

Establishment of Trimester-Specific Reference Intervals of Serum TSH & fT₄ in a Pregnant Indian Population at North Kolkata

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Received: 16 January 2013 / Accepted: 20 April 2013 / Published online: 15 May 2013
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Abstract Reference intervals (RIs) of serum thyroid stimulating hormone (TSH) and free thyroxine (fT₄) were determined in 402 healthy pregnant women by enzyme-linked immunosorbent assay (ELISA) technique after partitioning them into three trimesters. The reference population was chosen from a study population of 610 pregnant females by applying strict inclusion and exclusion criteria. The assays were done using proper quality control measures. RIs were calculated from the central 95 % of the distribution of TSH and fT₄ values located between the lower reference limit of 2.5 percentile and upper reference limit of 97.5 percentile value. 0.90 confidence intervals for the upper and lower reference limits were also determined. The reference intervals for TSH were 0.25–3.35 μIU/ml for the first trimester; 0.78–4.96 μIU/ml for the second trimester and 0.89–4.6 μIU/ml for the third trimester. Similarly, the reference intervals for fT₄ for first, second and third trimesters

were 0.64–2.0, 0.53–2.12 and 0.64–1.98 ng/dl respectively. The values thus obtained varied from those provided by the kit literature. In comparison to our derived reference intervals, the reference data from kit manufacturer under-diagnosed both subclinical hypo- and hyper-thyroidism within our pregnant reference population.

Keywords Reference interval · Reference range · Thyroid hormones · Trimester-specific · Pregnancy · ELISA

Introduction

Proper maternal thyroid function during pregnancy is vital for the health of the mother as well as the developing child. It also plays a critical role in the neonatal and child neurodevelopment [1]. Any thyroid disorder during gestation, whether hypo- or hyperthyroidism, may lead to varied obstetric complications with or without irreversible damage to the foetus [2, 3]. So, all pregnant women should ideally be screened with a valid biomarker of thyroid function having a distinct reference range. To ensure 100 % optimal maternal health it is also important to understand the maternal thyroid physiology during pregnancy. During gestation a number of hormonal and metabolic alterations occur, namely, rise in thyroxine binding globulins (TBG), surge of human chorionic gonadotropin (βHCG), altered iodide metabolism, haemodilution etc. At different trimesters a different set of factors predominate. These factors may act either synergistically or antagonistically to influence the thyroid hormone levels in blood [4]. So, the reference intervals (RI) for thyroid hormones in pregnant population naturally may not be the same as that

Electronic supplementary material The online version of this article (doi:10.1007/s12291-013-0332-1) contains supplementary material, which is available to authorized users.

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provided by the kit literature. This may lead to improper assessment of thyroid function during pregnancy.

A reference interval, according to IS015189, is the interval between, and including, 2 reference limits. Most frequently the central 95 % of the distribution located between the 2.5 and 97.5 percentiles are used for determining RI of a parameter [5–7]. For a valid comparison of an observed value with the reference, the subject must sufficiently resemble the reference individual in all respects [8]. As the physiology of thyroid hormones in a pregnant woman differs from one trimester to the other, trimester-specific reference intervals provide the best practical framework for clinical decision making regarding thyroid functions in pregnancy. This also ensures the adequacy of thyroid hormones throughout pregnancy [9].

There are reports of a few studies done worldwide on this subject. But these results cannot be extrapolated due to differences in ethnicity, iodine intake status, method of immunometric assay and statistical methodology used to derive the RIs in individual studies. Thus, a population based, trimester-specific RI is essential in everyday clinical practice for the correct interpretation of thyroid hormone values and accurate classification of thyroid disorders in pregnancy.

With this idea, the purpose of our study was to determine trimester-specific reference intervals of serum thyroid stimulating hormone (TSH) and free thyroxine (fT_4) in pregnant women of Indian origin at North Kolkata.

Materials and Methods

This study was conducted in the Department of Biochemistry in collaboration with the Antenatal Clinic of Department of Gynaecology and Obstetrics, R.G.Kar Medical College and Hospital, Kolkata from February 2012 to July 2012. It is a cross-sectional, descriptive and observational study in the reference population of pregnant women. Approval from the institutional Ethical Committee was obtained prior to undertaking the study.

Data was collected on the first and third Thursday of each month for a period of 6 months. 610 healthy pregnant women with history of consumption of iodised salt and having uncomplicated single intrauterine gestation were consecutively recruited. The gestational age was determined by last menstrual period (LMP) and ultrasonography report. Participants were interviewed, detailed history was taken and the case record form was filled. After an initial general survey, systemic examination was done. Also the records of their regular antenatal check up were noted. The women were properly counselled and provided their written informed consent.

From the study population the reference sample group was designed by applying the following exclusion criteria:-

1. History of thyroid dysfunction (goitre, hyper or hypothyroidism, thyroid carcinoma).
 2. Signs and symptoms of thyroid disorder like neck swelling, exophthalmos, hand tremors, tachycardia, palpitations cold sensitivity etc.
 3. Past or present history of intake of anti-thyroid drugs.
 4. Family history of thyroid illness.
 5. Women suffering from chronic medical disorder like hypertension, diabetes mellitus, asthma, inflammatory bowel disease, tumours, renal disease and other autoimmune diseases.
 6. Past history of spontaneous abortion.
 7. History of multiple or complicated pregnancy (hyperemesis, gestational diabetes, hypertension, perinatal infection or stillbirths).
 8. Regular use of any medication other than iron and folate.
 9. History of blood borne diseases like HIV, hepatitis B or C, syphilis.
- 8 ml of fasting venous blood was collected from each participant, serum separated and stored at $-20\text{ }^\circ\text{C}$. Analysis for fasting plasma glucose, serum liver function tests, serum renal function tests and serum lipid profile were done. Also serum antithyroperoxidase antibody (Anti-TPO) was measured using commercially available Enzyme-linked immunosorbent assay (ELISA) kits. On the basis of biochemical analysis, some population of pregnant women were excluded:
10. Elevated serum Anti-TPO ($>40\text{ IU/ml}$ according to kit literature) [10].
 11. Fasting plasma glucose $>140\text{ mg/dl}$ on 2 occasions.
 12. Impaired renal function tests, liver function tests and lipid profile.

The remaining study population (402) constituted the reference sample group. The reference sample group was then partitioned into 3 trimesters of pregnancy according to gestational age -First trimester: <13 weeks (125), Second: 13–27 weeks (151), and Third: >28 weeks (126).

Laboratory Hormonal Analysis

TSH, fT_4 and Anti-TPO antibody assays were done on commercially available ELISA kit namely Accubind (Monobind Inc, USA). TSH was estimated by Immunoenzymometric method type 3 [11, 12] while fT_4 with competitive enzyme immunoassay, analog method [13–15] sequential ELISA method was used to estimate anti-TPO antibody [16]. The analytical sensitivity for TSH and fT_4 assays were $0.10\text{ }\mu\text{IU/ml}$ and 0.15 ng/dl respectively. The

total and intra-assay precision values for TSH assays respectively were 8.2 and 5.6 % (for low serum range); 7.65 and 4.88 % (for medium serum range) and 7.1 and 4.3 % (for high serum range).

Similarly, for fT_4 these values were 10.98 and 9.81 % (for low serum range); 6.01 and 4.26 % (for medium serum range) and 7.3 and 3.25 % (for high serum range).

Statistical Analysis

All data were entered into MS Excel and analysed using SPSS 17. The nature of distribution of TSH and fT_4 values of each trimester were examined by inspecting histograms. The Anderson–Darling’s test as recommended by International Federation of Clinical Chemistry (I.F.C.C) was applied to evaluate the goodness-of-fit to Gaussian distribution [17, 18]. Extreme (highly deviating) values were identified using box plots. These identified probable outliers were tested by the two stage method of Horn et al. [19]. Where conversion of data to almost Gaussian distribution was not possible, Dixon’s Range test was applied to confirm outliers [7]. The confirmed outliers, if present, are eliminated from the reference sample group.

For data which were normally distributed parametric estimation was done: mean (x_m), standard deviation (s_x) and median of TSH and fT_4 were determined separately for each trimester. Reference intervals were estimated by determining the 2.5 and 97.5 percentile calculated as $x_m \pm 1.96 * s_x$. 0.90 Confidence Intervals (CI) were calculated by the formula: Percentile $\pm 2.81 * s_x/\sqrt{n}$. [5–7].

For data which were not normally distributed, transformation to Gaussianity was done either logarithmically or by square-root transformation and then the mean (y_m) and standard deviation (s_y) of transformed data were computed. RIs and 0.90 CI were estimated using previous formula. These values were reconverted to original data scale by Inverse ln or Inverse square root function [5, 7].

For data which could not be transformed by either method i.e., ln or square-root function; a nonparametric rank-based method (recommended by I.F.C.C) was used [20].

The Mann–Whitney U test was used to compare the differences between the first and second trimesters values and also the second and third trimesters values, for a level of significance $p < 0.05$.

Results

The study population comprised of 610 pregnant women out of which 195 (31.96 %) were in the first trimester, 205 (33.6 %) belonged to the second and 210 (34.43 %) in the third trimester. The age of the mothers varied from 16 to

37 years. After implementation of the exclusion criteria, a total of 208 women were excluded from the study, resulting to the final reference sample group of 402 women: first trimester—125 (31 %); second trimester—151 (37.4 %) and third trimester—126 (31.4 %). This final population was used to determine the reference limits and 0.90 confidence intervals of TSH and fT_4 for the three trimesters. The measures of central tendency, type of distribution and methodology of RI determination are given in Table 1. The RI of TSH as obtained in our study are 0.25–3.35 μ IU/ml for the first trimester; 0.78–4.96 μ IU/ml for the second trimester and 0.89–4.6 μ IU/ml for the third trimester (Table 2). Similarly, the RI of fT_4 for first, second and third trimesters were 0.64–2.0 ng/dl, 0.53–2.12 and 0.64–1.98 ng/dl respectively (Table 3).

Box plots of TSH and fT_4 values for the three trimesters (after outlier assessment), showing the sample minimum, sample maximum, 25th, 50th and 75th percentile along with the extreme values are depicted in Figs. 1 and 2 respectively. Significant difference was observed between first and second trimesters values of both TSH and fT_4 .

The RIs of TSH and fT_4 proposed by the manufacturer were 0.39–6.16 μ IU/ml and 0.8–2.0 ng/dl. These values when used to interpret thyroid function tests in our pregnant reference sample group under-diagnosed both subclinical hypothyroidism and subclinical hyperthyroidism (Table 4), when compared to our trimester-wise RIs. No case of either clinical hypo- or hyperthyroidism was found within our reference sample group.

Discussion

Recent studies have shown that untreated hypothyroidism and subclinical hypothyroidism during pregnancy increases the incidence of maternal anaemia, preeclampsia, postpartum haemorrhage and abruption placenta [21]. Related fetal complications are spontaneous abortion, low birth weight, prematurity, congenital malformation and lower intelligence quotient (IQ) in children. There is also a greater prevalence of subclinical hypothyroidism in women with delivery before 32 weeks [22]. Hyperthyroidism may lead to preeclampsia, still birth, preterm delivery, intrauterine fetal death and low birth weight [23].

Hence, thyroid function assessment during pregnancy is very vital for the wellbeing of both the mother and the foetus. This study is the first of its kind in eastern India and the second in our country. Marwaha et al. determined the reference ranges of fT_4 , free triiodothyronine (fT_3), and TSH in pregnant women in a primary care set up at Delhi by Electrochemiluminescence technique, and used the 5th and 95th percentile to define the reference ranges in the disease-free subjects [24].

Table 1 Descriptive statistics of reference population partitioned into three trimesters

Trimester	Parameter	Distribution	Mean \pm SD	Median	2.5 Percentile	97.5 Percentile
First trimester (N = 125)	TSH (μ IU/ml)	Normal (Parametric estimation)	1.81 \pm 0.79	1.8	0.25	3.35
	fT ₄ (ng/dl)	Not normal (Nonparametric rank based estimation)	1.36 \pm 0.35	1.4	0.64	2.0
Second trimester (N = 151)	TSH (μ IU/ml)	Not normal (Nonparametric rank based estimation)	2.22 \pm 1.10	1.84	0.78	4.96
	fT ₄ (ng/dl)	Not normal (Nonparametric rank based estimation)	1.27 \pm 0.38	1.28	0.53	2.02
Third trimester (N = 126)	TSH (μ IU/ml)	Not normal (Ln transformed parametric estimation)	2.2 \pm 0.88	2.07	0.9	4.6
	fT ₄ (ng/dl)	Normal (Parametric estimation)	1.31 \pm 0.34	1.33	0.64	1.99

Table 2 Trimester-wise reference data for TSH according to our study

Trimester	Reference interval of TSH in μ IU/ml	0.9 CI of lower reference limit of TSH in μ IU/ml	0.9 CI of upper reference limit of TSH in μ IU/ml
First	0.25–3.35	0.06–0.44	3.15–3.55
Second	0.78–4.96	0.64–0.87	4.7–5.07
Third	0.9–4.6	0.8–0.98	4.15–5.12

Table 3 Trimester-wise reference data for fT₄ according to our study

Trimester	Reference interval of fT ₄ in ng/dl	0.9 CI of lower reference limit of fT ₄ in ng/dl	0.9 CI of upper reference limit of fT ₄ in ng/dl
First	0.64–2.0	0.58–0.82	1.98–2.04
Second	0.53–2.02	0.44–0.61	1.93–2.1
Third	0.64–1.99	0.55–0.73	1.9–2.07

Our study provides the reference intervals of TSH and fT₄ in the first, second and third trimesters (Tables 2, 3). On comparing reference data of one trimester to the other by the Mann–Whitney U test, it was found that the first trimester TSH and fT₄ values varied significantly from those of the second but the change was not significant from second to the third. This justifies the partitioning of the TSH and fT₄ values into three trimesters while determining the reference intervals.

The RIs of TSH and fT₄ as supplied by the kit manufacturer was 0.39–6.16 μ IU/ml and 0.8–2.0 ng/dl. Before our study, these values were considered for the determination of thyroid disorders in pregnancy in our laboratory. On applying these RIs to diagnose thyroid disorders in our reference sample group, an appreciable number of pregnant women escaped the diagnosis of subclinical hypothyroidism using the manufacturer's RI; 3.2 % in the first, 2.6 % in second and 1.58 % in the third trimesters. Also, 1.98 and 3.96 % of pregnant women identified as subclinical hyperthyroidism (by our trimester-specific RIs) in second and third trimester respectively, failed to be diagnosed (Table 4). The fraction of population misdiagnosed would be much greater and would also include the spectrum of clinical hypo- or hyper-thyroidism, if these RIs were applied to the general population; acknowledging the fact that only the reference sample group has been taken into consideration here. This variation is because the RI

provided by the reagent manufacturer has been determined on a non-pregnant population and cannot be applied for the interpretation of thyroid function tests of a pregnant woman.

During pregnancy the best laboratory assessment of thyroid function to diagnose hypothyroidism is a TSH combined with fT₄ and for hyperthyroidism is fT₄, fT₃ combined with TSH, as the total hormone estimations are flawed due to the high TBG concentration during this period [25]. In our study fT₄ and TSH was estimated. The incidence of hyperthyroidism in pregnancy is quite uncommon (0.2 %) in contrast to hypothyroidism and most hyperthyroid cases are determined by fT₄ and TSH values [26]. Only in some hyperthyroid cases fT₃ is raised. So, fT₃ estimation was excluded from our study. According to I.F.C.C. expert panel on theory of reference values, the well-defined healthy population for reference interval determination must be based on specific exclusion criteria [27, 28]. So, we selected our reference population using a stringent criteria. To begin with, all mothers with any kind of thyroid abnormality were excluded since women positive for thyroid autoantibodies typically have higher TSH values and therefore affect and skew the upper reference limit [29, 30]. Of the antibodies, only anti-thyroperoxidase antibody was estimated. Anti-thyroglobulin antibody has little diagnostic value over TPO antibody measurement for autoimmune thyroid disease as it has been found in individuals without apparent thyroid

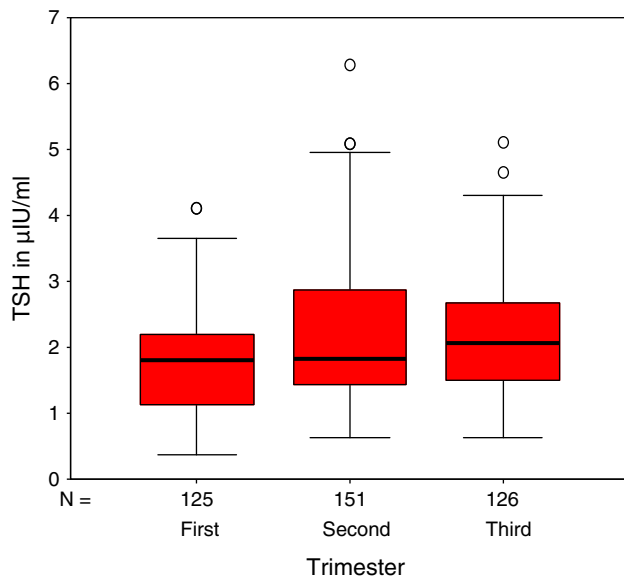


Fig. 1 Box plots for TSH values of three trimesters after outlier removal. Comparing between first and second trimester values: * $p = 0.015$ and second and third trimester values: $p = 0.417$

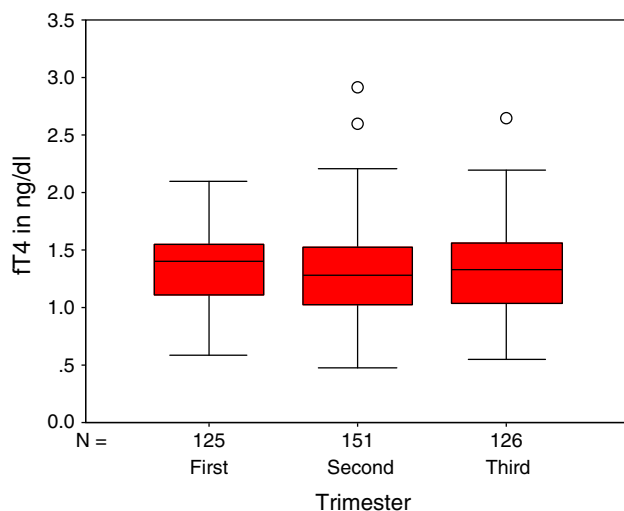


Fig. 2 Box plots for fT_4 values of three trimesters after outlier removal. Comparing between first and second trimester values: * $p = 0.029$ and second and third trimester values: $p = 0.202$

disease. Also, women with twin pregnancies or with hyperemesis gravidarum were also removed from the reference population due to their potential for low TSH values (higher serum β HCG) and interference with the lower limit of TSH reference range [31]. We also excluded mothers with positive history of autoimmune diseases like diabetes mellitus type1, based on the association between autoimmunity and thyroid dysfunction [32]. Exclusion of women with clinical as well as laboratory signs of medical disorders was also done. The final strictly defined reference population of

402 women was considered adequate for the estimation of reference intervals fulfilling the sample size requirements of National Committee for Clinical Laboratory Standards (NCCLS) [28].

Our laboratory estimations were done by validated, precise and accurate methods. The assays were done under proper quality control measures. The statistical strengths included examination of distributions, respective application of parametric or non-parametric methods and assessment of outliers.

Pregnancy is a very important stage in the life of a woman. Throughout the period of 280 days the mother harbours the growing child in her uterus. A lot of alterations occur in various biochemical parameters (within physiological limits) during this period. The normal development of the foetus and a smooth obstetric journey depends on these factors. Hence, along with routine antenatal checkups, an ultrasound imaging of the foetus and an array of maternal blood investigations are done to ensure the wellbeing of the child. Fasting plasma glucose, complete haemogram, platelet count, liver and renal function tests are commonly requested biochemical investigations. In the recent years many studies advocate the use of routine thyroid function tests for screening of thyroid disease in pregnancy [33, 34].

During gestation several hormonal changes and metabolic alterations occur resulting in complex effects on thyroid function. β HCG secretion from the placenta is enhanced during the first trimester. This thyrotropic effect of rising β HCG exerts negative feedback on TSH secretion [35]. As the pregnancy progresses from first to next, there is gradual rise of TBG (due to oestrogen stimulated hepatic synthesis). This results in an increase in total T_4 and total T_3 with simultaneous fall of free hormone concentration which causes feedback enhancement of TSH release from the pituitary [36, 37]. Additionally, due to hyperdynamic circulation during pregnancy, the iodine clearance by the kidney rises. This stimulates the thyroid gland to increase iodine uptake via increased TSH release from pituitary. With the onset of second trimester β HCG surge diminishes while the effect of rising TBG continues till the second and third trimester. The third factor, which intervenes later in gestation, is related to modifications in the peripheral metabolism of the thyroid hormones, particularly at the placenta level. All these factors lead to physiological adaptation in healthy women who are iodine-sufficient. Inadequate iodine intake and pathological conditions like autoimmune diseases alters this physiological balance which is reflected in the serum levels of thyroid hormones [38].

A number of cross-sectional studies have reported trimester-specific reference ranges for fT_4 and TSH among pregnant women [39–41]. But these reported ranges vary and these variations are due to differences in method of

Table 4 Thyroid clinical entities in the reference sample group using trimester-specific RIs derived from this study and that obtained from the assay kit literature

Trimester	Source of reference intervals	Subclinical hypothyroidism n (%)	Subclinical hyperthyroidism n (%)
First	Trimester-specific	4 (3.2)	0
	Kit literature	0	0
Second	Trimester-specific	4 (2.6)	3 (1.98)
	Kit literature	0	0
Third	Trimester-specific	2 (1.58)	5 (3.96)
	Kit literature	0	0

The different clinical entities are defined as

Normal thyroid function: TSH between reference limits and fT_4 between reference limits

Subclinical hypo-thyroidism, TSH over the upper limit of reference limits and fT_4 between reference limits

Subclinical hyper-thyroidism, TSH under the lower limit of reference limits and fT_4 between reference limits

Clinical hypo-thyroidism, TSH over the lower limit of reference limits and fT_4 under the lower limit of reference limits

Clinical hyper-thyroidism, TSH under the lower limit of reference limits and fT_4 over the lower limit of reference limits

immunometric assay, ethnic disparities [42] along with variations in iodine nutrition characteristics, inclusion criteria for determination of reference population, sample size and assessment of outliers.

To conclude, it is important that each laboratory determines its own population and method-based reference interval for diagnosing thyroid disorders in pregnancy. Moreover, these RI must preferentially be trimester-specific. To boost this concept we have determined our own laboratory-specific trimester-wise RI for TSH and fT_4 and intend to use them for screening of pregnant mothers for thyroid disorders, at any stage of gestation.

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