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Title page

Free thyroxine in early pregnancy is an independent negative predictor of 3rd trimester HbA1c. Odense Child Cohort.

Abbreviated title: Early pregnancy FT4 and late pregnancy HbA1c.

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Disclosure statement: The authors have nothing to disclose**Abstract**

Background: Lower thyroid function outside pregnancy is associated with increased risk of type 2 diabetes mellitus. The relationship between thyroid function in early pregnancy and glucose status in 3rd trimester has not been investigated.

Aims: To study the association between 1st trimester thyroid function and 3rd trimester glucose status.

Design: In the prospective study Odense Child Cohort (OCC), 1,041 women had 1st trimester blood samples analyzed for thyroid stimulating hormone (TSH), free T4 (FT4), thyroid peroxidase antibody, and HbA1c. Third trimester (week 28) fasting blood samples included plasma glucose, insulin and HbA1c. Oral glucose tolerance test (OGTT, 75 g glucose) was performed in 509 women. First trimester FT4 was dichotomized > vs. ≤ the 25th percentile (25p= 12.9 pmol/L). Homeostatic model assessment - insulin resistance (HOMA)-IR and HOMA-β were calculated

Results: Women with FT4 ≤ 25p had significantly higher HbA1c in 1st and 3rd trimesters and higher 3rd trimester fasting glucose, insulin, HOMA-IR and HOMA-β compared to women with FT4 > 25p. In multiple regression analyses, FT4 was an independent negative predictor of 3rd trimester HbA1c. FT4 levels in 3rd and 4th quartiles (high-normal FT4 levels) showed closest inverse associations with HbA1c (P-trend <0.001). TSH was not associated with 3rd trimester HbA1c.

Conclusion: Women with lower levels of FT4 in early pregnancy had higher HbA1c in 3rd trimester and FT4 was an independent negative predictor of 3rd trimester HbA1c.

Keywords: Pregnancy, FT4, HbA1c, glucose, insulin

Introduction

Pregnancy is a physiological state, which challenges thyroid function and glucose status [1]. Fetal thyroid gland begins hormone production after gestation week (GW) 12 and onwards, and maternal thyroid hormone is transferred to the fetus throughout pregnancy [2]. Maternal hypothyroidism is associated with suboptimal neurodevelopmental outcome in the offspring [3-5]. Thyroid stimulating hormone (TSH) is generally used as a screening tool for reduced thyroid function outside and inside pregnancy. According to the current American Thyroid Association guideline, T4 treatment is recommended in women with TSH above pregnancy specific reference ranges and positive TPO antibodies (TPOab) [2]. Furthermore, T4 treatment may be considered in women with positive TPOab and TSH concentrations >2.5 mU/L and below the upper limit of the pregnancy-specific reference range [2]. Third trimester is characterized by physiological insulin

resistance and some women develop transient hyperglycemia or gestational diabetes mellitus (GDM) [6]. GDM is diagnosed by fasting plasma glucose or 2-hour glucose during oral glucose tolerance test (OGTT) in 3rd trimester [7,8]. HbA1c is a simple way to assess the integrated diurnal glucose exposure [8]. HbA1c cannot be used to diagnose GDM, but HbA1c can be applied for metabolic risk assessment in women with GDM [9]. Offspring growth in pregnancy and BW are influenced by maternal glucose status. The glucose-related increase in offspring growth starts early in pregnancy and hyperglycemia in pregnancy results in higher BW and large for gestational age neonates. BW could also be affected by maternal thyroid function, as lower FT4 was associated with higher BW and higher risk of being large for gestational age (LGA) [10].

The aim of the present study was to examine associations between maternal thyroid function in early pregnancy and glucose status in 3rd trimester.

Material and methods

This study was part of Odense Child Cohort (OCC) [11]. OCC is a joint research study where 2,874 pregnant women within the municipality of Odense, Denmark, were recruited between 2010 and 2012 [11]. Women gave informed consent to receive and fill in questionnaires, to have additional blood samples performed and to be contacted prospectively. Participating women attended blood sampling during 1st-2nd trimesters (GW 7–16). Women had to give additional consent to attend for fasting blood samples around GW 25. Details regarding the study have been published previously [11].

Women pregnant with twins were not included in the present study cohort (multiple pregnancies, n=56) (Fig. 1). Furthermore, only women with available blood samples around GW 10 for measurement of thyroid hormone status were included, which excluded 1,764 women from the dataset. Five women were pregnant more than once within the inclusion period (only first pregnancy included) and eight women had diabetes diagnosed before week 20, which left 1,041 women in the present study cohort.

Glucose tolerance

GDM was diagnosed by a 75 g, 2h OGTT using a plasma glucose threshold ≥ 9.0 mmol/L according to Danish guidelines for antenatal care [12]. OGTT by indication: Women were subjected to a

selective screening strategy using risk factors for GDM i.e. BMI ≥ 27 kg/m^2, family history of diabetes mellitus, glucosuria during pregnancy, previous GDM, or previous delivery of a macrosomic child [12]. Pregnant women with two or more of these risk factors or with previous GDM were offered an early diagnostic OGTT between GW 14 and 20 and again in GW 28–30, whereas women with one risk factor were scheduled for the late OGTT only.

OGTT by randomization: According to the design of OCC [13], one random selected woman without risk factors for GDM was offered a late OGTT (week 28–30) for each woman undergoing an OGTT by indication. Randomization of women without risk factors for GDM was conducted consecutively throughout the study period. A total of 509 women had data on 3rd trimester OGTT in the present study (OGTT by indication, n= 255 and OGTT by randomization, n=254). Glucose levels were measured at 0h, 1h and 2h.

Covariates

Maternal age, pre-pregnancy BMI, parity, and smoking status were obtained from patient records using maternal civil registration numbers.

Assays

Fasting blood samples were analyzed for TSH (ACN code 10172), Free T4 (ACN code 10160) and TPOab (ACN code 10066) using electrochemiluminescence immunoassay on the E801 module at the Roche Cobas 8000 platform (Roche Diagnostics GmbH). The intra-assay coefficients of variation were for TSH: 7.2 and 4.6% at concentrations of 0.08 and 11 mU/l respectively, FT4: 3.6 and 3.8% at concentrations of 16.2 and 35.1 pmol/L respectively and TPOab: 15 and 12.5% at concentrations of 17.5 and 24 klU/L respectively. The cut off limit for positive TPOab was 34 kIU/L. HbA1c was analyzed using ion exchange high-performance liquid chromatography on a Tosoh G8 instrument (Tosoh Bioscience, Tokyo, Japan) set up for routine testing. Results were reported as International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) units in mmol/mol. Glucose levels were measured by the hexokinase method (Architect, Abbott), the intra-assay coefficient of variation (CV) was 5.2–5.4% and the inter-assay CV was 1.2–1.7%. Serum insulin was analyzed by an electro-chemiluminescence immunoassay (ECLIA, Cobas e411, Roche), the intra-assay CV was 0.8–3.7% and the inter-assay CV was 2.5–4.9%. Further details on HbA1c, glucose and insulin assays have been published, recently (10;16). Insulin resistance was estimated using

homeostatic model assessment of insulin resistance (HOMA-IR) as described by Matthews et al. [14] and calculated with the following formula: (Fasting plasma insulin in mIU/L × Fasting plasma glucose in mmol/L)/22.5. HOMA-β was calculated using the following formula: (Fasting plasma insulin * 20 mIU/L)/ (Fasting plasma glucose (mmol/L) − 3.5) [14].

Ethical approval

The study was performed in accordance with the Helsinki Declaration II and approved by the Regional Ethical Review Committee (Project ID S-20090130) and the Danish Data Protection Agency (j.no. 18/15692) [11]. All participants received written and oral information and provided their written consent for participation [11].

Statistical analyses

Baseline characteristics were reported as mean (SD), median (quartiles), or percentage (number). The normality of data was evaluated using histograms and the Skewness/Kurtosis test. In lack of international predefined cut off level for FT4, women were divided according to 1st trimester FT4 level < vs. ≥ the 25th percentile (12.9 pmol/L). Maternal characteristics were compared between subgroups (FT4 < vs. ≥25th percentile, TSH ≥ vs. <2.5 [15] mU/L, and GDM vs. normal glucose tolerance (NGT) [13] using Wilcoxon rank sum test for non-parametric continuous data, Student's t-test for parametric continuous data, and Chi squared test for categorical data. Spearman nonparametric correlations were calculated between FT4 and TSH levels and measures of insulin resistance and glucose tolerance. Linear regression models were based on results from bivariate associations with measures. Measures of glucose tolerance were entered as dependant variable and maternal thyroid status (FT4 or TSH), maternal age, maternal BMI and parity as predicting variables. Regression analyses were performed with maternal thyroid status (FT4 or TSH) entered as continuous variable, and analyses were repeated with maternal thyroid status divided into quartiles with the 1st (lowest) quartile as reference. The statistical assumptions underlying the models were thoroughly checked and validated. The assumption of linearity between predictors and the outcome variable was inspected by using scatter plots and augmented component-plus-residual plots. Normality of predicted residuals were checked visually with quantile-normal and probability-normal plots. Homogeneity of variance (homoscedasticity) of the residuals was checked by plotting the residual against the fitted (predicted) values. Finally, multicollinearity was

formerly assessed using variance inflation factors. A two-sided p-value <0.05 was applied in all analyses, including evaluation of effect modifiers. All data were analysed using STATA/IC (version 15.1, StataCorp LP, TX, USA).

Sensitivity analyses

We performed three sensitivity analyses where we omitted women with abnormal thyroid function defined by high TSH levels >3.6 mU/L [16], high T4 levels >19.1 pmol/L [16] and positive TPO-antibodies. In addition, we performed a sensitivity analysis where women with GDM were omitted. A total of 14 women were treated with thyroid related medication. Omitting these women from statistical analyses did not change significant results (data not shown).

Results

A flow chart of included women is presented in **Figure 1**. A total number of 1,041 women had available data on 1st trimester thyroid status and 3rd trimester HbA1c, fasting glucose and insulin. Data regarding 3rd trimester OGTT were available in 509 women. Characteristics of study participants vs. non participants in OCC have previously been described [11]. Included women did not differ from excluded women according to age, smoking status or educational level, but included women had a significant higher pre-pregnancy BMI and were more often nulliparous (data not shown)

Clinical and biochemical characteristics of included women and neonates is presented in **Tables 1A, B, C.** Women were dichotomized according to FT4 level above and below the 25th percentile (12.9 pmol/L). Women with low FT4 had significantly higher pre-gestational BMI and higher levels of TSH (1st trimester), HbA1c (1st and 3rd trimester), higher fasting glucose, insulin, HOMA-IR and HOMA-β (3rd trimester), compared to women with high FT4 levels.

Women with TSH above cut-off of (2.5 mU/L) were more often nulliparous and more often had positive TPOAb status compared to women with TSH values below cut off **(Appendix, Table 1A).** Thyroid status was comparable in women with a diagnosis of GDM compared to women with NGT, but no woman with GDM had positive TPOAb compared to 10% women with NGT (p= 0.08) **(Appendix, Table 1B).**

In bivariate association analyses, FT4 was inversely associated with pre-gestational BMI, HbA1c (1st and 3rd trimester), fasting insulin, fasting glucose and HOMA-IR (3rd trimester) and TSH was

inversely associated with 2h glucose during OGTT **(Table 2).**

Multiple linear regression analyses are presented in **Tables 3A, B.** FT4 was an independent negative predictor of HbA1c (3rd trimester), whereas only a trend was found for the models regarding fasting glucose and HOMA-IR. When FT4 quartiles were entered as a categorical variable in the regression model , 3rd and 4th quartile levels of FT4 showed the strongest inverse association with HbA1c (P-trend <0.001).

TSH was not a significant independent predictor of HbA1c (3rd trimester), P-value= 0.08, and the trend for TSH quartiles was only borderline significant (P-trend = 0.06). The 4th quartile of TSH showed a significant inverse association with 2h glucose during the OGTT (P-trend = 0.01).

Sensitivity analyses

We recalculated results in Tables 1 after omitting the following patients:

TSH above reference: TSH levels >3.6 mU/L were found in 45 women. After omitting these women, the difference in fasting glucose between women with FT4 below and above the 25th percentile became non-significant (p=0.32).

FT4 above reference: T4 levels >19.1 pmol/L were found in 15 women [16]. When these women were omitted from table 1A, the difference in fasting glucose between women divided according to the 25th percentile of FT4 became non-significant (p=0.13). Otherwise, significant results were unchanged.

Positive TPO-antibodies: A total number of 95 women had positive TPO-antibodies. When these women were omitted from analyses, the difference in HbA1c (1st trimester) became non-significant between women with FT4 below and above the 25th percentile (p=0.10).

GDM: We excluded 28 women with GDM. When these women were omitted from the analysis, the difference in HbA1c (1st trimester) between women with FT4 below and above the 25th percentile became non-significant (p= 0.13).

Discussion

This paper is the first to investigate associations between maternal thyroid function in early pregnancy and glucose status in 3rd trimester. Our main study finding was that low FT4 in early pregnancy was associated with higher levels of HbA1c during 3rd trimester, whereas early pregnancy TSH was not associated with HbA1c. Furthermore, women with FT4 ≤ 25th percentile had significantly higher HOMA-IR and HOMA-β during 3rd trimester compared to women with FT4

levels >25th percentile. We are only aware of one previous paper that reported associations between early pregnancy FT4 levels and glucose status in later pregnancy [17]. In accordance with our results, the authors found that FT4 during 1st trimester was negatively associated with HbA1c during 2nd trimester [17]. However, the aim of the paper was to establish reference intervals of 1st trimester thyroid function in West China and the authors did not include data on 3rd trimester glucose status [17]. Two papers reported that lower 3rd trimester FT4 levels were linked to impaired glucose status outcomes [18,19], higher HbA1c [18,19] and higher HOMA-IR [19]. However, data regarding 1st trimester thyroid function or glucose status were not available [18,19]. First trimester TSH was not associated with 3rd trimester glucose status in the present study, this finding was in accordance with data on TSH during 3rd trimester in two papers including 321 [18] and 956 [19] pregnant women. TSH levels are not stable during 1st trimester, as high HCG levels induce FT4 secretion, and these elevated FT4 levels inhibit TSH concentrations[20]. The variation in TSH levels may explain our finding of an inverse association between TSH and 2h glucose in 3rd trimester. We found that lower FT4 levels were associated with higher 3rd trimester HOMA-IR. These findings resemble the situation outside pregnancy, where hypothyroidism is associated with increased insulin resistance [21,22]. However, surprisingly, we reported higher HOMA-β in women with lower FT4 compared to women with high-normal FT4 levels. We are not aware of other human studies regarding associations between FT4 and HOMA-β during pregnancy. In a sheep model, hypothyroidism during pregnancy was obtained by removing the fetal thyroid gland in late pregnancy [23] and hypothyroidism was linked to higher pancreatic β–cell mass and more circulating insulin was measured in umbilical cord blood [23]. Furthermore, hypothyroidism *in utero* in mice [24] and rats [25,26] predisposed to T2D in adult offspring, especially if the offspring was challenged by metabolic stress, e.g. high fat diet [24]. These findings suggest that low thyroid function could be a mechanism for transgenerational transfer of metabolic risk. In OCC, the hypothesis of transgenerational transfer of early pregnancy FT4 levels and future health will be tested by measuring fat mass and HbA1c in children at the age of 7-9 years.

In the present study, GDM was diagnosed in 28 women [12]. FT4 levels were comparable during 1st trimester in women who developed GDM and women with NGT. Our major finding regarding FT4 as an independent negative predictor of 3rd trimester HbA1c remained significant after omitting women with GDM. In OCC, no woman with GDM had positive TPOAb. This finding contrasted two

previous studies [27,28] that combined assessment of early pregnancy thyroid status with medical record data regarding 3rd trimester GDM. The studies did not include blood samples regarding glucose status during 1st or 3rd trimester [27,28]. Available data increase the awareness of early low thyroid function as a risk marker of adverse glucose status during pregnancy. However, GDM is not an exact diagnosis [6,7], and inclusion of glucose data in the two papers would be necessary to acquire new clinical relevant knowledge.

First trimester thyroid function was not associated with BW in the present study. This finding seemed to contrast that lower FT4 levels during 2nd and 3rd trimester were associated with higher BW in 48,145 mother-child pairs [10]. However, our findings of a link between low-normal FT4 and higher integrated glucose load and higher insulin levels suggested that elevated glucose status could be a possible mediator for the observed association between low thyroid function and offspring growth [10]. The present study could be underpowered regarding detecting small differences in BW, and larger studies are needed to confirm this hypothesis. In OCC, HbA1c was measured on all study participants during early and late pregnancy, whereas OGTT was only performed in a sub-cohort [13].

Our finding of early FT4 as an independent predictor of 3rd trimester HbA1c contributes to the increasing recognition of HbA1c as a biomarker for glucose status also in pregnancy [29]. HbA1c assessment is relatively simple and no fasting is required. HbA1c is the integrated glucose assessment, however also in pregnancy, HbA1c concentrations may be affected by reduced erythrocyte lifespan [30] and the HbA1c analysis may not be readily available internationally [31]. Future studies will clarify possible associations between HbA1c in pregnancy and future offspring health also in pregnant women without diabetes mellitus.

Strength and limitations may apply in our study. Study strengths included the population-based design, availability of both fasting glucose, insulin and OGTT data, and blinding of participants and doctors regarding thyroid function and fasting glucose status, as samples were not analyzed during pregnancy, except 2h glucose samples in women with an OGTT by indication. The aim of OCC was to include all pregnant women in the population, but the prevalence of women with non-Caucasian origin participating in the study was lower than in the background population [11]. We used high standard assays for all analyses, but future studies may apply mass spectrometry for the

measurement of total T4 [32] and especially FT4. We measured early pregnancy thyroid function as well as early and late pregnancy HbA1c. However, oral glucose tolerance tests were only performed in part of the study subjects [13] and we had no access to the gold standard test regarding insulin sensitivity, the euglycemic hyperinsulinemic clamp.

In conclusion, lower levels of FT4 in early pregnancy was associated with higher HbA1c levels in 3rd trimester. Future studies are needed to investigate if FT4 is a better marker than TSH for early thyroid function in pregnancy.

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Appendix, Table 1A Clinical and biochemical characteristics in the study cohort divided according to 1st trimester TSH

	TSH ≥2.5 (n=158)	TSH <2.5 (n=883)	
Maternal			
Age (years)	31 (5)	30 (5)	0.45
BMI (kg/m^2)	24.4 (4.3)	24.4 (4.4)	0.87
Smoking (yes)	4% (4/155)	4% (32/870)	0.49
Western ethnicity (yes)	99% (149/151)	97% (814/840)	0.23
Nulliparous (yes)	70% (110/158)	56% (495/883)	**0.001***
1st trimester			
TSH (10^{-3} mU/L)	3.11 (2.82; 3.76)	1.23 (0.82; 1.71)	**<0.001****
FT4 (pmol/L)	13.4 (1.4)	14.2 (2.1)	**<0.001****
TPOab positive? (yes)	20% (33/158)	7% (62/883)	**<0.001****
HbA1c (mmol/mol)	31.4 (2.6)	31.8 (2.6)	0.08
3rd trimester			
HbA1c (mmol/mol)	29.9 (2.9)	30.4 (2.8)	0.06
HbA1c <32 (mmol/mol)	72% (98/136)	67% (506/751)	0.28
Fasting insulin (pmol/L)	67 (49; 94)	68 (48; 96)	0.91
Fasting glucose (mmol/L)	5.0 (0.5)	5.0 (0.4)	0.86
HOMA-IR	2.1 (1.5; 3.0)	2.1 (1.5; 3.2)	0.94
HOMA-β	139 (102; 177) (n=76)	133 (101; 174) (n=433)	0.50
2h glucose (mmol/L)	6.7 (1.4)	6.7 (1.4)	0.23
AUC glucose (mmol/L)	13.8 (2.3)	13.9 (2.4)	0.66
Offspring			
LGA (yes)	6% (10/115)	9% (77/870)	0.32
Boys (n=536)	(n=81)	(n=455)	
Birth weight (g)	3,598 (447)	3,559 (550)	0.54

Birth BMI (z-score)	0.13 (-0.50; 0.52)	-0.049 (-0.82; 0.71)	0.453
Girls (n=482)	(n=73)	(n=409)	4
Birth weight (g)	3,409 (444)	3,477 (509)	0.235
Birth BMI (z-score)	-0.15 (-0.82; 0.61)	-0.16 (-0.73; 0.66)	0.956

7

8 Abbreviations: AUC: area under the curve, FT4: free T4, LGA: large for gestational age, HOMA:
9 homeostatic model, TPOab: Thyroid peroxidase antibodies
10 *<0.05, **<0.001
11 Smoking, western ethnicity, parity, TPOab positive and LGA presented as percentage (number)
12 Continuous data presented as mean (SD) or median (quartiles).
13 P-value for differences between women with low and high TSH, using Wilcoxon rank sum test for
14 non-parametric continuous data, t-test for parametric continuous data, and Chi squared test for
15 categorical data.
16
17 **Appendix, Table 1B** Clinical and biochemical characteristics in the whole study in the study cohort
18 divided according to glucose tolerance status.

	GDM (n=28)	NGT (n=481)	GDM vs. NGT
Maternal			
Age (years)	31 (5)	30 (5)	0.09
BMI (kg/m^2)	29.2 (6.3)	25.6 (5.2)	**0.002***
Smoking (yes)	7% (2/28)	4% (21/488)	0.37
Western ethnicity (yes)	100% (28/28)	97% (465/479)	1.0
Nulliparous (yes)	60% (17/28)	41% (200/488)	1.0
1st trimester			
TSH (mU/L)	1.36 (0.83; 1.84)	1.48 (0.97; 2.13)	0.36
FT4 (pmol/L)	14.1 (2.0)	14.0 (1.9)	0.82
TPOab positive (yes)	0% (0/28)	10% (46/481)	0.08
HbA1c (mmol/mol)	32.9 (3.1)	34.7 (2.9)	**0.04***
3rd trimester			

HbA1c (mmol/mol)	33.6 (3.5)	30.6 (2.8)	**<0.001***	19
HbA1c <32 (mmol/mol)	29% (8/27)	65% (280/428)	**<0.001***	20
Fasting insulin (pmol/L)	97 (67; 152)	74 (51; 112)	**0.003***	21
Fasting glucose (mmol/L)	5.7 (0.7)	5.1 (0.4)	**<0.001***	22
HOMA-IR	3.6 (2.2; 6.0)	2.4 (1.6; 3.8)	**0.007***	23
HOMA-β	136 (114; 197)	138 (102; 183)	0.72	24
	(n=28)	(n=481)		25
2h glucose (mmol/L)	6.7 (1.4)	6.6 (1.3)	**<0.001****	26
AUC glucose (mmol/L)	18.5 (2.3)	13.7 (2.2)	**<0.001****	27
Offspring				28
LGA (yes)	14% (4/28)	10% (47/480)	0.44	29
Boys (n=272)	(n=16)	(n=256)		30
Birth weight (g)	3,514 (510)	3,591 (560)	0.59	31
Birth BMI (z-score)	0.15 (-0.13; 0.95)	0.15 (-0.67; 0.77)	0.89	32
Girls (n=233)	(n=12)	(n=221)		33
Birth weight (g)	3,450 (881)	3,486 (497)	0.81	34
Birth BMI (z-score)	0.13 (-0.37; 1.15)	-0.10 (-0.73; 0.70)	0.50	35
				36

Abbreviations: AUC: area under the curve, FT4: free T4, LGA: large for gestational age, HOMA: homeostatic model, TPOab: Thyroid peroxidase antibodies *<0.05, **<0.001

37 Smoking, western ethnicity, parity, TPOab positive and LGA presented as percentage (number)
38 Continuous data presented as mean (SD) or median (quartiles).
39 P-value for differences between women with low and high TSH, using Wilcoxon rank sum test for
40 non-parametric continuous data, t-test for parametric continuous data, and Chi squared test for
41 categorical data.

Table 1A Clinical and biochemical characteristics in the study cohort divided according to 1st trimester free T4 (FT4) levels

	All (n=1,041)	FT4 <25 percentile (n=284)	FT4 ≥25 percentile (n=757)	
Maternal				
Age (years)	30 (4)	30 (5)	30 (4)	0.44
BMI (kg/m^2)	24.3 (4.4)	25.2 (4.8)	24.1 (4.2)	**0.0003***
Smoking (yes)	4% (36/1,025)	4% (13/278)	3% (23/747)	0.25
Western ethnicity (yes)	97% (963/991)	97% (260/267)	97% (703/724)	1.0
Nulliparous (yes)	58% (605/1,041)	60% (169/284)	58% (436/757)	0.62
1st trimester				
TSH (10^{-3} IU/L)	1.43 (0.91; 2.10)	1.61 (1.15; 2.33)	1.31 (0.82; 1.92)	**<0.001****
FT4 (pmol/L)	14.1 (2.0)	12.0 (0.7)	14.8 (1.8)	**<0.001****
TPOab positive (yes)	9% (95/946)	10% (27/284)	9% (68/757)	0.79
HbA1c (mmol/mol)	31.7 (2.6)	32.1 (2.5)	31.6 (2.6)	**<0.001****
3rd trimester				
HbA1c (mmol/mol)	30.3 (2.8)	30.9 (2.9)	30.1 (2.8)	**<0.001****
Fasting insulin (pmol/L)	68 (48; 96)	72 (50; 107)	65 (48; 92)	**0.01***
HbA1c <32 (mmol/mol)	68% (604/887)	62% (141/227)	70% (463/660)	**0.03**
Fasting glucose (mmol/L)	5.0 (0.4)	5.1 (0.5)	5.0 (0.4)	**<0.001****
HOMA-IR	2.1 (1.5; 3.2)	2.4 (1.5; 3.6)	2.0 (1.5; 3.1)	**0.02***
HOMA-□	134 (102; 175) (n=509)	138 (103; 182) (n=150)	131 (102; 170) (n=359)	**0.04***
2h glucose (mmol/L)	6.7 (1.4)	6.8 (1.5)	6.7 (1.4)	0.18
AUC glucose (mmol/L)	13.9 (2.4)	14.2 (2.6)	13.8 (2.3)	0.28
Offspring				
LGA (yes)	8% (87/1,025)	10% (27/278)	8% (60/747)	0.39
Boys	(n=536)	(n=156)	(n=380)	
Birth weight (g)	3,564 (535)	3,559 (525)	3,567 (541)	0.86

Birth BMI (z score)	0.0053 (-0.78; 0.67)	0.044 (-0.73;0.73)	-0.02 (-0.79; 0.64)	0.66
Girls	(n=482)	(n=120)	(n=362)	
Birth weight (g)	3,465 (500)	3,457 (494)	3,468 (503)	0.83
Birth BMI (z score)	-0.16 (-0.75; 0.66)	-0.13 (-0.64; 0.66)	-0.17 (-0.76; 0.66)	0.70

Abbreviations: AUC: area under the curve, FT4: free T4, LGA: large for gestational age, HOMA: homeostatic model, TPOab: Thyroid peroxidase antibody

*<0.05, **<0.001

Smoking, western ethnicity, parity, TPOab positive and LGA presented as percentage (number)

Continuous data presented as mean (SD) or median (quartiles).

P-value for differences between women with below and above 25 percentile (12.9 pmol/L) using Wilcoxon rank sum test for non-parametric continuous data, t-test for parametric continuous data, and Chi squared test for categorical data.

Table 1B Clinical and biochemical characteristics in the study cohort divided according to 1st trimester TSH

	TSH ≥2.5 (n=158)	TSH <2.5 (n=883)	
Maternal			
Age (years)	31 (5)	30 (5)	0.45
BMI (kg/m^2)	24.4 (4.3)	24.4 (4.4)	0.87
Smoking (yes)	4% (4/155)	4% (32/870)	0.49
Western ethnicity (yes)	99% (149/151)	97% (814/840)	0.23
Nulliparous (yes)	70% (110/158)	56% (495/883)	**0.001***
1st trimester			
TSH (10^{-3} IU/L)	3.11 (2.82; 3.76)	1.23 (0.82; 1.71)	**<0.001****
FT4 (pmol/L)	13.4 (1.4)	14.2 (2.1)	**<0.001****
TPOab positive? (yes)	20% (33/158)	7% (62/883)	**<0.001****
HbA1c (mmol/mol)	31.4 (2.6)	31.8 (2.6)	0.08

3rd trimester			
HbA1c (mmol/mol)	29.9 (2.9)	30.4 (2.8)	0.06
HbA1c <32 (mmol/mol)	72% (98/136)	67% (506/751)	0.28
Fasting insulin (pmol/L)	67 (49; 94)	68 (48; 96)	0.91
Fasting glucose (mmol/L)	5.0 (0.5)	5.0 (0.4)	0.86
HOMA-IR	2.1 (1.5; 3.0)	2.1 (1.5; 3.2)	0.94
HOMA-□	139 (102; 177) (n=76)	133 (101; 174) (n=433)	0.50
2h glucose (mmol/L)	6.7 (1.4)	6.7 (1.4)	0.23
AUC glucose (mmol/L)	13.8 (2.3)	13.9 (2.4)	0.66
Offspring			
LGA (yes)	6% (10/115)	9% (77/870)	0.32
Boys (n=536)	(n=81)	(n=455)	
Birth weight (g)	3,598 (447)	3,559 (550)	0.54
Birth BMI (z-score)	0.13 (-0.50; 0.52)	-0.049 (-0.82; 0.71)	0.43
Girls (n=482)	(n=73)	(n=409)	
Birth weight (g)	3,409 (444)	3,477 (509)	0.23
Birth BMI (z-score)	-0.15 (-0.82; 0.61)	-0.16 (-0.73; 0.66)	0.95

Abbreviations: AUC: area under the curve, FT4: free T4, LGA: large for gestational age, HOMA: homeostatic model, TPOab: Thyroid peroxidase antibodies *<0.05, **<0.001 Smoking, western ethnicity, parity, TPOab positive and LGA presented as percentage (number)

Continuous data presented as mean (SD) or median (quartiles).

P-value for differences between women with low and high TSH, using Wilcoxon rank sum test for non-parametric continuous data, t-test for parametric continuous data, and Chi squared test for categorical data.

Table 1C Clinical and biochemical characteristics in the whole study cohort, and in the study cohort divided according to glucose tolerance status.

	GDM (n=28)	NGT (n=481)	GDM vs. NGT
Maternal			

Age (years)	31 (5)	30 (5)	0.09	42
BMI (kg/m^2)	29.2 (6.3)	25.6 (5.2)	**0.002***	43
Smoking (yes)	7% (2/28)	4% (21/488)	0.37	44
Western ethnicity (yes)	100% (28/28)	97% (465/479)	1.0	45
Nulliparous (yes)	60% (17/28)	41% (200/488)	1.0	46
1st trimester				47
TSH (10^{-3} IU/L)	1.36 (0.83; 1.84)	1.48 (0.97; 2.13)	0.36	48
FT4 (pmol/L)	14.1 (2.0)	14.0 (1.9)	0.82	49
TPOab positive (yes)	0% (0/28)	10% (46/481)	0.08	50
HbA1c (mmol/mol)	32.9 (3.1)	34.7 (2.9)	**0.04***	51
3rd trimester				52
HbA1c (mmol/mol)	33.6 (3.5)	30.6 (2.8)	**<0.001****	53
HbA1c <32 (mmol/mol)	29% (8/27)	65% (280/428)	**<0.001****	54
Fasting insulin (pmol/L)	97 (67; 152)	74 (51; 112)	**0.003***	55
Fasting glucose (mmol/L)	5.7 (0.7)	5.1 (0.4)	**<0.001****	56
HOMA-IR	3.6 (2.2; 6.0)	2.4 (1.6; 3.8)	**0.007***	57
HOMA-□	136 (114; 197) (n=28)	138 (102; 183) (n=481)	0.72	58 59
2h glucose (mmol/L)	6.7 (1.4)	6.6 (1.3)	**<0.001****	60
AUC glucose (mmol/L)	18.5 (2.3)	13.7 (2.2)	**<0.001****	61
Offspring				62
LGA (yes)	14% (4/28)	10% (47/480)	0.44	63
Boys (n=272)	(n=16)	(n=256)		64
Birth weight (g)	3,514 (510)	3,591 (560)	0.59	65
Birth BMI (z-score)	0.15 (-0.13; 0.95)	0.15 (-0.67; 0.77)	0.89	66
Girls (n=233)	(n=12)	(n=221)		67
Birth weight (g)	3,450 (881)	3,486 (497)	0.81	68
Birth BMI (z-score)	0.13 (-0.37; 1.15)	-0.10 (-0.73; 0.70)	0.50	69

Abbreviations: AUC: area under the curve, FT4: free T4, LGA: large for gestational age, HOMA: homeostatic model, TPOab: Thyroid peroxidase antibodies *<0.05, **<0.001 Smoking, western ethnicity, parity, TPOab positive and LGA presented as percentage (number) Continuous data presented as mean (SD) or median (quartiles).

P-value for differences between women with low and high TSH, using Wilcoxon rank sum test for

non-parametric continuous data, t-test for parametric continuous data, and Chi squared test for categorical data.

Table 2 Beta coefficients in bivariate regression analyses

	FT4	TSH
Maternal		
Age (years)	-0.04	-0.03
BMI (kg/m^2)	**-0.11***	-0.05
1st trimester		
TSH (10^{-3} IU/L)	**-0.29***	
FT4 (pmol/L)		**-0.29***
HbA1c (mmol/mol)	**-0.07***	**-0.10***
3rd trimester		
HbA1c (mmol/mol)	**-0.14***	-0.05
Fasting insulin (pmol/L)	**-0.10***	-0.01
Fasting glucose (mmol/L)	**-0.07***	-0.03
HOMA-IR	**-0.10***	-0.02
2h glucose (mmol/L)	-0.04	**-0.10***
AUC glucose (mmol/L)	0.06	-0.01
Offspring		
Boys		
Birthweight (g)	-0.002	-0.02

Girls		
Birthweight girls (g)	-0.020	-0.073

Abbreviations: AUC: area under the curve, FT4: free T4, LGA: large for gestational age, HOMA: homeostatic model, TPOab: Thyroid peroxidase antibodies

Spearman non-parametric associations between maternal FT4 and TSH and measures of glucose and insulin.

Data presented as Beta coefficients, those with P-values <0.05 presented in bold.

Table 3A Linear regression analyses between glucose related outcomes (3rd trimester) and T4 and TSH during 1st trimester.

	Model 1 HbA1c (N=874)	Model 2 Fasting glucose (N=1,021)	Model 3 logHOMA-IR (N=1,021)		Model 4 HbA1c (N=874)	Model 5 2h glucose (N=509)
FT4	**-0.10** **(-0.19; -0.02)** **(P=0.02)**	-0.01 (-0.24; 0.0008) (P=0.07)	-0.005 (-0.012; 0.0085) (P=0.08)	TSH	-0.15 (-0.32; 0.02) (P=0.08)	-0.10 (-0.22; 0.02) (P=0.09)
Age	**0.13** **(0.09; 0.17)** **(P<0.001)**	**0.01** **(0.004; 0.17)** **(P<0.001)**	-0.0018 (-0.007; 0.0014) (P=0.28)	Age	**0.13** **(0.09; 0.18)** **(P<0.001)**	**-0.05** **(0.02; 0.08)** **(P<0.001)**
BMI	**0.15** **(0.12; 0.20)** **(P<0.001)**	**0.03** **(0.03; 0.04)** **(P<0.001)**	**0.0029** **(0.0025; 0.0031)** **(P<0.001)**	BMI	**0.16** **(0.12; 0.20)** **(P<0.001)**	**0.07** **(0.05; 0.10)** **(P<0.001)**

Parity	-0.28	-0.03	**-0.03**	Parity	-0.32	-0.11
	(-0.67; 0.10)	(-0.09; 0.02)	**(-0.06; -0.002)**		(-0.72; 0.06)	(-0.37; 0.15)
	(P=0.15)	(P=0.23)	**(P=0.04)**		(P=0.72)	(P=0.40)

Abbreviations: AUC: area under the curve, FT4: free T4, HOMA: homeostatic model

Data represent β-coefficients (95% CI). P-values <0.05 in bold.

All models adjusted for maternal age, maternal BMI and parity.

Model 1: HbA1c entered as the dependent variable and FT4, age, BMI, and parity entered as independent variables.

Model 2: Fasting glucose entered as the dependent variable and FT4, age, BMI, and parity entered as independent variables.

Model 3: LogHOMA-IR entered as the dependent variable and FT4, age, BMI, and parity entered as independent variables.

Model entering 2h glucose as the dependent variable did not show significant R^2 (not shown).

Model 4: HbA1c entered as the dependent variable and TSH, age, BMI, and parity entered as independent variables.

Model 5: 2h glucose entered as the dependent variable and TSH, age, BMI, and parity entered as independent variables.

Models entering Fasting glucose and LogHOMA-IR as the dependent variables did not show significant R^2 (not shown).

Table 3B Adjusted associations between T4, TSH, and glucose related outcomes

Model 1 HbA1c (N=874)	Model 2 Fasting glucose	Model 3 logHOMA-IR (N=1,021)	Model 4 HbA1c (N=874)	Model 5 2h glucose (N=509)

		(N=1,021)				
FT4 Continuous	**-0.10 (-0.19; -0.02) (P=0.02)**	-0.01 (-0.24; 0.0008) (P=0.07)	-0.005 (-0.012; 0.0085) (P=0.08)	TSH Continuous	-0.15 (-0.32; 0.02) (P=0.08)	-0.10 (-0.22; 0.02) (P=0.09)
FT4 Categorical				TSH Categorical		
1st quartile (≤12.9)	ref	ref	ref	1st quartile (≤0.90)	ref	Ref
2nd quartile (13-13.7)	-0.15 (-0.67; 0.36) (P=0.56)	0.03 (-0.05; 0.10) (P=0.47)	0.002 (-0.04; 0.04) (P=0.89)	2nd quartile (0.907-1.42)	-0.27 (-0.77; 0.22) (p=0.28)	-0.15 (-0.48; 0.19) (P=0.39)
3rd quartile (13.8-14.9)	**-0.80 (-1.30; -0.31) (P=0.001)**	-0.03 (-0.10; 0.04) (P=0.41)	-0.015 (-0.05; 0.02) (P=0.41)	3rd quartile (1.43-2.07)	-0.46 (-0.96; 0.03) (P=0.06)	-0.15 (-0.49; 0.19) (p=0.37)
4th quartile (≥15)	**-0.78 (-1.26; -0.29) (P=0.001)**	-0.03 (-0.10; 0.04) (P=0.39)	-0.021 (-0.06; 0.02) (P=0.25)	4th quartile (≥2.08)	-0.42 (-0.92; 0.07) (P=0.09)	**-0.44 (-0.78; -0.10) (P=0.01)**
P-trend	**<0.001**	0.39	0.20	P-trend	0.06	**0.01**

Abbreviations: AUC: area under the curve, FT4: free T4, HOMA: homeostatic model

Data represent β-coefficients (95% CI). P-values <0.05 in bold.

All regression models adjusted for maternal age, maternal BMI and parity.

Top panel: T4 and TSH entered as continuous variables.

Bottom panel: T4 and TSH divided into quartiles and entered as categorical values with 1st quartile as reference (ref=reference).

Examples:

Model 1: HbA1c entered as the dependent variable and T4, age, BMI, and parity entered as

independent variables.

Model 4: HbA1c entered as the dependent variable and TSH, age, BMI, and parity entered as independent variables.

Figure 1 Flowchart of study population in Odense Child Cohort (OCC)