

**LONGITUDINAL TRAJECTORIES OF GESTATIONAL THYROID FUNCTION: A NEW APPROACH
TO BETTER UNDERSTAND CHANGES IN THYROID FUNCTION**

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Abstract

Context

Most studies of thyroid function changes during pregnancy use a cross-sectional design comparing means between groups rather than similarities within groups.

Objective

Latent class growth analysis (LCGA) is a novel approach to investigate longitudinal changes that provide dynamic understanding of the relationship between thyroid status and advancing pregnancy.

Design

Prospective observational study with repeated assessments

Setting

General community

Patients

1100 healthy pregnant women who were included at their first antenatal visit at 12 weeks gestation

Main outcome measures

Statistically defined distinct groups based on determined specific changing trajectories by LCGA of both fT4 and TSH at each trimester.

Results

LCGA revealed three trajectory classes. Class 1 (n = 1019, 92.4%), a '*Low increasing TSH*' reference group, had a gradual increase in TSH throughout gestation (from 1.1 to 1.3 IU/l). Class 2 (n = 30, 2.8%), '*High increasing TSH*', displayed the largest increase in TSH (from 1.9 to 3.3 IU/l); Class 3 (n = 51, 4.6%), '*Decreasing TSH*', had the largest fall in TSH (from 3.2 to 2.4 IU/l). (Sub)clinical hypothyroidism at 12 weeks occurred in up to 60% of class 3

women and was accompanied by elevated TPO-Ab titres (50%) and a parental history of thyroid dysfunction (23%). 70% of class 2 women were nulliparous compared to 46% and 49% in classes 1 and 3.

Conclusion

LCGA revealed distinct trajectories of longitudinal changes of fT4 and TSH levels during pregnancy in 7.4% of women. These trajectories were correlated with parity and TPO-Ab status and followed patterns that might reflect differences in pregnancy-specific immune tolerance between nulliparous and multiparous women.

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65 Latent class growth analysis shows different trajectories of gestational thyroid function

66 changes taking both fT4 and TSH, assessed at each trimester, into account.

INTRODUCTION

Pregnancy causes profound changes in regulation of the hypothalamus-pituitary-thyroid axis and extra-thyroidal thyroid hormone metabolism. (1) Consequently, results of thyroid function tests differ in pregnant women compared to non-pregnant women, and trimester-specific reference ranges of TSH and free T4 (fT4) have been proposed. (2) Usually reference ranges are obtained using blood samples taken from groups of TPO-Ab negative women in their 1st, 2nd or 3rd trimesters rather than by sampling the same women in each trimester. (2) Although this cross-sectional approach succeeds in obtaining reliable trimester-specific reference ranges for the population, it obscures longitudinal changes in thyroid function that occur in individual women during the course of their pregnancy.

Each individual has a unique set-point of the hypothalamus-pituitary-thyroid axis and genetic studies demonstrate that the thyroid status is inherited as a complex trait. (3,4) Thus, circulating TSH and fT4 concentrations within an individual vary across a rather narrow range, which is smaller than 50% of the population-based reference range. (5) Moreover, a large study in over 150,000 subjects showed the TSH-fT4 relationship is not inverse log-linear and is also age- and sex-dependent (6). Cross-sectional measurements of TSH and fT4 have been related in many studies to various obstetric outcomes including gestational diabetes, GHT and abnormal fetal position, as reviewed elsewhere. (2,7) Some studies have observed an association between outcomes and both TSH and fT4, whereas others have reported a relationship either with TSH but not fT4, or *vice versa*. (2,7) Similarly, studies investigating the consequences of maternal thyroid dysfunction on infant neurodevelopment have reported disparate findings. (2,7) Such inconsistencies have always been difficult to reconcile but may result from the use of thyroid function reference ranges derived from cross-sectional data. Also, as recently summarized, the reliability of fT4 assessments during pregnancy is still subject to debate, especially during late gestation due to the physiological changes related to pregnancy (increase of TBG and decrease of albumin concentrations. (2) It is likely, therefore, that more accurate information can be obtained if longitudinal TSH and

ft4 measurements from the same women during each trimester are used in epidemiological studies.

Longitudinal mixture models, including latent class growth analysis (LCGA), have been advocated as sophisticated statistical approaches that can accommodate temporal changes within heterogeneous population samples. (8) For example, studies in perinatal psychiatry reported various trajectories of perinatal depressive symptoms. (9)

We studied, therefore, whether LCGA can be used as an alternative approach to mitigate the limitations of cross-sectional studies on thyroid function by classifying individuals into groups based on their differing longitudinal trajectories of TSH and ft4 that occur during pregnancy.

We also investigated whether these longitudinal trajectories shared different obstetric complications. Finally, we executed post-hoc analyses to validate the findings of the LCGA.

In a randomly selected subsample of respondents (n=568) hCG was also assessed at 12 weeks gestation to estimate the effect of hCG on thyroid function in the possible different trajectories.

Materials and methods

We undertook a prospective study of the course of thyroid function in a large cohort of pregnant women, in whom TSH, fT4 and TPO-Abs were determined. We applied LCGA with trimester repeated assessment of thyroid function. The primary outcome was identification of trajectories of thyroid function that integrate changes in TSH and fT4 during the three trimesters. Secondary outcomes were to evaluate the role of TPO-Abs and determine the occurrence of thyroid dysfunction. This study is reported in line with the STROBE guidelines (10).

Subjects

During a period of two years (2002-2004), 1702 women who booked for antenatal visits at 12 weeks gestation, were followed in five community midwife practices in the iodine sufficient vicinity of Eindhoven, Netherlands. (11) Only Dutch Caucasian women (n = 1507) were invited to participate, and 1198 women (79.5%) consented. Exclusion criteria similar to those of the NHANES survey (such as previous thyroid disease, thyroid medication) were present in 49 women. (12) 1149 Women were eligible and followed up at 24 and 36 weeks gestation. Complete data were missing for 49 women and data analysis was performed in the remaining sample of 1100 (Table 1). At 36 weeks gestation, thyroid data from 40 women were lacking because they had already given birth. To maximise statistical power, thyroid parameters (fT4, TSH and TPO-Ab status) in these 40 women were estimated with the multiple imputation algorithm. The variables age, parity, parental history of thyroid dysfunction, smoking, pre-pregnancy BMI, history of miscarriage, and thyroid parameters at 12 and 24 weeks gestation (fT4, TSH and TPO-Ab status) were used for imputation modelling and pooled estimates were reported. (13)

The study was approved by the Medical Ethical Committee of Máxima Medical Centre in Eindhoven/Veldhoven and all women gave written informed consent.

Assessments

Serum TSH was measured using a solid-phase, two-site chemiluminescent enzyme immunometric assay (IMMULITE Third generation TSH, Diagnostic Products Corporation, Los Angeles USA). Inter-assay coefficients of variation are 5.0% and 4.4% at concentrations 0.22 mIU/L and 2.9 mIU/L, respectively. Trimester-specific TSH reference ranges were defined using 2.5th and 97.5th cut-offs in TPO-Ab negative women. The serum fT4 concentration was measured with a solid-phase immunometric assay (IMMULITE Free T4). Inter-assay coefficients of variation are 6.7% and 4.4% at concentrations of 11.6 pmol/L and 31.5 pmol/L, respectively. Trimester specific fT4 reference ranges were defined using 2.5th and 97.5th cut-offs in TPO-Ab negative women.

Serum TPO-Abs were determined using the IMMULITE Anti-TPO-Ab kit. Inter-assay coefficients of variation were 9% and 9.5% for concentrations of 40 IU/ml and 526 IU/ml, respectively. The anti-TPO assay is standardized in relation to the International Reference Preparation for anti-TPO MRC 66/387. A TPO-Ab titre > 35 IU/ml at 12 weeks gestation was defined as TPO-Ab-positive, and this cut-off identified 95 TPO-Ab positive women (8.6%, Table 1) and 1005 TPO-Ab negative women.

Human chorionic gonadotropin (hCG) was assessed at 12 weeks gestation in a random subsample of 568 participants by the immulite technique (IMMULITE HCG, Diagnostic Corporation, Los Angeles USA). The coefficient of variation was 5.8% at a concentration of 370 IU/l.

Statistical analysis

Descriptive statistics were performed in SPSS version 24 (IBM). To study longitudinal trajectories of gestational thyroid function, we performed LCGA, a longitudinal mixture modelling technique that can identify homogeneous classes within a larger heterogeneous longitudinal data sample. (14). We used MPlus version 7.4. (15) To model thyroid function we included TSH and fT4 at all trimesters. Because TSH values were positively skewed with

an abundance of scores close to zero, we modeled trajectories of gestational thyroid function using the MLR option in MPlus. (16) This option is robust to non-normality and preferred over transforming variables to approximate a normal distribution. (17) The starting point was a 1-class model, after which models were fitted to increasing numbers of classes. To determine the optimal number of classes, we applied the following fit indices: Bayesian Information Criterion (BIC), Lo-Mendell-Rubin Likelihood Ratio Test (LMR-LRT), and Bootstrapped Likelihood Ratio Test (BLRT). (18) Better fitting models have lower BIC values, and significant LMR-LRT and BLRT values indicate that a model with an additional class is a better fit to the data. The BLRT is superior to the LMR-LRT for identification of the correct number of classes. (18) Apart from these fit indices, we also considered entropy, with entropy values closer to 1 indicating clearer delineation of classes. (19) We further accounted for parsimony and interpretability of the models, and average posterior probabilities (APPs) of their classes. We also took into account whether additional classes included more than 1% of the total sample.

Obstetric complications

We also investigated whether different trajectories were associated with poor obstetric outcomes. These included preterm birth (PTB), gestational hypertension (GHT: pregnancy induced hypertension / pre-eclampsia) and poor fetal growth. Gestational age was calculated from the date of the last menstrual cycle and from an ultrasound scan during what was presumed to be the 12th week of pregnancy. If a discrepancy of more than seven days was seen between these two measurements, a second ultrasound scan was performed within two weeks to reassess gestational age. Pre-term birth was defined as a gestational age at birth < 37 weeks. Gestational hypertension (GHT) was defined as development of systolic blood pressure ≥ 140 mmHg and/or diastolic blood pressure ≥ 90 mm Hg after 20 weeks gestation (at least two BP readings) in previously normotensive women (+ pre-eclampsia in case of additional presence of proteinuria). The

Netherlands perinatal registry was used to define small for gestational age (SGA) neonates. In this registration system >95% of all hospital and home births in the Netherlands are registered. The definition of SGA was based on population based birth weight percentiles using the lowest 10th percentile cut-off (20). Although the cut-off for the definition of SGA and intra-uterine growth restriction is arbitrary, in most large studies the lowest 10th percentile is used (21). In order to define SGA appropriately birth weight is corrected for: gestational age, sex of the baby and parity. Differences between possible classes were compared using chi-square test for categorical and *t*-test/ANOVA for continuous variables with Bonferroni correction for multiple testing. Non-parametric testing of variables with considerable departure from normal distribution was performed using Mann-Whitney-U and Kruskal-Wallis tests.

RESULTS

Baseline characteristics of the sample of 1100 pregnant women at 12 weeks gestation are presented in Table 1. 95 women (8.6%) were considered to be TPO-Ab positive (TPO-Ab >35 kU/L). The reference range of normal thyroid function (between 2.5th – 97.5th percentiles) in 1005 TPO-Ab negative women was between 0.15 and 2.9 IU/l for TSH and between 11.9 – 20.8 pmol/l for fT4 at 12 weeks gestation. At 24 weeks gestation these values were 0.4 – 2.7 IU/l and 10.2 – 17.8 pmol/l and at 36 weeks gestation 0.42 – 3.2 IU/l and 9.5 – 17.0 pmol/l, respectively. Trimester specific overt thyroid dysfunction was defined as a TSH and fT4 outside these reference ranges while sub-clinical thyroid dysfunction referred to women with trimester specific TSH level outside the reference range but a normal fT4 concentration. In total, there were 55 (5%) women who delivered pre-term. Of these 55 PTB women, 6 were TPO-Ab + (11%), 4 (7.3%) had sub-clinical hypothyroidism and 4 (7.3%) had sub-clinical hyperthyroidism at some time point during gestation. Similar figures were found in the remaining 1045 women who delivered at term: 8.5% were TPO-Ab+, 69 (6.6%) had sub-

clinical hypothyroidism and 48 (4.6%) sub-clinical hyperthyroidism. GHT occurred in 61 (5.5%) women and was significantly more frequent in nulliparous women (45 out of 513 = 8.8%) than in multiparous women (16 out of 587 = 2.7%, $X^2(1) = 19$, $p < 0.001$). There were no differences of TPO-Ab + status, sub-clinical hypothyroidism or sub-clinical hyperthyroidism between GHT and normotensive women (data not shown). The 77 women who gave birth to SGA neonates had similar levels of TPO-Ab+ and sub-clinical hyperthyroidism (data not shown) but developed sub-clinical hypothyroidism more frequently during gestation compared to the remaining 1023 women: 10 (13%) versus 63 (6.1%) ($X^2(1) = 5.3$, $P = 0.02$).

Insert Table 1 about here

The mean hCG in the sub-sample of 568 women was 57.714 IU/l (SD: 29.380) with a median of 52.733 IU/L, an a range from 7043 to 196.211 IU/L. The 90th and 95th percentile of hCG were 100.691 IU/L and 111.317 IU/L. This sub-sample showed no statistically different baseline characteristics (age, parity, lifestyle habits) compared to the total sample (data not shown). The mean (SD) fT4, mean (SD) TSH and median (range) logTSH of this sub-sample were: 16.3 pmol/l (2.5), 1.28 mIU/L (0.81) and 0.043 mIU/L (range: -.2.00 to 0.84 mIU/L) and 50 (8.8%) women were TPO-Ab positive. These numbers were statistically no different compared to the total sample (Table 1).

Trajectories of gestational thyroid function

Based on the BIC, LMR-LRT and BLRT statistics, the 3-class model was chosen. This 3-class model had adequate class sizes and entropy, and high APPs (.84-.98, Supplemental Table S1). These model estimates were replicated using convergence checks, supporting a 3-class model as the optimal solution. (18)

The three longitudinal thyroid function trajectories that formed each class are shown in Figure 1. All share a gradual fT4 decline.

Insert figure 1 about here

Class 1 (n = 1019, 92.4%) was labeled '*Low increasing TSH*' and regarded as the reference group. Individuals had a gradual increase in TSH towards the end of gestation (TSH at 36 weeks was 1.3 IU/l). Class 2 (n = 30, 2.8%) was labeled: '*High increasing TSH* ', displaying the highest increase in TSH towards the end of gestation (TSH at 36 weeks was 3.3 IU/l); individuals had an intermediate mean fT4 (15.8 pmol/l) at 12 weeks. Class 3 (n = 51, 4.6%) was labeled '*Decreasing TSH*' and had the highest TSH (3.2 IU/l) and the lowest fT4 (mean 15.3 pmol/l) at 12 weeks gestation. These women displayed a pattern of decreasing TSH throughout gestation to a mean TSH of 2.4 IU/l and mean fT4 of 13.2 pmol/l at 36 weeks gestation. Thyroid and other characteristics in relation to the three trajectories of TSH and fT4 are shown in Table 2.

Insert Table 2 about here

Overt and sub-clinical hyperthyroidism occurred only in class 1, whereas overt and sub-clinical hypothyroidism occurred predominantly in class 3. Using trimester specific reference ranges, in total, there were 73 (6.6%) women with sub-clinical hypothyroidism at any trimester of whom 28 (38%) were TPO-Ab+ and 52 (4.7%) with sub-clinical hyperthyroidism of whom 7 were TPO-Ab+ (13.5%). Sub-clinical hypothyroidism resolved by 24 and 36 weeks gestation in 20 (of whom 50 were TPO-Ab+) of 32 class 3 women who showed sub-clinical hypothyroidism at 12 weeks (Table 2). Within class 3, 5 women had sub-clinical hypothyroidism in all trimesters (60% TPO-Ab+). In class 2, the 4 women with sub-clinical hypothyroidism at 12 weeks had sub-clinical hypothyroidism in all trimesters (100% TPO-

Ab+). Of the remaining 14 women in class 2 who developed sub-clinical hypothyroidism by 36 weeks, 4 were TPO-Ab+ (28%). A family history of thyroid dysfunction occurred more often in class 3 ($\chi^2(2) = 13$, $p = 0.003$) and TPO-Ab were also most prevalent in class 3. Multi-parity occurred less frequently in class 2 compared to class 1 ($\chi^2(1) = 6.8$, $p = 0.0093$) or class 3 ($\chi^2(1) = 3.4$, $p = 0.066$). PTB was equally distributed in all classes: 4.8% in class 1, 6.3% in class 2 and 5.8% in class 3. In class 1 there were 66 (6.5%) SGA neonates, in class 2 five (16.6%) and in class 3 six (11.7%), which was significantly different: $\chi^2(2) = 6.5$, $p = 0.039$. Compared to the reference group (class 1), class 2 had an O.R. of 2.8 (95% CI: 1.13 – 7.3) for SGA. With regard to GHT, LCGA did not identify a trajectory of change in thyroid function that was associated with increased risk: $\chi^2(2) = 3.8$, $p = 0.15$. Table 3 shows similar trajectories in the sub-sample of 568 women in whom also hCG was assessed as well as mean (SD), median (range) of hCG.

Insert Table 3 about here

The mean hCG concentrations were the highest in class 1 and the lowest in class 2 but not significantly different (Anova: $F(2) = 1.40$, $p = 0.22$). Similarly, the median hCG was the highest in class 1 and the lowest in class 2 but not significantly different (Kruskal-Wallis: $\chi^2(2) = 2.5$, $p = .28$). In the reference group (class 1), the maximum hCG level was 196.211 IU/L while this figure was 84.243 IU/L in class 2 (representing the 83th percentile of class 1) and 98.292 IU/L in class 3 (representing the 89th percentile of class 1). The numbers (%) of TPO-Ab positive women in these sub-sample trajectories were similar to that (Table 2) of the total sample: 32 (6.1) in class 1, 3 (17.6) in class 2 and 15 (51.7) in class 3. Also the numbers of women with overt hyperthyroidism (six, 1%) and sub-clinical hyperthyroidism (eleven, 1.9%) were comparable to that of the total sample (Table 2) and occurred only in class 1. Of the six women with overt hyperthyroidism of class 1, none had an hCG

concentration > 90th percentile while in the eleven women with sub-clinical hyperthyroidism, five (55%) had an hCG level > 90th percentile.

Because 70% of the women in class 2 were nulliparous compared to 49% and 46% in class 3 and 1 respectively, we repeated LCGA in nulliparous and multiparous women separately.

In nulliparous women (Figure 2A, n = 513), three classes were again identified; their characteristics were similar to those of the whole group of 1100 women. Class 1 (n = 486, 94.7%) '*Low increasing TSH*' (reference group), class 2 (n = 13, 2.6%) '*High increasing TSH*' and class 3 (n = 14, 2.7%) '*Decreasing TSH*'. (Parameter estimates are shown in Supplemental Tables S2.)

Insert figure 2 about here

In multiparous women, however, only two classes were identified (Figure 2B, N = 587). Class 1 (n = 553, 94%) '*Low increasing TSH*' (reference group), and class 3 (n = 34, 6%) '*Decreasing TSH*' with similar characteristics to class 3 in the whole cohort and the nulliparous group (Parameter estimates are shown in Supplemental Tables S3).

Thyroid and other characteristics in relation to longitudinal trajectories of TSH and fT4 in nulliparous and multiparous women are shown in Table 4. The prevalence of TPO-Ab in class 1 subjects did not differ between nulliparous and multiparous women, and remained stable throughout pregnancy.

Insert Table 4 about here

The prevalence of TPO-Ab in class 3 decreased in nulliparous women by 14% (from 21% at week 12 to 7% at week 36), whereas a significantly greater decrease of 38% (from 41% at week 12 to 2.9% at week 36) was observed in multiparous women ($X^2(1) = 11.8$, $p = 0.001$).

The highest prevalence of sub-clinical hypothyroidism occurred in class 2 nulliparous women

(69%). The number of women reporting a parental history of thyroid dysfunction was also highest in class 2 nulliparous women. Throughout gestation, the highest concentrations of TPO-Ab were present in nulliparous women. The prevalence of TPO-Ab was highest (54%) at week 12 in nulliparous class 2 women, and remained high until late gestation (31%). The prevalence of TPO-Abs in nulliparous class 2 women was higher than in class 3 (7%) and class 1 (6.1%) ($X^2(2)=12.0$, $p=0.006$) nulliparous women and in class 3 (2.9%) and class 1 (5.4%) multiparous women at all stages of pregnancy.

The prevalence of GHT among nulliparous women was not significantly different between classes ($X^2(2)=2.1$, $p = 0.36$): 15.4% (2 out of 13) in class 2, 8.8% (43 out of 486) in class 1, and no cases in class 3. In multiparous women these figures were significantly different between classes ($X^2(1) = 5.0$, $p = 0.025$): 8.8% (3 out of 34) in class 3 and 2.4% (13 out of 553) in class 1. Of the 55 women with PTB, 36 occurred in nulliparous and 19 in multiparous women ($X^2(1) = 8.3$, $P = 0.002$). Table 4 shows that in nulliparous women the prevalence of PTB was not different between class 1, 2 and 3 (6.3%, 15.4% and 21.4%, respectively) but the absolute numbers were rather low ($X^2(2) = 6.1$, $P = 0.12$). In multiparous women, all the 19 (3.4%) PTB occurred in class 1 and none in class 3.

DISCUSSION

We performed a prospective study of longitudinal changes in thyroid function in individual women during pregnancy. Latent class growth analysis (LCGA) enabled women to be grouped into novel classes according to longitudinal trajectories of thyroid status. Three classes were identified, a distinction that could not have been made if only cross-sectional data analysis was performed. LCGA longitudinal analysis and classification thus provides new pathophysiological understanding of the relationship between thyroid status, thyroid autoimmunity and the progression of pregnancy.

Three longitudinal trajectories of thyroid function

The physiological decrease in fT4 and rise in TSH as pregnancy progresses was seen in over 90% of women in class 1, the reference group. Low TSH levels in the first trimester result from the thyroid-stimulating effect of high hCG levels.(1) The rise in TSH during late gestation, when the thyroid-stimulating effect of hCG has subsided, likely reflects the capability of the thyroid gland to meet increasing demands for thyroid hormone during pregnancy. Such increased demands result from the fetal need for maternal T4 in the first trimester, increased levels of serum thyroxine-binding globulin, increased renal iodide clearance, and increased degradation of thyroid hormones by placental deiodinase type 3. (1,7) Nevertheless, even though TSH concentrations increase as pregnancy progresses they remain at low-normal levels, suggesting the occurrence of subclinical and overt hyperthyroidism in class 1 women is related to high levels of hCG, especially since the concentration of TPO-Ab decreases in the 2nd and 3rd trimester. This was partially supported by hCG assessment in a sub-sample with limited epidemiological power: none of the six women with overt hyperthyroidism had an hCG level >90th percentile, while 55% of women with sub-clinical hyperthyroidism had an hCG level >90th percentile.

The trajectory in class 3 women may reflect the thyroidal response in pregnant women who are prone to develop or already have autoimmune thyroiditis. Thus, 20% of class 3 women had a family history of thyroid disease and 50% were TPO-Ab positive. Women in class 3 had a higher TSH concentration in the first trimester, reflecting thyroid autoimmunity, but it is notable that the TSH concentration decreased with advancing gestation in class 3 women compared to class 1 and 2 women in whom TSH increased in the 2nd and 3rd trimesters. It seems therefore that up-regulation of the HPT-axis to meet the increased thyroid hormone demands of pregnancy is compromised in class 3 women. In support of this interpretation, their fT4 concentrations are lower throughout gestation, consistent with recent findings that the thyroidal response to hCG is restricted in TPO-Ab positive pregnant women. (22)

Nevertheless, the outcome of hCG assessment in a sub-sample could also suggest another

mechanism. Up to 50% of women in class 3 had elevated TPO-Ab titres, which were already present before conception. An elevated TPO-Ab titer is one of the most important determinants of high TSH. So, these women will also likely have an increased pre-conception TSH concentration. The beta-unit of hCG has a TSH like effect, and so it is possible that due to higher pre-conception TSH levels in these women, hCG stimulation of the TSH receptor is less pronounced compared to “healthy” TPO-Ab negative women (class 1, reference group).

The prevalence and concentration of TPO-Abs decreases in the 2nd and 3rd trimesters, but is less pronounced in class 3 women compared to classes 1 and 2. Immune tolerance of the partial allograft in pregnancy is largely dependent on CD4+ regulatory T-cell's (T-regs), which increase in number and function during gestation and are crucial inhibitors of autoimmunity. (23,24, 25) Thus, augmentation of Tregs is viewed as an important mechanism behind the observed decrease of thyroid antibodies and improvement of autoimmune thyroid diseases during gestation. (26) The modest decrease in TPO-Abs in class 3 women underlines the increased prevalence of thyroid autoimmunity in these women compared to class 1 and 2. We propose, therefore, that class 3 women might have an impaired increase in Tregs during pregnancy. Indeed, deficient numbers of Tregs have been documented in non-pregnant women with Hashimoto's thyroiditis. (26) Women in class 2 have an intermediate prevalence of hypothyroidism, family history of thyroid disease and TPO-Ab compared to classes 1 and 3. Thus, it can be concluded that class 2 comprises women with less advanced thyroid autoimmunity, in whom Treg mechanisms might remain able to prevent worsening of thyroid function in pregnancy. Interestingly, class 2 and 3 women of the sub-sample showed the lowest hCG levels compared to the reference group (although not significantly lower because of insufficient power). hCG is known to attract T-reg cells at the fetal-maternal interface and has the potential to provoke the conversion of non-Treg cells into Treg cells. (27) Class 2 and 3 women showed the highest numbers of TPO-Ab with the lowest levels of hCG and the highest numbers of pregnancy complications such as SGA and GHT. However, the number

of women with an hCG sub-sample analysis was too low to differentiate further for parity and / or fetal sex. GHT predominantly occurs in primiparous women (over-represented in class 2) and hCG seems only to induce T suppressor cells in lymphocytes from females but not from males. (27) Overall, therefore, lower concentrations of hCG in class 2 and 3 women could result in decreased production of Treg cells resulting in impaired fetal tolerance. Because, by definition, SGA is corrected for parity, we were able to investigate a possible association between SGA and thyroid function trajectory in the main LCGA analysis with adequate epidemiological power. Women in class 2 had a 3-fold increased risk to deliver SGA neonates. In a large cohort study from Rotterdam, an association between high maternal FT4 during early gestation and SGA was identified, with no effect of maternal TSH or TPO-Ab status (28). This conclusion was based on a single assessment of thyroid function, whereas in the current study LCGA was performed using three sequential measures of thyroid function and indicated that women in class 2, with the highest increase of TSH throughout gestation and fT4 levels above the reference group, had the highest incidence of SGA neonates. Also, class 2 women had a trend towards the highest incidence of PTB, further linking high TSH with modestly low fT4 levels throughout gestation to poor obstetric outcome. However, LCGA also showed that the highest incidence of GHT occurred in class 3 women with the highest TSH and lowest fT4 at early gestation. A possible explanation could be that women in class 3 (as shown in the analyses of the sub-sample) have lower hCG during early gestation, resulting in a lower rise in fT4 and a higher TSH concentration. Moreover, by increasing the number of uterine natural killer cells, hCG in general contributes to a proper remodeling of the maternal spiral arteries crucial for sufficient fetal nourishment. (27). Impaired spiral artery development (because of lower hCG) results in poor placentation which is a major risk factor of GHT. (7) Another explanation could be that class 3 women had a 3-fold higher rate of TPO-Ab+ compared to class 2: it has recently been suggested that the fT4 stimulating effect of hCG on fT4 production is less prominent in TPO-Ab + women (22). High hCG levels in healthy pregnant women will result in higher FT4

levels and thereby indirectly to lower TSH levels. In TPO-Ab positive women, the thyroid gland may already be compromised to a certain extent and the response of the thyroid gland to hCG may be compromised as well (lower release of T4 in response to the same amount of hCG). Alternatively, SGA and GHT may result from differing pathogenetic mechanisms.

Nulliparous versus multiparous women

LCGA analysis demonstrated an absence of class 2 trajectory in multiparous women, whereas the characteristics of classes 1 and 3 in multiparous women were not different to the whole cohort of 1100 women. Although all three classes of thyroid function trajectory were observed in nulliparous women, the highest prevalence of overt and subclinical hypothyroidism, TPO-Ab and family history of thyroid disease were present in class 2 women compared with class 3 women in the whole cohort of 1100. These findings suggest differences in immune regulation between nulliparous and multiparous women. During a first pregnancy, the pregnancy-related immune stimulation induces type 2 cytokines and down-regulates type 1 cytokines. Thus, in normal pregnancy, there is a skew towards type 2 immunity. (29,30) This type of immune stimulation also activates a subset of memory T-cells that has the potential for long-term survival. In the case of re-exposure to antigens in a second pregnancy (from the same father), memory T-cells undergo rapid expansion and mediate more robust T-effector functions. (29,30) Thus, multiparous women will more readily respond to a second fetal allograft with another type 2 immune reaction or from the opposite perspective: the skewed type 2 immune response is less pronounced in nulliparous women. The finding that GHT occurred more frequently in nulliparous women compared to multiparous women is consistent with previous reports, and likely results from difference in immune stimulation at conception. (30) Furthermore, class 3 multiparous women with the highest prevalence of elevated TPO-Abs, had a four-fold higher incidence of GHT compared to the reference group 1. In class 3 nulliparous women, in whom the prevalence of elevated

TPO-Abs dropped from 21% at 12 weeks to 7% at 36 weeks, it is notable that no cases of GHT were reported, whereas in class 2 women with the highest levels of TPO-Abs at 12 and 36 weeks, 15.4% developed GHT. These differences in the prevalence of GHT between the individual classes of nulliparous and multiparous women correlate with the concentration of TPO-Abs and also re-emphasize the differences in type 2 immune responses that occur during pregnancy in nulliparous and multiparous individuals. (31)

Strengths and limitations

The major strengths of this study are (i) its prospective nature, (ii) application of LCGA on thyroid status (TSH + fT4) assessed in each trimester of individual women to allow identification of individual trajectories of changes in thyroid function during pregnancy, and (iii) use of a large cohort representative of Caucasian pregnant women living in an iodine-sufficient region. Although the classification into three longitudinal trajectories met all the criteria for adequate LCGA (including the sub-analyses), classes 2 and 3 were rather small in absolute numbers of pregnant women. Furthermore, we only assessed hCG in a sub-set of the total sample. We found substantial differences of hCG levels and patterns between the different trajectories but they were not significantly different due to low power. Therefore, we were unable to stratify further for parity (GHT is predominantly found in primiparous women) or fetal sex (hCG related induction of T suppressor cells only occurs in lymphocytes from females). (27) Incorporating hCG levels in LCGA would, however, require a much larger sample of about 2000-3000 women and could further contribute to explain differences of thyroid function patterns and obstetric complications in different longitudinal trajectories. Iron status was also not assessed, and it has been demonstrated that low iron status is associated with a higher prevalence of elevated TPO-Ab titres (and hence a higher rate of increased TSH levels) (32, 33). Thus, it is possible that differences in iron status could account for differences in TPO-Ab and hence TSH patterns between the three identified

classes of women. Finally, Tg-Abs were not determined and so their relationships with different classes of women and possible effects on pregnancy outcome could not be investigated (34) and there is an ongoing discussion on the reliability of fT4 assessments during late gestation (2).

Implications

In 5-8% of apparently healthy pregnant women, thyroid function during gestation does not follow the classical trajectory of slightly increasing TSH and decreasing fT4 (class 1), but follows trajectory 3 (decreasing TSH) or trajectory 2 (pronounced increase of TSH) associated with thyroid autoimmunity and hypothyroidism. It might be that pregnancy outcomes correlate better with the three identified longitudinal trajectories of thyroid function than with thyroid function tests obtained from cross-sectional studies. The same has recently been suggested for correlations with infant neurodevelopment. (35) Such possibilities require further investigation with special focus on the issue of screening of thyroid function.

Although the LCGA according to parity identified differences between nulliparous and multiparous women, the sub groups were small and larger more detailed studies will be required to investigate the possible interacting roles of iodine intake, ethnicity and iron status on thyroid trajectory, antibody status, and pregnancy outcome. The attenuated immune response in subsequent pregnancies should also be explored in relation to thyroid autoimmunity in more detail. The adaptive response has recently been proposed as the immunological basis for partner-specific protection against complications in subsequent pregnancies compared with the first pregnancy. (30) One could postulate this phenomenon to be related to the lower prevalence of GHT in multiparous women and to be a possible explanation for the observed association between (subclinical) hypothyroidism and GHT. (2) Furthermore, in normal pregnancy it has become clear that microvesicles of the syncytiotrophoblast enter the peripheral maternal circulation, a process called: microchimerism (the presence of small amounts of foreign particles or DNA detectable in a

genetically distinct individual) (36). Microchimerism is thought to play a role in recurrent abortion and also to explain differences of pregnancy related immune response between primi- and multiparous women (37). In addition, it has been suggested that several pregnancy complications (including GHT, fetal growth restriction and PTB) show a gender-specific pattern. In a recent study of the Generation-R project it was demonstrated that male sex was associated with poor maternal vascular adaptation to pregnancy. (38) A very recent meta-analysis including over 3 million participants showed that male sex was associated with increased risk of GHT in the non-Asian population. (39) Future research looking at difference of pregnancy complications between thyroid function trajectories adjusted to parity should thus take gender into account.

Finally, identification of a longitudinal thyroid function trajectory that a particular pregnant woman follows can only be recognised at the end of pregnancy. The future challenge will be to determine whether it is possible to identify early pregnancy risk factors that predict women with specific longitudinal thyroid function trajectories that are associated with adverse outcomes. A possible candidate could be pulsed Doppler ultrasound of the umbilical and uterine arteries to assess flow velocity forms as a reflection of placental vascular resistance. Thyroid hormone receptors are expressed in the trophoblast cells and thyroid hormone is important for appropriate placentation. (40) Higher vascular resistance has been related to GHT, SGA and PTB. A recent study of the Generation-R project showed fT4 in the higher range – but not TSH – to be associated with higher vascular resistance. (41) The authors suggested this discrepancy to be hCG related. Another approach could be LCGA in which possible different trajectories of the combination of fT4, TSH and hCG function should be compared with pulsed Doppler ultrasound measures. A limitation of the Doppler ultrasound method is that it is only reliable from 20 weeks gestation making it less applicable to detect at early gestation (10-12 weeks) women at risk for longitudinal thyroid trajectories associated with poor obstetric outcome.

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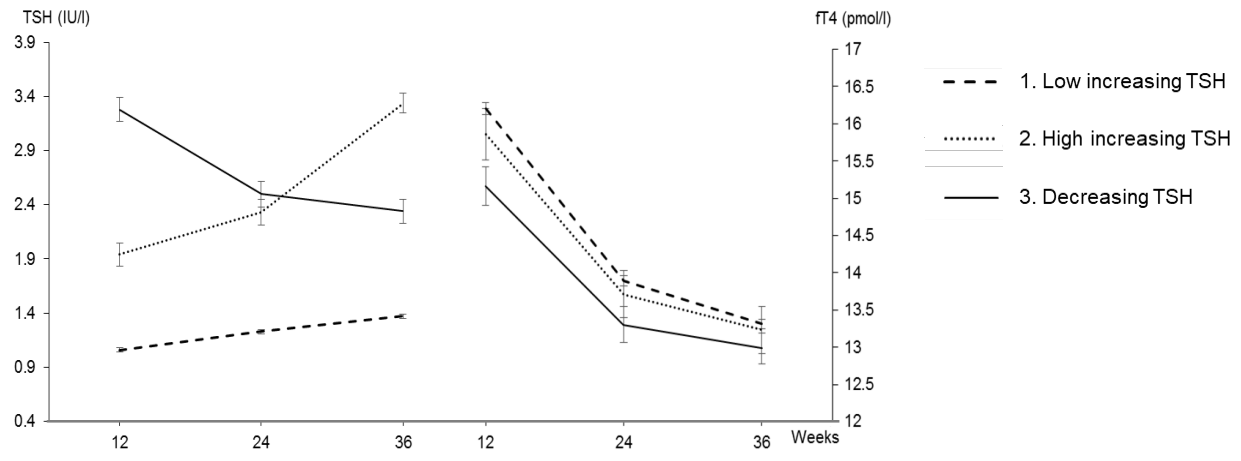
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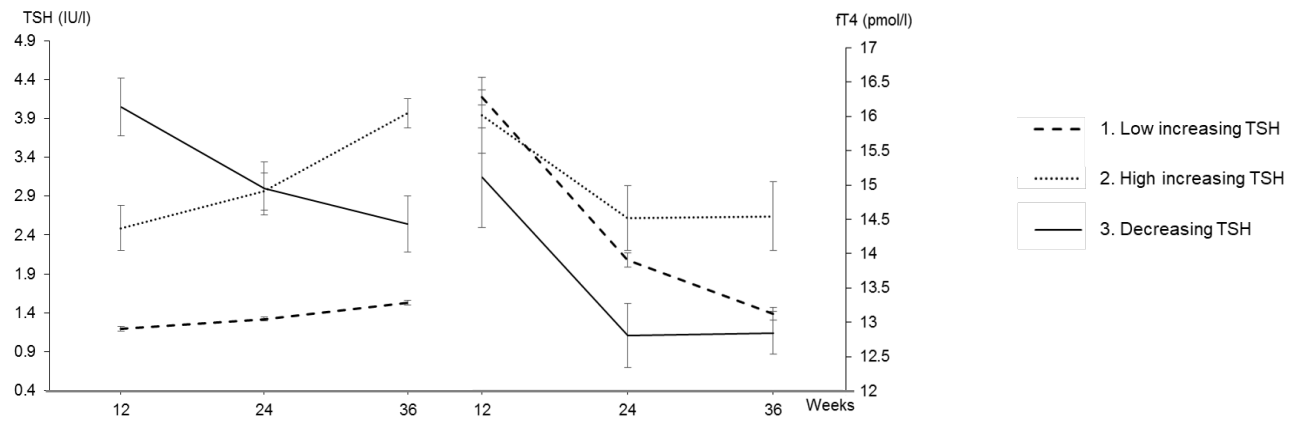
Figure 1. Three trajectories of TSH and fT4 change from first to third trimester of pregnancy.

Note. For ease of interpretation, the figure displays raw means for TSH and fT4 levels, instead of estimated means derived from the LCGAs. Estimated means from the LCGAs can be found in Supplementary Table S1. Women in class 1 (n = 1019, 92.4%) are characterized by the lowest initial TSH levels that showed a gradual increase towards the end of gestation and the highest fT4 levels at baseline that gradually decreased further at 36 weeks gestation. Women in class 2 (n = 30, 2.8%) had intermediate TSH levels at baseline that increased towards the end of gestation. Women in class 3 (n = 51, 4.6%) are characterized by highest initial TSH levels that decreased during pregnancy and lowest fT4 levels at baseline that gradually further decreased to the lowest levels at 36 weeks gestation.

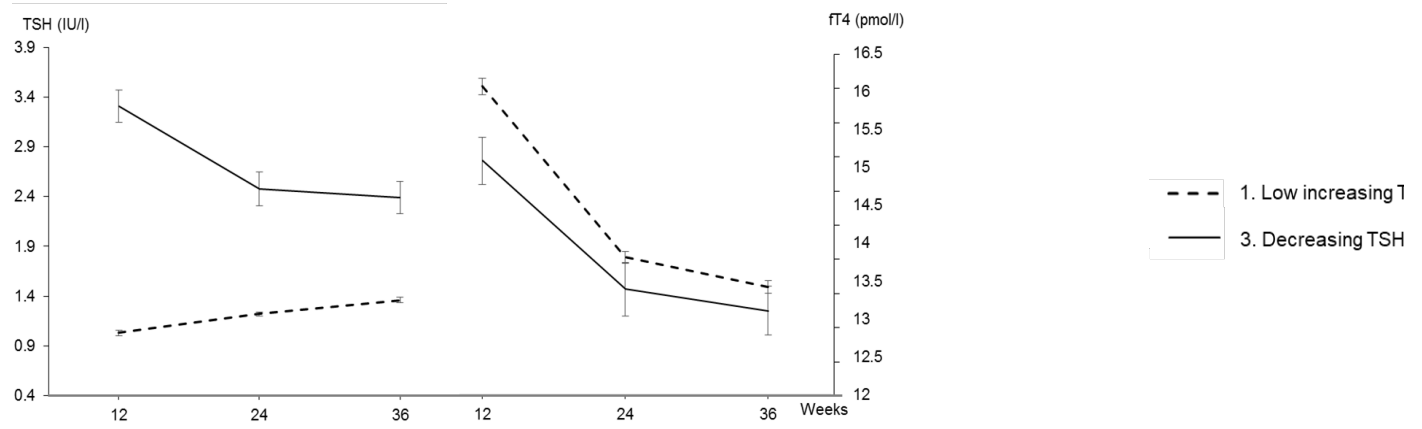
Figure 2. Trajectories of TSH and fT4 change from first to third trimester of pregnancy separate for nulliparous (a) and multiparous women (b).

Note. For ease of interpretation, the figure displays raw means for TSH and fT4 levels, instead of estimated means derived from the LCGAs. Estimated means from the LCGAs can be found in Supplementary Table S2 and S3.





(a)



(b)

Table 1 Baseline characteristics of a sample of 1100 women at 12 weeks gestation.

	Mean (SD)	N (%)
Age	30.5 (3.6)	
Life style habits		
currently smoking		143 (13)
alcohol use in pregnancy		123 (10)
BMI	25.6 (4.7)	
Obstetric features		
Parity		
Primiparity		513 (47)
Multiparity		587 (53)
1		464 (79)
2		106 (18)
>2		17 (3)
Spontaneous miscarriage earlier in life		178 (16)
Obstetric complications		
Pre-term birth (< 37 weeks) (PTB)		55 (5)
Gestational hypertension (GHT)		61 (5.5)
Small for gestational age (SGA)*		77 (7)
Thyroid function		
Mean TSH (SD) mIU/l	1.32 (0.81)	
Median logTSH (range) mIU/l	0.041 (-2.09 - 0.90)	
Mean fT4 (SD) pmol/l	16.1 (3.5)	
Thyroid function parameters		
Parental history of thyroid dysfunction		87 (7.9)
TPO-Ab > 35		95 (8.6)

* SGA: birth weight < 10th percentile adjusted for term at birth, parity and sex

Table 2

Sub-groups of thyroid dysfunction according to the three sub-classes. Total N = 1100

	Class 3 n = 51		Class 2 n = 30		Class 1 n = 1019	
	N(%)	Mn (SD)	N(%)	Mn (SD)	N(%)	Mn (SD)
Thyroid function at 12 weeks						
Overt hypothyroidism	3 (5.8)		1 (3.3)		0	
Sub-clinical hypothyroidism	32 (62.7)		4 (13.3)		1 (0.1)	
Overt hyperthyroidism	0		0		11 (1.1)	
Sub-clinical hyperthyroidism	0		0		21 (2.1)	
Thyroid function at 24 weeks						
Overt hypothyroidism	0		0		1 (0.1)	
Sub-clinical hypothyroidism	17 (33.3)		12 (40)		1 (0.1)	
Overt hyperthyroidism	0		0		2 (0.2)	
Sub-clinical hyperthyroidism	0		0		27 (2.6)	
Thyroid function at 36 weeks						
Overt hypothyroidism	1 (1.9)		0		1 (0.1)	
Sub-clinical hypothyroidism	8 (15.4)		18 (56.3)		0	
Overt hyperthyroidism	0		0		5 (0.5)	
Sub-clinical hyperthyroidism	0		0		22 (2.1)	
Thyroid related parameters at 12 weeks						
Multiparity	26 (51)		9 (30)		552 (54)	
1	18 (69)		8 (89)		438 (79)	
2	7 (27)		1 (11)		98 (18)	
>2	1 (4)		0		16 (3)	
Spontaneous miscarriage	9 (17.6)		4 (13.3)		209 (20.5)	
Age		31.0 (3.6)		30.3 (3.9)		(30.5 3.5)
Smoking	5 (9.8)		4 (13.3)		120 (11.8)	
Alcohol intake	6 (11)		3 (10)		135 (13)	
Pre-pregnancy BMI		26.1 (4.9)		24.7 (3.6)		25.5 (4.5)
Parental history of thyroid dysfunction	11 (21.6)		2 (6.7)		74 (7.3)	
TPO-Ab						
Median TSH (mIU/l) values at:						
12 weeks	3.20		2.05		1.00	
24 weeks	2.30		2.45		1.20	
36 weeks	2.30		3.45		1.40	
Median fT4 values (pmol/l) at:						
12 weeks	14.8		15.85		16.0	
24 weeks	13.1		14.0		13.7	
36 weeks	13.0		13.5		13.0	
Women with TPO-Ab > 35 at:						
12 weeks	25 (49)		4 (13.3)		66 (6.4)	
24 weeks	22 (43)		3 (10)		53 (5.2)	
36 weeks	19 (37)		1 (3.3)		46 (4.5)	
TPO-Ab titers at:						
12 weeks						
median	25		9		9	
minimum	9		9		9	
maximum	3600		140		1900	
24 weeks						
median	16		9		9	
minimum	9		9		9	
maximum	2400		67		920	
36 weeks						
median	15		9		9	
minimum	9		9		9	
maximum	1549		53		580	
Obstetric complications						
Pre-term birth (< 37 weeks) (PTB)	3 (5.9)		2 (6.7)		50 (4.9)	
Gestational hypertension (GHT)	2 (3.9)		4 (13.3)		55 (5.4)	
Small for gestational age (SGA)*	6 (11.7)		5 (16.6)		66 (6.5)	

class 3: 'decreasing TSH'. class 2: 'high increasing TSH'; class 1: 'low-increasing TSH'.

reference range thyroid function assessed in 1026 TPO-Ab negative women: TSH 0.15 – 2.9 IU/l and fT4 11.9 – 20.8 pmol/l

bold characters represent statistically significant differences between groups.

* SGA: birth weight < 10th percentile at delivery, adjusted for term of gestation, parity and sex.

Table 3 hCG concentrations (IU/L) at 12 weeks gestation in a subset sample (N=568) according to three trajectories of thyroid function (TSH and fT4) throughout gestation.

	Class 3, n = 29	Class 2, N = 17	Class 1, n = 52
hCG level (IU/L)			
Mean (SD)	52.423 (19.418)	48.555 (18.796)	58.306 (30.060)
median	52.232	48.123	53.448
minimum	26.408	19.869	7.043
maximum	98.292	84.243	196.211
range	72.884	64.374	189.168

class 3: 'decreasing TSH'. class 2: 'high increasing TSH'; class 1: 'low-increasing TSH'

Table 4 Trajectories of thyroid dysfunction in nulli-parous (n = 513) and multi-parous (n = 587) women.

A. Nulliparous: N = 513		Class 3 n = 14	Class 2 n = 13	Class 1 n = 486
		N(%)	N(%)	N(%)
Thyroid function at 12 weeks				
Overt hypothyroidism		0	3 (23)	0
Sub-clinical hypothyroidism		3 (21)	9 (69)	7 (1.4)
Overt hyperthyroidism		0	0	2 (0.4)
Sub-clinical hyperthyroidism		0	0	7 (1.4)
Thyroid related parameters at 12 weeks				
Parental history of thyroid dysfunction		0	3 (23)	38 (7.8)
TPO-Ab				
Women with TPO-Ab > 35 at:				
12 weeks		3 (21)	7 (54)	35 (7.2)
24 weeks		2 (14)	6 (46)	34 (6.9)
36 weeks		1 (7)	4 (31)	30 (6.1)
Median TSH (mIU/l) values at:				
12 weeks	3.26		1.95	1.10
24 weeks	2.30		2.65	1.20
36 weeks	2.20		3.45	1.40
Median fT4 values (pmol/l) at:				
12 weeks	14.5		16.05	16.2
24 weeks	13.2		13.9	13.8
36 weeks	12.7		13.35	13.2
TPO-Ab titers at:				
12 weeks				
median		9	72	9
minimum		9	9	9
maximum		2100	3600	1900
24 weeks				
median		9	33	9
minimum		9	9	9
maximum		1600	2400	920
36 weeks				
median		9	16	9
minimum		9	9	9
maximum		1000	1549	350
Obstetric complications				
Pre-term birth (< 37 weeks) (PTB)		3 (21.4)	2 (15.4)	31 (6.3)
Gestational hypertension (GHT)		0	2 (15.4)	43 (2.4)
B. Multiparous: N = 587		Class 3 n = 34	-----	Class 1 n = 553
		N(%)		N(%)
Thyroid function at 12 weeks				
Overt hypothyroidism		1 (7)		0
Sub-clinical hypothyroidism		18 (53)		0
Overt hyperthyroidism		0		7 (1.3)
Sub-clinical hyperthyroidism		0		14 (2.5)
Thyroid related parameters at 12 weeks				
Parental history of thyroid dysfunction		6 (18)		40 (7)
Median TSH (mIU/l) values at:				
12 weeks	3.20			0.96
24 weeks	2.20			1.12
36 weeks	2.30			1.30
Median fT4 values (pmol/l) at:				
12 weeks	14.8			15.8
24 weeks	13.8			13.7
36 weeks	12.9			13.3
TPO-Ab				
Women with TPO-Ab > 35 at:				
12 weeks		14 (41)		36 (6.5)
24 weeks		2 (5.9)		34 (6.1)
36 weeks		1 (2.9)		30 (5.4)

TPO-Ab titers at:		
12 weeks		
median	16	9
minimum	9	9
maximum	1200	1300
24 weeks		
median	10	9
minimum	9	9
maximum	830	790
36 weeks		
median	9	9
minimum	9	9
maximum	470	580
Obstetric complications		
Pre-term birth (< 37 weeks) (PTB)	0	19 (3.4)
Gestational hypertension (GHT)	3 (8.8)	13 (2.4)

class 1: 'Decreasing TSH trajectory'. class 2: 'High increasing TSH trajectory'; class 3: 'Low increasing TSH trajectory'.
Reference range thyroid function assessed in 1026 TPO-Ab negative women: TSH 0.15 – 2.9 IU/l and fT4 11.9 – 20.8 pmol/l
bold characters represent statistically significant differences between groups.