lead to SGA. The effects of low hCG on fetal growth were limited to a specific gestational period, whereas the effects of high hCG on the risk of pre-eclampsia were not time-dependent or this could not be detected due to potential lack of power given that effect modification may be difficult to detect in dichotomous outcomes [56]. Both associations exhibited fetal sex-specific characteristics, implying that these differences need to be considered when studying the effects of hCG. This thesis adds a novel component to the understanding of the physiology of pregnancy specific hormone - hCG, and its role in multifactorial pregnancy complications that underlie a great portion of maternal and fetal morbidity and mortality.

Thyroid function in childhood

The essential tool for clinicians in diagnosing thyroid disorder is a proper reference range. A certain variability in the reference range cutoffs might not affect diagnosing overt disease but may largely aggravate diagnosing mild forms of thyroid dysfunction. The literature overview provided in Chapter 4.1 of this thesis shows a considerable variability in the reported pediatric reference ranges for TSH and FT4, especially in the first week of life. Even in children aged ≥1 year, the variability was relatively large, as is exhibited, for example, by the reported upper limit for TSH (2.36 to 6.57 mU/L). Besides the differences across age-categories and studies using different assays, differences were also present between studies using a similar assay [57-59]. Moreover, the population size and selection of the study population may be suboptimal, as some studies lack the sufficient size for calculation of reference ranges or do not exclude the individuals with diseases that affect thyroid function [2]. A principal factor that complicates the interpretation of childhood reference ranges overall, is the lack of consensus on the calculation of reference ranges. For instance, while some studies use the non-parametric approach, utilizing 2.5th-97.5th range or the 5th-95th reference range, some studies use a (semi-)

parametric approach defining normality based on +/- 1.96 or 2 standard deviations from the mean [60]. This complicates the utilization of the reference ranges in the clinical practice and hampers the general conclusion from the literature summary, which could be overcome by standardization of the methodology in the reference range calculation.

The determinants of thyroid function are important in understanding childhood physiology and differences compared to adult population. In the population-based cohort of children investigated in Chapter 4.1, a stable association of FT4 with TSH is demonstrated, suggesting that the hypothalamic-pituitary-thyroid (HPT) axis in children at the age of 6 is likely not subject to pathology. Future studies should longitudinally investigate the potential changes in this association later in childhood and adolescence, to gain further insight in the crucial time-points when HPT axis becomes vulnerable to pathological changes. Of interest would also be to investigate how is this association affected by genetic variation. The major determinants of childhood thyroid function were age, sex, ethnicity, anthropometric characteristics and time of venipuncture and the between-individual differences in these characteristics were shown to largely influence the variation in TSH and FT4 values. Therefore, these determinants should be taken into account for interpretation of thyroid function tests.

Although there is substantial evidence on the role of thyroid function in the cardiovascular system development [61-63], little epidemiological data are available on this matter. Given that early-life determinants may shape the development of the cardiovascular system and affect its function throughout adulthood [64-68], knowledge on these determinants could have predictive value for cardiovascular health. The results shown in Chapter 4.2 suggest that higher FT4 is associated with a lower left ventricular mass at the age of 6 years. Our results suggest that the effects of FT4 on the left ventricular mass may be mediated via effects of FT4 on the lean body mass, which is a strong determinant of left ventricular mass [69,70]. Therefore, it is likely that in this young population, we could examine the developmental influence of thyroid hormone. Future studies should investigate whether the effects on the left ventricular mass persist to a later age and whether they might to any extent program clinically relevant entities, such as left ventricular hypertrophy. Longitudinal measurements of the cardiovascular function would enable an investigation of the variability in this association over time and long follow-up would permit an assessment of the crucial time point when cardiac structure is subject to adverse effects of thyroid (dys)function.

Investigation of early determinants of blood pressure is encouraged by the longitudinal studies that have shown that blood pressure tracks from childhood to adulthood [68]. In line with the previous reports [71,72], the results of Chapter 4.2 show that TSH is associated with higher blood pressure in childhood. It has been speculated that the association of TSH with blood pressure is a result of the common genetic factors that are associated with both thyroid function and blood pressure [73]. A novel finding of this thesis is that

FT4 is associated with blood pressure potentially via effects on arterial stiffness. The mechanism underlying this association is likely to be different from the association of TSH with blood pressure, as FT4 (but not TSH) was associated with the measurement of arterial stiffness. This suggests that the effects of thyroid hormone on the arterial wall potentially via initiation of endothelial dysfunction and effects on arterial stiffness [74-77], may create structural changes that could have effects on blood pressure already in childhood. This is further substantiated by the evidence of atherosclerotic changes already in early childhood [78]. Future studies should quantify the potential role of thyroid hormone in early arterial changes and elucidate other contributive factors for high blood pressure that may be modifiable and could be implemented in preventive strategies.

Longitudinal studies have shown that bone mineral density (BMD) tracks from childhood to adulthood, suggesting that low BMD at early age may be a risk factor for osteoporosis in adulthood [79]. While adverse effects of childhood hyperthyroidism on bone accrual are known [80,81], little epidemiological data are available on the influence of mild thyroid variations on bone development. The results shown in Chapter 4.3 suggest that higher thyroid hormone concentrations are associated with lower BMD throughout childhood. Another concept worth of discussion originates from reports from animal studies, showing a direct role of TSH in the skeletal metabolism [82], while translation of these results into human population setting is controversial [83]. In Chapter 4.3 an association of TSH at the age of 6 with BMD was detected cross-sectionally, but not at the age of 10, and was overall not present in the European subgroup, suggesting that this association could be subject to residual/unmeasured confounding effect. Future studies should investigate to what extent detected associations of thyroid function with BMD persist in later age and whether this may have an impact on the clinically relevant entities related to osteoporosis. The differences in the associations between total and European population detected in Chapter 4.3 deserve special investigation, particularly regarding the potential role of genetic polymorphism.

Chapter 4 of this thesis provides a summary of the available studies on thyroid function reference ranges for pediatric population and quantifies the differences in the reference values based on the age, assay, population and calculation methodology. Age, sex, ethnicity, anthropometric characteristics and time of venipuncture were identified as major determinants of TSH and FT4 in childhood. The novel findings described in Chapter 4 add to the current knowledge on the physiology of childhood and early effects of thyroid hormone on major target organs, which may prove useful in early prediction of clinically relevant events related to cardiovascular and bone health.

METHODOLOGICAL CONSIDERATIONS

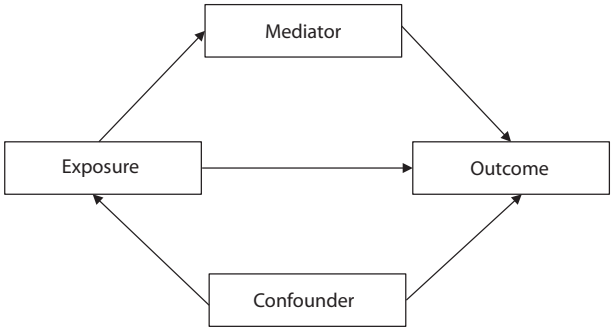
There are several methodological aspects of this thesis that are worth of discussion. First of all, the response rate at baseline for the Generation R cohort was 61% based on the number of children at birth and women that participated in the cohort had fewer pregnancy complications compared to women that did not participate [84], suggesting a selection towards a healthier population. This may lead to lower prevalence rates of for example pre-eclampsia and consequently reduce statistical power and generalizability of the results to other less healthy populations. However, studies have shown that selective participation at baseline does not affect the studied associations within population-based cohorts [85,86], suggesting that it is not likely that the associations detected in this thesis are biased due to non-response at baseline. On top of this, hCG measurements were available in 89% of women, whereas thyroid function was measured in early pregnancy serum samples that were available in 71% of women [87], leaving a possibility of biased estimates in the studies investigating the associations of hCG and thyroid function with different outcomes. This might be overcome in future population-based cohorts by earlier inclusion of women in the study, preferably pre-conceptionally, so that a larger percentage of early-pregnancy blood measurements is available. Another relevant point is loss to follow-up, as at 6 years of age, approximately 70% of children that visited the research center had their blood drawn successfully, and the missing measurements were mainly due to a non-consent for venipuncture or failure of the procedure. A sensitivity analysis performed by comparison of main characteristics between children with and without available thyroid function measurements in this thesis showed no systematic differences in the outcomes of interest, which may represent an argument against biased estimates.

In the studies included in this thesis, differential misclassification is not a likely source of bias as the parents and data collectors were not aware of the specific research question. In addition to this, data on exposures were measured from the blood samples, and data on the outcomes were obtained from hospital registries or via direct measurement of placental function, fetal ultrasound or cardiovascular function and body composition. Nonetheless, non-differential misclassification might have occurred, for example in case of women's report on folic acid use during pregnancy, as some women have likely used combined multivitamin formulas, which potentially could have led to an underestimation of the effect estimates. Future studies could overcome this misclassification by specifying in the questionnaire which type of vitamin supplementation did women take during pregnancy.

Although the studied associations in this thesis were adjusted for multiple potential confounders, residual or unmeasured confounding cannot be excluded due to observational nature of this study. This might, for example, include the influence of environ-

mental factors, genetic susceptibility or the interaction of the two. In some cases, one variable can be both a confounder and a mediator (Figure 1) and this was explored in this thesis. Several potential (partial) mediators were identified, including placental function in the association of thyroid hormone with pre-eclampsia and birth weight, and lean body mass in the association of thyroid hormone with left ventricular mass. In the first case, the mediating effect estimates were small and not consistent across pregnancy outcomes and placental ultrasound measurements, whereas, in the latter case, effects were more robust. This suggests that the association of thyroid function with pregnancy complications is largely explained by either direct effects of thyroid hormone or by other unmeasured or unknown mediators. However, it is still possible that in some cases, the methodology used for mediation analyses was not sensitive enough to accurately examine and detect the potential mediating effects. The possibility that one variable could be both a confounder and a mediator was explored for example in case of body composition, that could both mediate and confound the association of thyroid function with cardiovascular outcomes [69]. This is also due to the complicated and potentially bidirectional association of thyroid function with BMI [88] whereas not much is known on the association of thyroid function with specific body composition components in childhood. This was explored in Chapter 4.2 where we identified lean body mass (and not fat mass) as a mediator in the association of thyroid hormone with left ventricular mass. This stepwise approach shows the importance of combining the biology and statistical methodology in disentangling clinically relevant associations.

Figure 1. A statistical mediation and confounding model



CLINICAL IMPLICATIONS

This thesis provides several concepts that could improve the clinical perspective and healthcare during pregnancy and in early childhood and adds to translation of the experimental studies into clinically relevant associations. In line with that, this thesis

shows that higher thyroid hormone concentrations in early pregnancy are associated with higher placental vascular resistance and with higher homocysteine concentrations, which have both been associated with adverse pregnancy outcomes. This underlines the importance of optimization of thyroid hormone concentration in pregnancy and may add to the understanding of the potential adverse effects of levothyroxine (over)treatment during pregnancy. Furthermore, the results of this thesis that show associations of hCG concentrations with risks of fetal growth restriction and pre-eclampsia, may be incorporated in clinical prediction models. This means that by using hCG concentration profile together with other risk factors, the probability of adverse events in pregnancy could be analyzed. Finally, the results of this thesis concerning thyroid function in early childhood may improve clinical perspective of the pediatric population in several aspects; by summarizing all the available reports on thyroid function reference ranges in one study, the knowledge that is necessary for clinicians to properly diagnose thyroid dysfunction and assess whether a reference interval is generalizable to a specific population, becomes easier accessible. The knowledge on the determinants of childhood thyroid function described in this thesis may improve identification and/or exclusion of the mechanisms underlying an abnormal thyroid function test. In addition, the results showing the association of thyroid function with cardiovascular function and bone density in childhood may help to identify determinants of clinical events in adulthood. This thesis adds novel aspects to the understanding of the physiology of growth and development and the functionality of HPT axis in early age. Childhood studies described here are important from the perspective of improvement of the knowledge about the differences between pediatric and adult population.

FUTURE PERSPECTIVES

Different study designs, such as randomized control trial (RCT) or Mendelian randomization studies may be used to gain a better insight into causality. For verification of the associations of maternal thyroid hormone metabolism with placental function and homocysteine concentrations, of particular interest would be to perform analysis of RCTs that investigate effects of levothyroxine treatment. In some cases, an RCT is not feasible, but Mendelian randomization studies may prove useful, such as for example in the case of the association of hCG with pregnancy outcomes. Future studies should also investigate the (patho)physiological mechanisms underlying the associations studied in this thesis. Of particular interest would be experimental studies that would investigate our results obtained on the association of thyroid function with placental hemodynamics.

More detailed measurements could provide further insight into the strength and consistency of the studied associations. For instance, repeated measurements of hCG

would enable studying inter-individual differences in the change of hCG concentrations during pregnancy and whether that potential variation affects the association with pre-eclampsia and fetal growth. Longitudinal measurements of childhood thyroid function are lacking, and the field could benefit from this type of data particularly for studying potential tracking pathways of thyroid function from childhood to adolescence, and to gain insight into the consistency of the associations with body composition components, cardiovascular function and bone density in later stages of childhood. Long-term follow-up observational cohort studies are necessary to study the physiology of variation in thyroid function over the life course and potential consequences for clinically relevant events in adulthood, such as cardiovascular events and osteoporosis.

CONCLUSION

The results of this thesis show that hCG and thyroid function are important endocrine determinants in early life. The studied associations of hCG concentrations with pregnancy outcomes suggest that this hormone may be an important factor and/or marker of the pathophysiology of adverse pregnancy outcomes that underlie a great portion of maternal and fetal morbidity. The associations of thyroid function with the functional and metabolic outcomes of pregnancy and developmental outcomes in childhood emphasize its essential role in human physiology from the conception onwards.

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CHAPTER 6

Summary and Samenvatting

SUMMARY

This thesis investigates developmental and metabolic aspects of hormonal influence in early life. The focus was on pregnancy as well as on early childhood. The associations of hCG and thyroid function with pregnancy outcomes were examined and we aimed to investigate potential underlying mediating pathways and delineate the effects of thyroid hormone and hCG. We investigated the functionality of hypothalamic-pituitary-thyroid axis and determinants of thyroid function in early childhood. The effects of childhood thyroid function on major target organs such as the cardiovascular system and bone were investigated.

Chapter 1 provides the background on the physiology of pregnancy and childhood and the endocrinological aspects relevant for both pregnant women and the offspring. The aims of this thesis are described and the setting of the studies is presented.

Chapter 2 focuses on the effects of thyroid function in pregnancy in terms of placental function and metabolism of homocysteine. In Chapter 2.1, we show that high thyroid hormone in early pregnancy is associated with high placental vascular resistance in the second and third trimester, in both maternal and fetal placental compartment. Our results also suggest that these effects might have a limited mediating role in the effects of thyroid function on pre-eclampsia and birth weight. Chapter 2.2 describes the association of thyroid function with homocysteine concentrations in pregnant women and in neonates. High thyroid hormone was associated with high homocysteine concentrations and the results suggest that the depleting effects of thyroid hormone on folate and vitamin B12 may have a role in this association.

Chapter 3 investigates the associations of hCG concentrations with fetal growth patterns and the risk of pre-eclampsia. Chapter 3.1 shows an association of high total hCG with a high risk of pre-eclampsia, which could be partially explained by the effects of hCG on the balance between pro- and anti-angiogenic factors, PlGF and sFlt-1. Chapter 3.2 unravels the pattern of the association of hCG with fetal growth and shows that association of low total hCG in the late first trimester with low birth weight arises due to a decrease in fetal growth in these pregnancies. In both Chapter 3.1 and 3.2, sex-specific differences in the associations of hCG with the outcomes of interest were detected.

Chapter 4 focuses on the aspects of thyroid function in childhood and investigates major determinants of childhood TSH and FT4 concentration, as well as the variability in existing pediatric reference ranges and the effects of thyroid function on major target organs. Chapter 4.1 provides a literature overview of published reference ranges for thyroid function in children, showing differences across age categories, assay use and population size. Child age, sex, ethnicity and anthropometric characteristics were identified as major thyroid function determinants and a stable association of FT4 and TSH is shown. Chapter 4.2 shows that thyroid function is a determinant of left ventricular mass

and this was partially mediated by the effects of FT4 on the lean body mass. TSH and FT4 were positively associated with blood pressure, with a potential role of arterial stiffness in the latter case, suggesting different mechanisms via which these hormones are associated with blood pressure. In Chapter 4.3, we show a consistent inverse association of FT4 with bone density and show that the association of TSH with bone density is not consistent .

In **Chapter 5**, a reflection of main findings of this thesis is provided and methodological considerations, clinical implications of the detected associations, as well as future perspectives for the field are discussed.

SAMENVATTING

Dit proefschrift onderzoekt ontwikkelings- en metabole aspecten van hormonale invloeden in het vroege leven. De zwangerschap en de vroege kinderjaren stonden hierin centraal. De associatie tussen hCG en schildklierfunctie enerzijds en zwangerschapsuitkomsten anderzijds werden onderzocht. Daarnaast zochten wij naar mogelijk onderliggende oorzaken die invloed hebben op deze associaties en hebben we de effecten van schildklierhormoon en hCG onderzocht. We onderzochten de functionaliteit van de hypothalamus-hypofyse-schildklier -as en determinanten van schildklierfunctie tijdens de vroege kinderjaren. Tot slot werd er gekeken naar het effect van de schildklierfunctie op de kinderleeftijd in relatie tot grote orgaansystemen zoals het cardiovasculaire systeem en de botten.

Hoofdstuk 1 geeft een beschrijving van de fysiologie van de zwangerschap en de kinderleeftijd en van de endocrinologische aspecten die relevant zijn voor zowel zwangere vrouwen als hun kinderen. Tevens worden de doelstellingen van dit proefschrift beschreven en de setting van de studies uiteengezet.

Hoofdstuk 2 richt zich op de effecten van de schildklierfunctie tijdens de zwangerschap in relatie tot de placentafunctie en het metabolisme van homocysteïne. In Hoofdstuk 2.1 tonen we aan dat een hoge concentratie schildklierhormoon in de vroege zwangerschap geassocieerd is met een hoge vasculaire weerstand van de placenta in het tweede en derde trimester. Deze associatie wordt gezien aan zowel het maternale als het foetale gedeelte van de placenta. Onze resultaten suggereren ook dat deze effecten mogelijk een mediërende rol spelen in de relatie tussen schildklierfunctie enerzijds en pre-eclampsie en geboortegewicht anderzijds. Hoofdstuk 2.2 beschrijft de associatie van de schildklierfunctie met homocysteïne concentraties in zwangere vrouwen en neonaten. Een hoge concentratie van het schildklierhormoon is geassocieerd met een hoge concentratie homocysteïne en de resultaten suggereren tevens dat de relatie van schildklierhormoon op folaat en vitamine B12 hier een rol kan spelen.

Hoofdstuk 3 onderzoekt de associaties van hCG concentraties met foetale groeipatronen en het risico op pre-eclampsie. Hoofdstuk 3.1 geeft een weergave van de associatie van een hoge hCG concentratie met een verhoogd risico op pre-eclampsie, welke deels verklaard kan worden door het effect van hCG op de balans tussen de pro- en anti-angiogene factoren PlGF en sFlt-1. Hoofdstuk 3.2 ontrafelt het patroon aangaande de associatie van hCG met foetale groei en toont aan dat de associatie van een lage concentratie totaal hCG laat in het eerste trimester met een laag geboortegewicht voortkomt uit een verminderde foetale groei in deze zwangerschappen. In zowel Hoofdstuk 3.1 als Hoofdstuk 3.2 werden geslachts-specifieke verschillen aangetoond in de associatie van hCG met de eerdergenoemde uitkomsten.

Hoofdstuk 4 richt zich op de aspecten van de schildklierfunctie in de kinderjaren en onderzoekt belangrijke determinanten van TSH en FT4 concentraties tijdens de kinderleeftijd. Daarnaast wordt ook de variabiliteit onderzocht in bestaande pediatrische referentiecurves en de effecten van de schildklierfunctie op grote orgaansystemen. Hoofdstuk 4.1 geeft een weergave van de literatuur van gepubliceerde referentiecurves van schildklierhormoon in kinderen, waarbij verschillen worden aangetoond tussen leeftijdscategorieën, analyse technieken en populatiegrootte. Leeftijd, geslacht, etniciteit en antropometrische karakteristieken van het kind bleken belangrijke determinanten te zijn voor de schildklierfunctie. Tevens is er een stabiele associatie van FT4 en TSH aangetoond. Hoofdstuk 4.2 laat zien dat de schildklierfunctie een determinant is van linker ventrikel massa en dat deze deels werd gemedieerd door de effecten van FT4 op spiermassa. TSH en FT4 zijn positief geassocieerd met bloeddruk. In de relatie van FT4 met bloeddruk is er een potentieel mediërende rol voor arteriële stijfheid gevonden. Dit suggereert dat er verschillende mechanismen werkzaam zijn die de associatie van TSH en FT4 met bloeddruk verklaren. In Hoofdstuk 4.3 tonen we een consistente inverse associatie aan van FT4 met de botdichtheid en van TSH met de botdichtheid, waarbij de associatie van TSH niet consistent is tussen Europese subgroepen.

In **Hoofdstuk 5** wordt een interpretatie gegeven van de belangrijkste bevindingen van dit proefschrift. Daarnaast worden methodologische overwegingen, klinische implicaties van de gevonden associaties en toekomstige perspectieven in dit onderzoeksgebied besproken.

CHAPTER 7

Appendix

Authors' affiliations
List of publications
PhD portfolio
About the author
Words of gratitude

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LIST OF PUBLICATIONS

Barjaktarovic M, Korevaar TI, Jaddoe VW, de Rijke YB, Visser TJ, Peeters RP, Steegers EA. Human chorionic gonadotropin (hCG) concentrations during the late first trimester are associated with fetal growth in a fetal sex-specific manner.
Eur J Epidemiol. 2017 Feb;32(2):135-144.

Barjaktarovic M, Korevaar TI, Chaker L, Jaddoe VW, de Rijke YB, Visser TJ, Steegers EA, Peeters RP. The association of maternal thyroid function with placental hemodynamics.
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Barjaktarovic M, Korevaar TI, Gaillard R, de Rijke YB, Visser TJ, Jaddoe VWV, Peeters RP. Childhood thyroid function, body composition and cardiovascular function.
Eur J Endocrinol. 2017 Oct;177(4):319-327.

Barjaktarovic M, Steegers EA, Jaddoe VW, de Rijke YB, Visser TJ, Korevaar TI, Peeters RP. The association of thyroid function with maternal and neonatal homocysteine concentrations.
J Clin Endocrinol Metab. 2017 (in press)

Onsesveren I, **Barjaktarovic M,** Chaker L, de Rijke YB, Jaddoe VW, van Santen HM, Visser TJ, Peeters RP, Korevaar TI. Childhood thyroid function reference ranges and determinants: a literature overview and a prospective cohort study.
Thyroid. 2017 Nov;27(11):1360-1369.

Barjaktarovic M, Korevaar TI, Jaddoe VW, de Rijke YB, Visser TJ, Peeters RP, Steegers EA. Human chorionic gonadotropin (hCG) and the risk of pre-eclampsia.
Submitted

Veldscholte K, **Barjaktarovic M**, Trajanoska K, Jaddoe VWV, Visser TJ, de Rijke YB, Peeters RP, Rivadeneira F, Korevaar TI. The association of thyroid function with bone density during childhood.
Submitted

PHD PORTFOLIO

PhD student	Mirjana Barjaktarovic
Erasmus MC Department	Internal Medicine, Endocrinology, Academic Center for Thyroid Diseases
PhD period	August 2014 - February 2018
Promotors	Prof. Dr. Robin P. Peeters Prof. Dr. Eric A.P. Steegers
Co-promotor	Dr. Tim I.M. Korevaar

Training	Year	ECTS
Master of Science in Clinical Epidemiology, NIHES, Rotterdam, the Netherlands	2014-2015	70
General courses		
Study Design	2014	4.3
Biostatistical Methods I: Basic Principles	2014	5.7
Clinical Epidemiology	2014	5.7
Methodologic Topics in Epidemiologic Research	2014	1.4
Biostatistical Methods II: Classical Regression Models	2014	4.3
Principals of Research in Medicine	2014	0.7
Methods of Clinical Research	2014	0.7
Methods of Public Health Research	2014	0.7
Clinical Trials	2014	0.7
Health Economics	2014	0.7
Conceptual Foundation of Epidemiologic Study Design	2015	0.7
Introduction to Global Public Health	2015	0.7
Causal Inference	2015	0.7
Social Epidemiology	2015	0.7
The Practice of Epidemiologic Analyses	2015	0.7
Causal Mediation Analyses	2015	0.7
Fundamentals of Medical Decision Making	2015	0.7
Advanced courses		
Epidemiology of Infectious Diseases	2015	1.4
Repeated Measurements in Clinical Studies	2015	1.4
Psychiatric Epidemiology	2015	1.1
Principles of Epidemiologic Data-analysis	2015	0.7
Maternal and Child Health	2015	0.9
Skill courses		
English Language	2014	1.4
Introduction to Medical Writing	2015	1.1
Academic courses		
Research Integrity	2016	0.3

Conferences – oral presentations		
Dutch Endocrine Meeting, Noordwijk	2016	0.7
European Congress of Endocrinology, Munich	2016	0.7
Science Days Internal Medicine, Antwerp	2017	0.7
Dutch Endocrine Meeting, Noordwijk	2017	0.7
Endocrine Society, Orlando	2017	0.7
European Thyroid Association, Belgrade	2017	0.7
Seminars and meetings		
Thyroid Lab Meetings	2014-2018	1.0
Generation R Research Meetings	2014-2018	1.0
Maternal and Child Health Meetings	2014-2018	1.0
Seminars Epidemiology	2014-2018	1.0
Dutch Thyroid Club Annual Meetings, Amsterdam	2014-2017	0.7
Teaching		
Ibrahim Onsesveren, MSc student – *Childhood thyroid function reference ranges and determinants*	2016	1.0
Karlien Veldscholte, medical student – *Thyroid function and bone density in childhood*	2017	1.0
Teaching Assistant, Biostatistical Methods I	2016	0.3
Other Activities		
Peer Reviews for *Circulation, Netherlands Journal od Medicine*	2017	0.5
Data collection tasks	2015-2016	60
Grants and awards		
ERAWEB grant for a PhD at Erasmus MC in Rotterdam, the Netherlands	2014	
European Society of Endocrinology – travel grant	2016	
Outstanding Abstract Award – Endocrine Society	2017	
Goodlife Healthcare Travel Grant of the Dutch Endocrine Society	2017	
Erasmus Trust Fond Travel Grant	2017	

ABOUT THE AUTHOR

Mirjana Barjaktarovic was born on November 4th, 1989 in Sombor, Serbia. Mirjana completed high school in Belgrade in 2008 and started Medical School at the University of Belgrade in the same year. During her medical studies, Mirjana has worked as a demonstrator in pathology and was involved in a research project concerning treatment outcomes of patients with infectious diseases. At her final year of medical school, Mirjana started growing an interest in developing her skills as a clinical researcher and explored possibilities of working in large population-based cohorts such as Generation R. In 2014 Mirjana obtained a degree of Doctor of Medicine and moved to the Netherlands as she received the ERAWEB grant for a full-time PhD candidate position at Erasmus MC in Rotterdam, under supervision of Professor Dr. Robin Peeters and Dr. Tim Korevaar. In 2015, Mirjana obtained Master of Science degree in Clinical Epidemiology at the Netherlands Institute of Health Sciences. During her research training, Mirjana carried out multiple research projects that are encompassed in this PhD thesis 'Early life impacts of thyroid function and human chorionic gonadotropin (hCG)'. After obtaining her PhD degree, Mirjana will pursue a clinical career in internal medicine.

WORDS OF GRATITUDE

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"The glory of medicine is that it is constantly moving forward, that there is always more to learn. The ills of today do not cloud the horizon of tomorrow, but act as a spur to greater effort."

William James Mayo

Early Life Impacts of Thyroid Function and Human Chorionic Gonadotropin (hCG)

Propositions

1. The association of high thyroid function with pregnancy complications is partially explained via effects on placentation. – *This thesis*

2. High thyroid function is associated with high homocysteine concentrations. The metabolic pathways underlying this association are different between adults and neonates. – *This thesis*

3. The two extremes of the hCG range contribute to pregnancy complications via different mechanisms. – *This thesis*

4. Although physiology of thyroid hormone and hCG is interrelated, these two hormones have different pathways in the regulation of pregnancy and fetal growth. – *This thesis*

5. Early childhood thyroid function influences cardiovascular health in adult age. – *This thesis*

6. Translation of data obtained from in vitro experiments and animal studies into human population setting is crucial for unravelling physiological pathways and identification of clinically relevant associations.

7. The role of genetics in endocrinology is becoming increasingly recognized, and the combination of genetic analyses and clinical tools may allow early diagnosis and subsequent improvement of clinical outcomes.

8. Paternal influences on offspring's health are undervalued. More investigation of the effects of paternal genetic material and lifestyle factors on fetal development is needed.

9. Comprehension of the patterns of childhood growth and development necessitates more efforts in the investigation of the processes that occur even before conception.

10. The value of observational studies lies in detection of modifiable pathophysiological factors and identification of high-risk groups.

11. "It is paradoxical, yet true, to say, that the more we know, the more ignorant we become in the absolute sense, for it is only through enlightenment that we become conscious of our limitations." – *Nikola Tesla*

Mirjana Barjaktarović,
Rotterdam, January 24th 2018