"ESTABLISHMENT OF REFERENCE RANGE OF SERUM THYROID STIMULATING HORMONE IN FIRST AND SECOND TRIMESTER OF PREGNANCY IN A TERTIARY CARE HOSPITAL – A CROSS SECTIONAL STUDY"

REG.NO. BC0111002

Dissertation

Submitted to the

KLE University, Belgaum, Karnataka.

In partial fulfilment
of the requirements for the degree of

M. D. (DOCTOR OF MEDICINE)
IN
BIOCHEMISTRY

DEPARTMENT OF BIOCHEMISTRY,

J. N. MEDICAL COLLEGE, KLE UNIVERSITY,

BELGAUM – 590 010

APRIL - 2014

KLE UNIVERSITY, BELGAUM,

KARNATAKA.

Endorsement by the Head Of Department, Principal/ Head of the institution

This is to certify that the dissertation entitled **"ESTABLISHMENT OF REFERENCE RANGE OF SERUM THYROID STIMULATING HORMONE IN FIRST AND SECOND TRIMESTER OF PREGNANCY IN A TERTIARY CARE HOSPITAL – A CROSS SECTIONAL STUDY"** is a bonafide research work done by **REG.NO. BC0111002.**

Dr. ANURADHA B. PATIL$_{MD.}$
Professor and Head, Department
of Biochemistry,
J. N. Medical College, KLE
University, Belgaum – 590010

Date:
Place: Belgaum

Dr. A. S. GODHI, **MS, FICS**
Principal,
J. N. Medical College,
Belgaum – 590010.
Karnataka, India.

Date:
Place: Belgaum

ABBREVIATIONS

µg	-micro gram (10^{-6} gram)
µL	-micro liter (10^{-6} liter)
^{o}C	-degree celcius
+ve	-positive
-ve	-neagative
β-hCG	-β-subunit of human chorionic gonadotrophin
AB+ve	-antibody-positive
Anti-TG /TgAb	-Anti-thyroglobulin antibody
Anti-TPO/ TPO-Ab	-Anti-thyroid peroxidise antibody
ATA	-anti-thyroid antibodies
BSA	-bovine serum albumin
c^2	-chi-square
CI	-confidence interval
EDTA	-ethelyn di-amine tetra-acetic acid
ELISA	-enzyme-linked immmunosorbent assay
ESD	-extreme studentized deviate
Fig.	-figure
FITC	-fluorescent isothiocyanate
fT3	-free tri-iodothyronin
fT_4	-free thyroxin
GTD	-gestational trophoblastic disease
GTT	-gestational thyrotoxicosis
hCG	-Human chorionic gonadotrophin

HG	-hyperemesis gravidarum
H_2O_2	-hydrogen peroxide
IQ	-Intelligent quotient
IU/L	-international unit/ litre
KI	-potassium iodide
LMP	-last menstrual period
L-T_4	-levo-thyroxin
mAB	-monoclonal antibody
mcIU/ml or µIU/ml	-micro-international unit per millilitre
MEIA	-micro-particle enzyme immunoassay
min	-minute
mIU/L	-milli-international unit per litre
ml	-millilitre
N	-number
N-linked	-nitrogen linked
NACB	-National Academy of Clinical Biochemistry
OH	-overt hypothyroidism
O-linked	-oxygen-linked
OPD	-Outpatient department
OR	-odds ratio
p	-level of significance
pmol/L	-pico-mole per litre
ppm	-parts per million
PPV	-positive predictive value
PRW	-pregnant woman

RNI	-recommended nutrient intake
RLU	-relative light unit
SCH	-sub-clinical hypothyroidism
SD	-standard deviation
SFD	-small-for date
Sl. no.	-serial number
T_3	-tri-iodothyronine
T_4	-thyroxin
TBG	-thyroxin-binding globulin
TFT	-Thyroid function test
TPO	-Thyroid peroxidase
TRAb	-Anti-thyroid hormone receptor antibody
TRH	-Thyrotropin-realising hormone
TSH	-Thyroid stimulating hormone
USG/ US	-Ultrasonography
USI	-Universal salt ionization
Vs./ vs.	-versus
wks	-weeks
yr/ yrs.	-year/ years
Z-score	-standard score

ABSTRACT

Objective of the study:

- Establishment of 'gestational age-specific' and 'method-specific' reference intervals for 'Thyroid stimulating hormone' in first and second trimester spontaneous, healthy, singleton pregnancy.
- To examine the implication of this reference intervals for the interpretation of 'thyroid function tests' in pregnant women.

Methodology:

We had enrolled 147 pregnant women attending ante-natal OPD of KLES Dr. Prabhakar Kore Hospital and MRC in their 8-14 gestational weeks. Pregnant women having history of thyroid disease or medication/supplementation or abortion in previous pregnancy were excluded. From rest 117 pregnant women we collected serum sample and tested for serum TSH and anti-TPO. 17 pregnant women were excluded as they are anti-TPO +ve (thyroid autoimmunity). Reference range was calculated based on the result from 100 samples. Among them those who come back after 6-8 week of their 1st sample collection (in their 15th-21st gestational week) we have collected 2nd trimester sample to calculate 2nd trimester reference range and compared 1st and 2nd trimester TSH value in same patients.

Results:

Reference range was calculated by parametric method as sample size was less than 120. So, we have made TSH values normalized after doing square-root transformation. The calculated reference range by parametric method after doing back-transformation for 100 pregnant women for late 1st trimester is 0.01-3.65

mcIU/ml. We got 2nd trimester TSH values normalized by doing box-cox transformation. Calculated reference range for 2nd trimester after back-transformation by parametric method is 0.11-4.04 mcIU/ml. The difference between 1st and 2nd trimester values is statistically significant (p=0.0008)

Conclusion:

22% of pregnant women in their 1st trimester would have been misclassified as hyperthyroid when they were actually euthyroid and 4% of them would have missed their diagnosis of hypothyroidism if we had applied the normal non-pregnant women reference range of TSH (0.55-4.78 mcIU/ml) to interpret thyroid function test in them. So, our study justified the need of establishment of separate method-specific reference range for pregnant women, (especially at late 1st trimester). Though we couldn't comment the same about early second trimester, but for that further study are needed involving more samples. But we can tell the TSH values are significantly different in late 1st and early 2nd trimester. The advantages of our study are consecutive collection of 2nd trimester sample from same subjects, exclusion of overt thyroid dysfunction and anti-TPO +ve subjects and immediate analysis of samples. The disadvantages of our study are less sample size and problem in follow-up.

Key-words: TSH, trimester-specific, reference range, chemiluminescent, pregnant women, Anti-TPO antibody

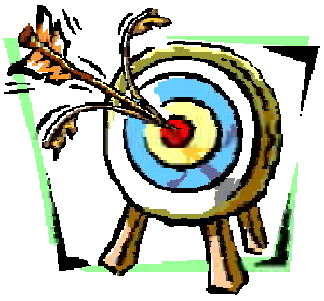
TABLE OF CONTENTS

LIST OF FIGURES

LIST OF GRAPHS

LIST OF TABLES

Introduction



Objectives

Review of Literature

Basic Sciences

Methodology

Results

Discussion

Conclusion

Summary

Bibliography

Annexure-I

Annexure-II

Annexure-III

Annexure-IV

INTRODUCTION

Thyroid stimulating hormone (thyrotropin/TSH) is secreted by thyrotrope cells of the anterior pituitary which has significant role in control of thyroid and if we have to choose a single parameter to assess thyroid function TSH is the best physiological marker. Though it has diurnal variation, assessing its circulating level single estimation of TSH is adequate as it has relatively long plasma half-life (50 min).[1] Hypothyroidism, though a common clinical entity, in most cases hypothyroidism is difficult to diagnose clinically as the presenting symptoms are very much non-specific. Clinical or overt hypothyroidism is diagnosed when an abnormally high serum TSH level is accompanied by an abnormally low thyroxin level. Subclinical hypothyroidism is defined by an elevated serum TSH level with normal serum thyroxin. Severe hypothyroidism is often associated with infertility and increased miscarriage rate, so we can assume that it is an uncommon entity during pregnancy, though same can't be told about mild hypothyroidism.[1,2]

` Serum TSH measurement is the single most reliable test to diagnose all common form of hypothyroidism and hyperthyroidism. Serum TSH is the only parameter whose concentration is raised in both overt and mild hyperthyroidism.[3]The overall incidence of hypothyroidism in pregnancy is 2.5%. Overt thyroid deficiency was found in 1.3/1000 and subclinical disease in 23/1000. Overt hypothyroidism is associated with excessive adverse perinatal outcome, i.e. preeclampsia, abruptio placentae, cardiac dysfunction, low birth weight and stillbirth. Maternal hypothyroidism may impair fetal neuropsychological development. Pregnant women with subclinical hypothyroidism have significantly higher incidence of preterm birth, placental abruption and admission of infants to the intensive care nursery.[2]

Human chorionic gonadotropin (hCG) is a glycoprotein hormone having an α-subunit which is nearly identical to some of other glycoprotein hormone and a hormone-specific β-subunit. The β-subunit of both TSH and hCG possess 12 half-cysteine residues at highly conserved positions. The partial structural similarity provides an indication that hCG may stimulate the secretion of thyroid hormones. During a normal pregnancy, we observe a partial TSH suppression at the end of first trimester which result from feedback inhibition by small and transient increase in free thyroxin (T_4) level which is induced by the direct stimulatory effect of hCG on the thyroid which is peaked this time.[4,5]

A study in western Australian women shows that applying the conventional TSH reference interval to pregnant women results in misclassification of thyroid status in over 20% of women. Some 16% of women would have been mistakenly classified as having subclinical and mild hyperthyroidism, which may result unnecessary clinical investigation or treatment, and maternal anxiety. In a further 4.5% of the women, TSH was elevated according to the first-trimester-specific reference range which means they are actually hypothyroid, these women would have missed diagnosis with conventional reference interval.[6] Another study in Geneva, Switzerland also shows that the results of Thyroid Function Test (TFT) would have been potentially misclassified if non-pregnant reference intervals were used.[7]

As a consequence, the United State National Academy of Clinical Biochemistry (NACB) recommends that 'trimester-specific reference values should be used when reporting thyroid test values for pregnant patients' and the NACB guidelines specifically recommend that specimens used for such studies should not

contain thyroid auto-antibodies (TPO-Ab).[8] UK guidelines also suggest that ' trimester and method-specific reference interval should be used when reporting thyroid test values for pregnant women'.[9]

Here lies the rationale of establishing the trimester and method-specific reference ranges of TSH in different trimesters of pregnancy in Indian pregnant women so that subclinical hypothyroidism patients can be identified and can be treated accordingly to prevent adverse outcome and misclassification of patients as hyperthyroid can also be prevented.

OBJECTIVES

1. **Primary objective:** Establishment of ‘gestational age-specific’ and ‘method-specific’ reference intervals for ‘Thyroid stimulating hormone’ in first and second trimester healthy, singleton pregnant women.

2. **Secondary objective:** To examine the implication of these reference intervals for the interpretation of ‘Thyroid function tests’ in pregnant women.

REVIEW OF LITERATURE

Thyroid hormone, TSH and its function

The thyroid, a butterfly-shaped gland that is present in the neck, has its middle connecting lobe in front of trachea. The gland has two important functions. Firstly, it secretes the thyroid hormones, which is necessary for maintaining the level of metabolism in the tissues that is needed for them to function properly. Thyroid hormones stimulate oxygen consumption in most of the cells in the body, help control intermediary

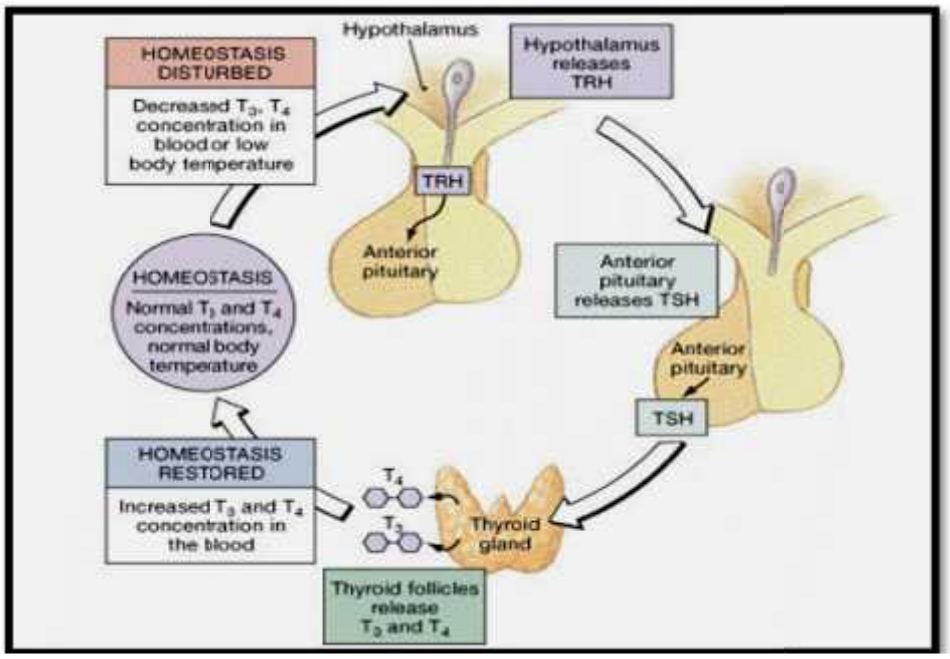


Figure 1: homeostasis and feedback control of thyroid hormone secretion

metabolism, and thereby influence body mass and higher mental activity. Usually as early stage of life thyroid dysfunctions occurs, the effects are more severe. Its absence or hypo function during fetal and neonatal life results in severe mental retardation and dwarfism. In adults, hypothyroidism is accompanied by sluggish mental and physical activity and poor resistance to cold. Conversely, hyperthyroidism leads to body wasting, nervousness, tremor, tachycardia, and

excess heat production. Thyroid function is controlled by the thyroid-stimulating hormone (TSH/thyrotropin) secreted from anterior pituitary. The secretion of thyrotropin is in regulated by thyrotropin-releasing hormone (TRH) from the hypothalamus and is also subject to negative feedback control by high circulating levels of thyroid hormones acting on the anterior pituitary and the hypothalamus.[10]

Human TSH is a glycoprotein containing 211 amino acid residues, along with hexose, hexosamines, and sialic acid. It is composed of two subunits, designated α and β. The common α-subunit is encoded by a gene on chromosome 6 and the hormone-specific β-subunit by gene on chromosome 1. The α-subunit and β-subunit attached non-covalently before being secreted from thyrotropes. The thyroid gland produces two related hormones, thyroxin (T_4) and tri-iodothyronine (T_3). Acting through nuclear receptors, these hormones play a critical role in development during cell differentiation and help maintain thermogenic and metabolic homeostasis in the adult. [1]

Thyroid hormones control the final stage of brain differentiation such as growth of dendron and axon, formation of synapse, neuronal migration and myelination.[11] In hypothyroid rats neuropil in the cerebral cortex and the cerebellar Purkinje cells can't develop properly. Neuronal cell bodies are smaller and more densely packed, there is reduced branching and elongation of dendron, as well as altered distribution of dendritic spines and delayed cell proliferation and migration.[12] The fetus get its thyroid hormones from two potential sources- its own thyroid and the thyroid of its mother. Human fetuses acquire the ability to self-production of thyroid hormones at roughly 12 weeks intrauterine. Thyroid hormone receptors are widely distributed in the fetal brain, and even present before they start

synthesizing thyroid hormones.[13] Fetus receives only thyroxin from mother up to 12 weeks through placental circulation but as TSH or fT_3 are not able to cross placenta. fT_4 is partially converted to fT_3 and combines with receptors in fetal brain because fT_3 is needed for fetal brain development. From 12th week, placental changes prevent thyroxine passage to fetus and fetal pituitary thyroid axis start functioning like adult.[13]

Physiological changes in pregnancy affecting thyroid function

In pregnancy, different hormonal and metabolic changes take place that help to ensure the continuation of pregnancy, resulting in complex changes in maternal thyroid function.

1. These changes are directed by the production of polypeptide hormones by the placenta and fetal pituitary gland, as well as by steroid production in the fetal adrenals and gonads. Human chorionic gonadotropin (hCG) concentrations increase rapidly following implantation, ensuring sufficient progesterone concentrations to maintain the pregnancy until placental production is adequate. Thus, hCG concentrations increase dramatically during the first trimester of pregnancy, and plateau gradually thereafter.[14]

Human chorionic Gonadotrophin (hCG) is a member of the glycoprotein hormone family that is consisting a common α -subunit non-covalently attached with hormone-specific β -subunit. The α-subunit of hCG consists of a polypeptide chain of 92 amino acid residues containing 2 N-linked oligosaccharide side-chains. The β- subunit of hCG consists of 145 residues with 2 N-linked and 4 O-linked oligosaccharide side-chains. The β-subunit of TSH is composed of 112

residues and 1 N-linked oligosaccharide. The α-subunits of both hCG and TSH possess 12 half-cysteine residues at highly conserved positions. Cystine-knot structure is produced by 3 disulfide bond, which is identical in both TSH and hCG and is essential for binding to their receptor A single gene on chromosome 6 encodes for the common α-subunit, while the genes that encode for the β-subunits are clustered on chromosome 19, with seven genes (but only three actively transcribed) coding for β-hCG.[4,5] The structural similarity between hCG and TSH provides already an indication that hCG may act as a thyroid stimulating agent, by overlap in their functions. hCG possesses an intrinsic (albeit weak) thyroid-stimulating activity and perhaps even a direct thyroid-growth-promoting activity.[4,15]

In 1967, Burger reported that urinary hCG had thyroid-stimulating activity in a mouse bioassay.[16] In normal pregnancy, a small and transient increase occurs in free thyroxine level near the end of the 1st trimester (the time of peak value of circulating hCG) induced by its thyrotropic activity and, in turn, causes a partial TSH suppression.[15] When tested in bioassays, hCG has very weak thyrotropic activity compared to TSH during normal pregnancy. This is the reason why, in normal pregnant women, the thyrotropic activity of hCG not become evident and thyroid function tests mostly unaltered.[4]

The thyrotropic role of hCG in normal pregnancy is illustrated in (fig.2) The figure shows the inverse relationship between serum hCG and TSH concentrations, at the end of the first trimester which is the time of peak hCG level is associated with maximum depression in TSH level producing a characteristic mirror image pattern. At this period during normal pregnancy, even those women, who otherwise

don't have any thyroid dysfunction have a transiently lowered serum TSH, even below the lower limit of the reference range for normal non-pregnant women.[4]

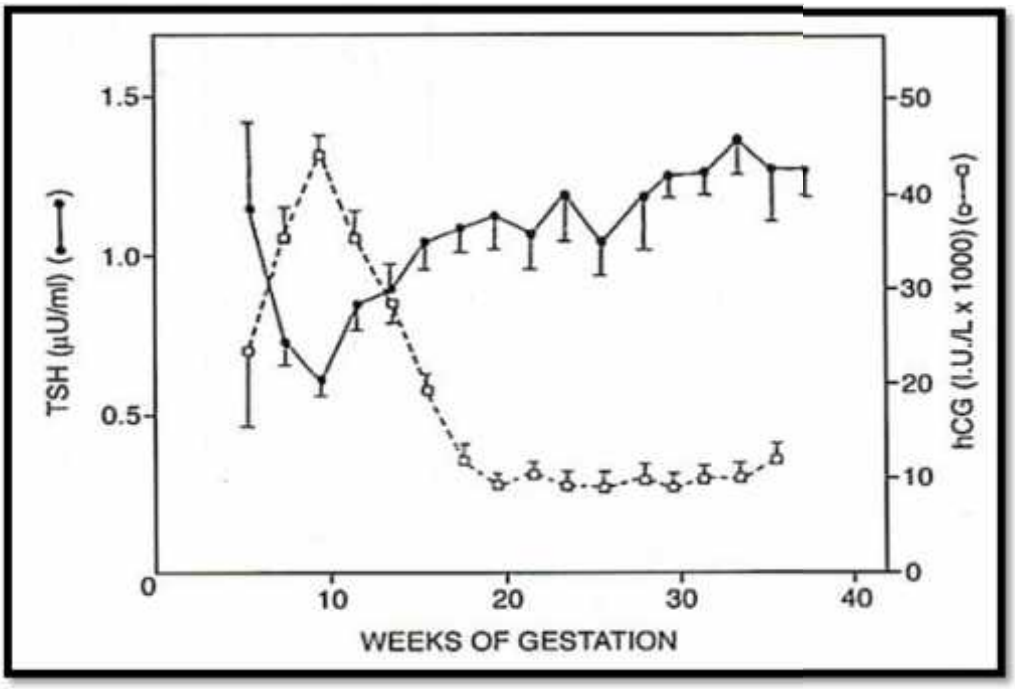


Figure 2. Serum TSH and hCG as a function of gestational age showing characteristic mirror image pattern at the end of 1st trimester[17]

Experimental studies with desialylated and deglycosylated hCG, to assess its thyrotropic effect, using secretion of T_3 as indicator (in a serum-free culture system with human thyroid follicles), have shown that native hCG can be transformed into such hCG variants which can act as thyroid stimulating super-agonists just removing the sialic acid or carbohydrate residues.[18] The human thyroid gland stimulated excessively by high level of hCG can be found in studies of patients with hydatidiform mole and choriocarcinoma gives us further evidence to support the thyrotropic role of hCG. In these conditions, clinical and biochemical manifestations of hyperthyroidism usually seen, which is as per this explanation and this hyperthyroidism is rapidly relieved after appropriate surgical treatment.[19]

2. Increased blood concentrations of Thyroxine-binding globulin (TBG): TBG is the most important among proteins that transport thyroid hormones in blood because it has the highest affinity for T_4 (thyroxine) of the group. Increased levels of TBG bind to more amount of T_4 which lead to reduced free T_4 level, which decrease feedback inhibition on TSH secretion, so TSH secretion is increased by the pituitary and, as a result, enhanced production and secretion of thyroid hormones. The ultimate effect of increased TBG synthesis is to set a new equilibrium between free and bound thyroid hormones and thus a significant increase in total T4 and T3 levels. The increasing need for thyroid hormones is reached its peak by about 20 weeks of gestation and persists until term.[11]

The cause of the marked increase in serum TBG is probably multifactorial. The animal studies showed that TBG biosynthesis was increased in primary cultures of hepatocytes when primed with estradiol.[20] However, the lack of increase of other binding proteins by estrogen in HEP-G2 cells raised the possibility that other factors might be operative in the pregnant state.[21] Studies on estrogen-induced changes of glycosylation of TBG have indicated that the increase in circulating levels of TBG was probably be due in a large part to a reduction of its plasma clearance.[21] There is a marked increase in the more heavily sialylated fractions of TBG in the sera of pregnant or estrogen-treated individuals. This increase in TBG's sialic acid content inhibits the uptake of the protein by specific asialylo-glycoprotein receptors on hepatocytes, and the more heavily sialylated proteins from pregnant sera have therefore a longer plasma half-life.[22] Such alterations in sialylation is not found in TBG isolated from patients with congenital TBG elevation, the latter being due to a true over-production of the protein.[23] Thus, in addition to the stimulatory estrogen effects of estrogen on TBG synthesis, a major contribution to the increased TBG

concentration during pregnancy is the reduced clearance of the protein. This explanation is attractive since it would also also account for the increases observed in concentrations of other circulating glycoproteins in hyperestrogenemic states. Delivery leads to a rapid reversal of this process and serum TBG concentrations return to normal within 4-6 weeks. With that, serum T_4 and T_3 also return to pregestational serum levels. In addition to the 2 to 3-fold increase in serum TBG, modest decreases in both serum transthyretin and albumin are commonly found in pregnancy, but the physiological impact of these changes, if any, is unknown.[24]

Another proposed mechanism for this increase in total thyroid hormone concentrations is production of type III deiodinase from the placenta. This enzyme, which converts T4 to reverse T3, and T3 to diiodotyrosine , has extremely high activity during fetal life.[25]

3. GFR is increased during pregnancy, which in turn causes constant elevation in renal clearance of iodine. This happens early in pregnancy. This is a very important factor laeding to decrease in plasma inorganic iodine concentration.[26] Euthyroid state is maintained by production of sufficient thyroid hormone. Actually this is a compensation made by thyroid gland by enlarging own size and the plasma clearance of iodine. Whether or not subsequent development of a goiter ensues depends upon the plasma concentration of inorganic iodide, as thyroid volume varies as a function of iodine intake.[14]

4. Numerous studies have evaluated changes in thyroid size and function during gestation. These have confirmed an increased prevalence of pregnancy-associated goiter in geographic regions of relative iodine deficiency[14] In a large study of 606 pregnant women in an area of marginal iodine intake (50 to 80

mg/day), thyroid volume increased as determined by ultrasonography by an average increment of 20%.[27] An inverse

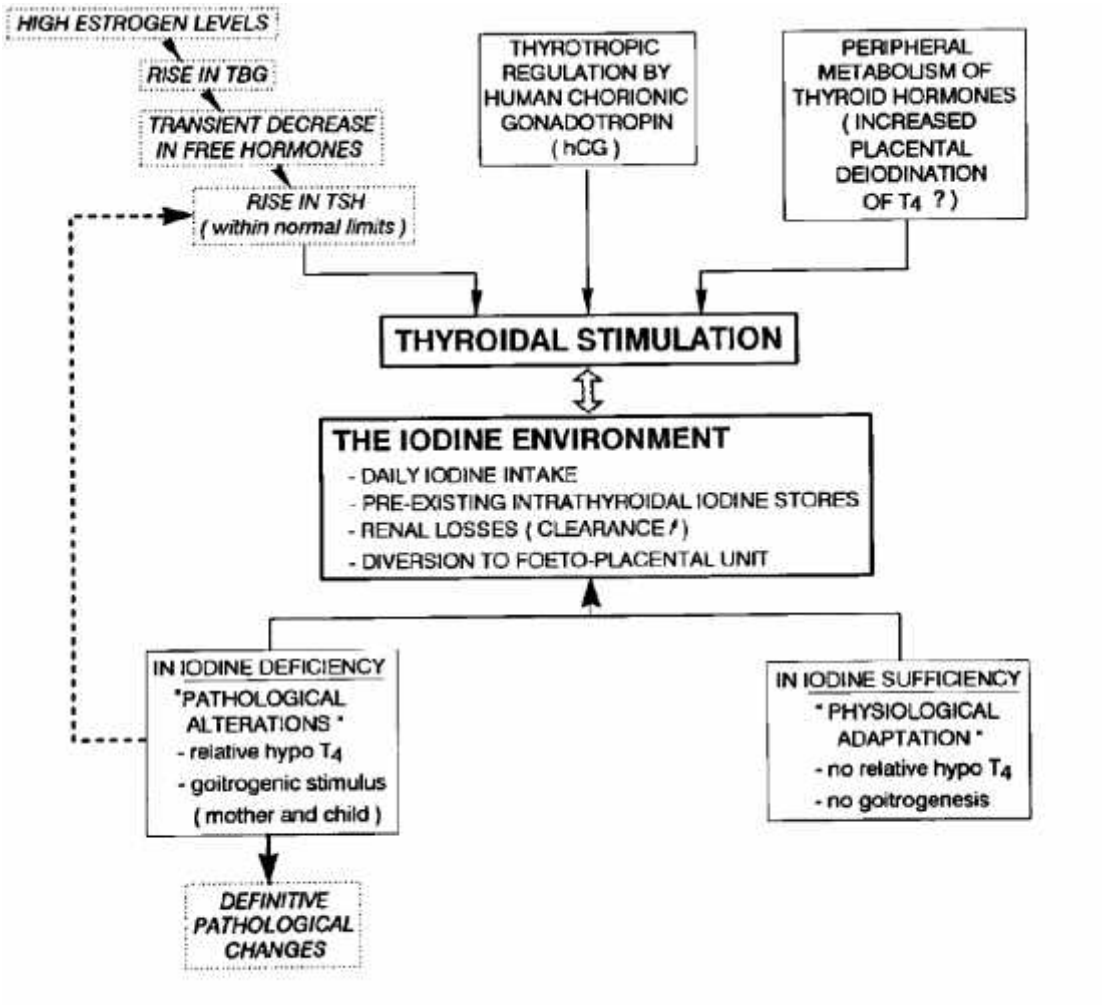


Figure 3: From physiological adaptation to pathological alterations of the thyroidal economy during pregnancy. The scheme illustrates the sequence of events occurring for the maternal thyroid gland, emphasizing the role of iodine deficiency to stimulate the thyroidal machinery[27]

Relationship has been found between iodine intake and thyroid blood flow. In a cross-sectional study done in Scotland, an area of relative iodine deficiency, 70% of pregnant women were diagnosed as having a goiter, in contrast to 38% of non-pregnant women.[28] But a recent prospective study in the iodine-replete Netherlands

also failed to demonstrate a significant change in thyroid volume/size during pregnancy.[29] These studies provide ample evidence that thyroid volume increases during pregnancy in regions of moderate or marginal iodine intake, while no significant changes in thyroid size or volume occur in iodine-replete conditions. Intervention studies suggest that thyroid enlargement during pregnancy is a physiological, compensatory adaptation to the increased demands for iodine associated with pregnancy.

The American Thyroid Association has also recently recommended that North American women receive dietary supplements containing 150 µg iodine daily during pregnancy and lactation and that all prenatal vitamins contain 150 µg of iodine, recommendations that have not been adopted.[30] In areas of marginal iodine intake (i.e., 50 µg /day), supplementary iodine (160 µg /day) given to pregnant women reduced neonatal goiter from 33% to 7%.[14]

The increased plasma concentration of TBG, together with the increased plasma volume, results in a corresponding increase in the total T_4 pool during pregnancy. While the changes in TBG are most dramatic during the first trimester, the increase in plasma volume continues until delivery. Thus, for free T_4 concentration to remain unaltered, the T_4 production rate must increase (or its degradation rate decrease) to allow for additional T_4 to accumulate. Globally, it is accepted that there is a ~50 % increase in the production of T_4 during gestation.[27]

5. The human placenta in addition to this cellular barrier also regulates the amounts of thyroid hormones passing from the mother to the fetus through its expression of placental thyroid hormone transporters, thyroid hormone binding proteins, iodothyronine deiodinases, sulfotransferases and sulfatases.[31,32]

Hypothyroidism in pregnancy

Clinical or overt hypothyroidism is diagnosed when an abnormally high serum thyrotropin level is accompanied by an abnormally low thyroxin level. Subclinical hypothyroidism is defined by an elevated serum thyrotropin level with normal serum thyroxin.[2]. The prevalence of overt and subclinical hypothyroidism in pregnancy is estimated at 0.3-0.5% and 2-3% respectively.[33] Endemic iodine deficiency is the most common cause of hypothyroidism seen in pregnant women worldwide. However the main cause of hypothyroidism in iodine-replete populations is chronic autoimmune thyroiditis.[34] Other causes include post-surgical, post-radioiodine ablation and hypothyroidism secondary to pituitary disease which, although rare, can include lymphocytic hypophysitis occurring during pregnancy or postpartum.[35] Overt hypothyroidism in pregnancy may present classically but is oftentimes subtle and difficult to distinguish from the symptoms of normal pregnancy. A high index of suspicion is therefore required especially in women with a predisposition to thyroid disease such as a personal or family history of thyroid disease, the presence of goiter or the co-existence of other autoimmune disorders like type 1 diabetes[36] which are all predictive factors of high risk of autoimmune thyroid disease. A recent analysis showed that the overall prevalence of hypothyroidism was 2.2% to 3.4%, and the prevalence of thyroid antibodies ranged from 25% to 77% of hypothyroid pregnant women, with a mean prevalence of 46%. Thyroid autoimmunity was 5.2-fold more frequent in women with a diagnosis of hypothyroidism, compared with euthyroid controls (mean of 48.5% versus 9.2%).[37]

The subclinical hypothyroid pregnant women have no clinical features and are often asymptomatic, but 50–60% will have evidence of autoimmune thyroid disease

(positive TPOAbs and or thyroglobulin antibodies, TgAbs) in iodine-sufficient areas. It should be noted however that endemic iodine deficiency is the most common cause of hypothyroidism seen in pregnant women worldwide. The prevalence of autoimmune thyroid disease in the pregnant population is comparable to that found in the general female population with a similar age range, i.e. between 5-15%. Careful study of women with thyroid antibodies during pregnancy has shown that despite the expected decrease in antibody titers during gestation, thyroid function gradually deteriorated towards hypothyroidism in a significant fraction of such women. [38, 39]

Symptoms and signs may raise clinical suspicion of hypothyroidism during pregnancy (weight increase, sensitivity to cold, dry skin, etc.) but others may go unnoticed (asthenia, drowsiness, constipation, etc.). Because many women remain asymptomatic, particular attention is required from the obstetrical care providers for this condition to be diagnosed and to evaluate more systematically thyroid function when women attend the prenatal clinic for the first time. Only thyroid function tests confirm the diagnosis. A serum TSH elevation suggests primary hypothyroidism and measurement of serum free T4 levels further distinguish between subclinical hypothyroidism (SCH) and overt hypothyroidism (OH), depending on whether free T4 is normal or clearly below normal for gestational age. Determination of thyroid antibodies, thyroperoxidase (TPO-Ab) and thyroglobulin (TG-Ab) antibodies, confirms the autoimmune origin of the disorder.[34, 40, 41]

Despite the known association between decreased fertility and hypothyroidism, the latter condition does not preclude the possibility to conceive. This is probably the main reason why, until a few years ago, hypothyroidism had been considered –

wrongly – to be relatively rare during pregnancy.[42, 43] Abalovich et al. showed that 34% of hypothyroid women became pregnant without thyroxine treatment: 11% of them had overt and 89% are subclinical hypothyroidism (155)[44] Overt hypothyroidism is associated with excessive adverse perinatal outcome, i.e. pre-eclampsia, abruptio placentae, cardiac dysfunction, low birth weight and stillbirth. Maternal hypothyroidism may impair fetal neuropsychological development. Pregnant women with subclinical hypothyroidism have significantly higher incidence of preterm birth, placental abruption and admission of infants to the intensive care nursery.[2]

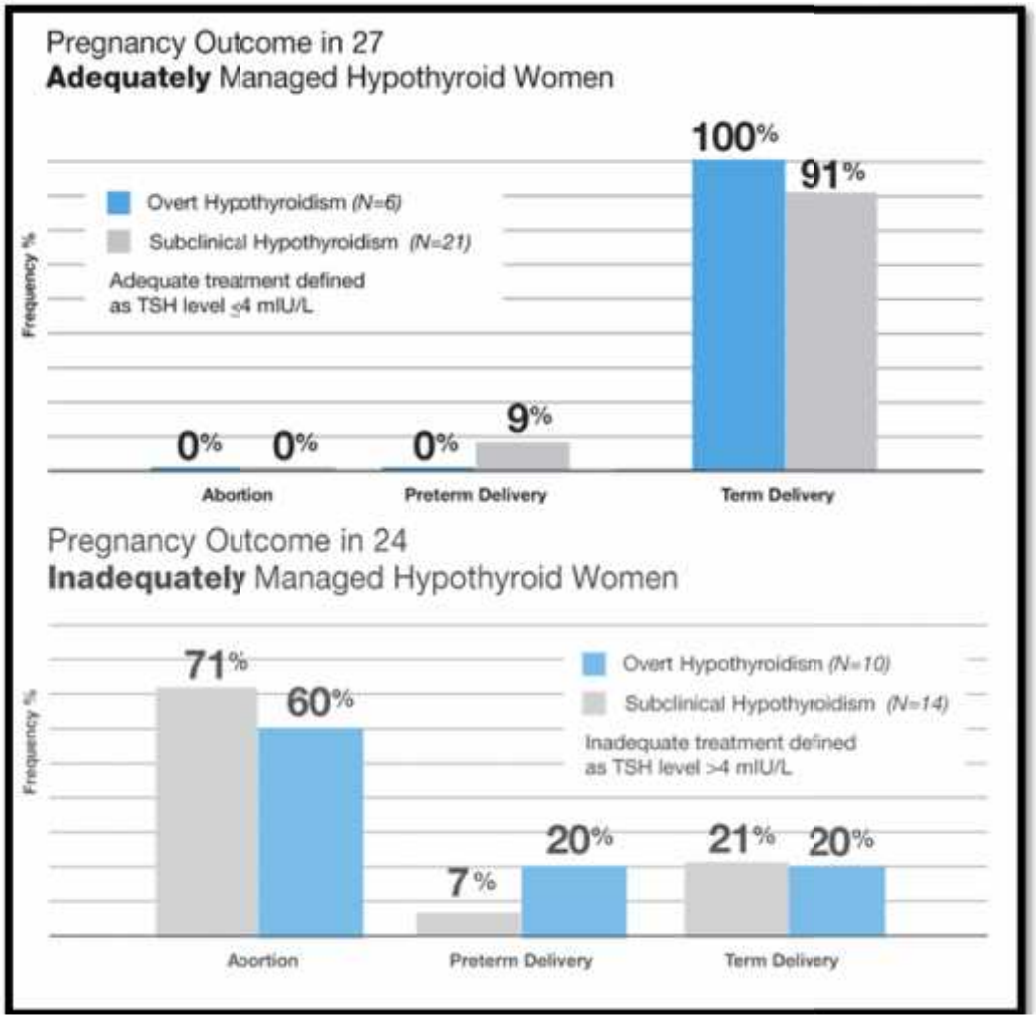


Fig.4. comparison of outcome in patient who are adequately and inadequately treated for overt and subclinical hypothyroidism in pregnancy[46]

One Indian study has demonstrated that hypothyroidism has statistically significant relationship with recurrent pregnancy loss in first trimester.[45] In a study at San Francisco,(fig.4) Tao To et al. has shown that pregnant women with treated hypothyroidism comparing with without hypothyroidism are not at any increased risk for perinatal morbidity.[46]

Maternal hypothyroxinemia is potentially damaging for neurodevelopment of the fetus throughout pregnancy, but especially so before mid-gestation, as the mother is then the only source of T_4 for the developing brain. Despite a highly efficient uterine–placental 'barrier' to their transfer, very small amounts of T_4 and tri-iodothyronine (T_3) of maternal origin are present in the fetal compartment by 4 weeks after conception, with T_4 increasing steadily thereafter. A major proportion of T_4 in fetal fluids is not protein-bound: the 'free' T_4 (fT_4) available to fetal tissues are determined by the maternal serum T_4, and reaches concentrations known to be of biological significance in adults. Despite very low T_3 and 'free' T_3 (fT_3) in fetal fluids, the T_3 generated locally from T_4 in the cerebral cortex reaches adult concentrations by mid-gestation, and is partly bound to its nuclear receptor. Experimental results in the rat strongly support the conclusion that thyroid hormone is already required for normal corticogenesis very early in pregnancy. The first trimester surge of maternal fT_4 is proposed as a biologically relevant event controlled by the conceptus to ensure its developing cerebral cortex is provided with the necessary amounts of substrate for the local generation of adequate amounts of T_3 for binding to its nuclear receptor. Women unable to increase their production of T_4 early in pregnancy would constitute a population at risk for neurological disabilities in their children. As mild–moderate iodine deficiency is still the most widespread cause of maternal hypothyroxinemia in Western societies, the birth of

many children with learning disabilities may already be preventable by advising women to take iodine supplements as soon as pregnancy starts, or earlier if possible.[47]

In 1969, Man and Jones suggested that mild maternal hypothyroidism alone was associated with lower IQ levels in the offspring; their study involved a cohort of 1349 children, and measurements of serum butanol extractable iodine were used to distinguish between euthyroidism and hypothyroidism in women.[48] A study by Matsuura and Konishi in 1990 documented that fetal brain development is adversely affected when both the mother and fetus have hypothyroidism caused by chronic autoimmune thyroiditis[49] A study by Haddow et al. shows that full-scale IQ scores of children of 48 mothers who had not been treated for hypothyroidism averaged 7 point lower than those of the 124 matched control children (P=0.005)[50]

For hypothyroidism in pregnancy British thyroid guideline recommends:

- The thyroid status of hypothyroid patients should be checked with TSH + fT_4 during each trimester. Measurement of T_3 is not appropriate.
- Normal TSH and fT_4 concentrations for the gestational age should be maintained.
- In hypothyroid patients the TSH should be checked and the thyroxine dose should be adjusted as soon as pregnancy is diagnosed.
- The dose of thyroxine will usually require a small increase, to ensure that the fT_4 level is in the (upper) reference range and the TSH in the low/normal range (0.4 – 2.0mU/L would be appropriate as this is normal for pregnancy)

- An increase in the dose of T_4 is especially important for women who have been treated for thyroid cancer, to ensure that the TSH remains fully suppressed
- After delivery the TSH should be checked (eg at 2 to 4 weeks post-partum, at which time the dose of thyroxine can usually be reduced back to the pre-pregnancy level.
- Ideally, the following sequence of TFT should be performed in the hypothyroid woman during pregnancy:
    - before conception if possible
    - **at time of diagnosis of pregnancy**
    - **at antenatal booking**
    - at least once in second and third trimesters and again after delivery (e.g. 2-4 weeks post-partum)
    - the newly diagnosed hypothyroid patient will need to be tested frequently (e.g. every 4-6 weeks) until stabilized.[9]

Pregnancy is a strong goitrogenic stimulus for both the mother and fetus, even in areas with only a moderate iodine restriction or deficiency. Maternal goiter formation can be directly correlated with the degree of prolonged glandular stimulation that takes place during gestation. Goiters formed during gestation only partially regress after parturition, and pregnancy therefore constitutes one of the environmental factors that may help explain the higher prevalence of goiter and thyroid disorders in women, compared with men. Most importantly, goiter formation also takes place in the progeny, emphasizing the exquisite sensitivity of the fetal thyroid to the consequences of maternal iodine deprivation, and also indicating that the process of goiter formation already starts during the earliest

stages of the development of the fetal thyroid gland.[4] The effect of iodine deficiency on thyroid function in African women during pregnancy and postnatal period was examined. Results show that the level of serum free thyroxine index was elevated very significantly at late gestation ($p<.001$) but the women were not hyperthyroid, suggesting a marked disturbance in binding of T4 with TBG during pregnancy. Five women with SFD babies were found in 'compensated hypothyroid state' and showed a significant depression ($p<.01$) in serum T_4/TBG,T_4,FT_4I and T_3 levels to a low normal range, with a concurrent significant rise ($p.<.001$) in TSH level (above normal range) throughout pregnancy. Incidence of small for date (SFD) babies was higher ($p. <.001$) in iodine deficiency zone (15.2%) compared to control zone (9.8%). It is concluded that a state of maternal 'subclinical (compensated) hypothyroidism' during pregnancy possibly plays an important role in the aetiopathogenesis of SFD babies in Africans.[51] In countries without an efficient USI program, or with an established USI program where the coverage is known to be only recent or partial, complementary approaches are required to reach the recommended nutrient intake (RNI) for iodine. Such approaches include the use of iodine supplements in the form of potassium iodide (100-200 µg/day) or the inclusion of KI (125-150 µg/day) in vitamin/mineral preparations manufactured for pregnancy requirements.[52] A cross sectional study was carried out to find out the prevalence of goiter in a rural community of Belgaum district. The prevalence of goiter among rural population was found to be 16.6%. Goiter of grade 1 was 15.7% and that of grade 2 was 0.9%. Prevalence among males and females were 7.2% and 21.8%, respectively. The prevalence of goiter was highest among adolescents. Estimation of iodine content in the salt sample revealed that 50% of samples had adequate iodine content (≥15 ppm). 'Multiple logistic regression analyses revealed

that females of the age group 10-49 years were independently associated with goiter. From the study it is concluded that prevalence of goiter was relatively high and therefore constituted a public health problem in this region.[53]

Hyperthyroidism in pregnancy and thyroid autoimmunity

An elevated serum TSH concentration is present in both overt and mild hyperthyroidism. In the latter, the serum fT_4 concentration is, by definition, normal. Virtually all types of hyperthyroidism encountered in clinical practice are accompanied by suppressed serum TSH concentrations, typically less than 0.1 mIU/L.[3] Hyperthyroidism has a significant short-term morbidity and long-term morbidity and mortality. The prevalence of hyperthyroidism in women is 0.5-2% and is also ten times more common in women than in men.[8]

The incidence of hyperthyroidism in pregnant women has been estimated at 0.2%.[54] Most women have symptoms before pregnancy, but some will demonstrate symptoms for the first time during pregnancy. The most common cause is Graves disease, which accounts for 85–90% of all cases[54, 55] Diagnosis of hyperthyroidism during pregnancy is important because untreated or poorly treated hyperthyroidism can lead to adverse obstetrical outcomes. These include first-trimester spontaneous abortions, high rates of still births and neonatal deaths, two- to threefold increases in the frequency of low birth weight infants, preterm delivery, fetal or neonatal hyperthyroidism, and intrauterine growth retardation[54, 56] Diagnosis of Graves disease can be difficult because healthy pregnant women may exhibit tachycardia, palpitations, mild heat intolerance, emotional lability, diaphoresis, and warm, moist skin. For these reasons, diagnosis of hyperthyroidism during pregnancy needs to be made on careful clinical observations and well-conceived laboratory testing.

Clinical symptoms such as weight loss or inappropriately low weight gain for gestational age, goiter, lid lag, muscle weakness, heart rate 100, and onycholysis may help to differentiate symptoms of hyperthyroidism from the hypermetabolic effects of pregnancy.[54] Laboratory testing is similar to that for nonpregnant women in that it should include measurement of serum TSH. Evaluation should not include total T_4 or T_3 because these will be increased in healthy pregnant women, and should instead include an assessment of free hormone values either directly or via a calculated index. Routine laboratory tests in hyperthyroid patients may show mild leukopenia, hypercalcemia (10% of patients), increased alkaline phosphatase, and occasional mild increases in other liver enzymes[54]

Thyroid anti-microsomal antibodies (also known as thyroid peroxidase antibodies or TPO-Ab) are increased in most (80–90%) patients with Graves disease, and thyroid hormone receptor antibodies (TRAbs) are increased in 80% or more of patients.[54, 57] Therefore, measurement of these antibodies can be useful in establishing a diagnosis of Graves disease. Although the presence of TPO antibodies favors a diagnosis of autoimmune hyperthyroidism over other etiologies, the presence of TRAbs is more specific for Graves disease. In addition, the TRAbs have prognostic implications for fetal and neonatal hyperthyroidism, as discussed later. It is important to note that the natural course of Graves disease is altered during pregnancy, with an aggravation in the first trimester because of increased thyroid activity, amelioration in the second half of pregnancy because of immune suppression, and aggravation in the postpartum period as the immune system rebounds.[58] TRAbs can cross the placenta and, at high enough concentrations, can bind to TSH receptors and stimulate the fetal thyroid. High titers of TRAbs in maternal serum during the third trimester are predictive of fetal or neonatal

dysfunction. Therefore, it has been suggested that TRAbs be measured early in pregnancy and again in the last trimester. Values 500% of baseline are considered high and are a predictor of fetal or neonatal disease.[54]

Thyroid peroxidase (TPO) is a key enzyme in the synthesis of thyroid hormone. TPO is involved in thyroid hormone synthesis (organification and coupling reactions). TPO is a major antigen corresponding to thyroid-microsomal autoantibodies. Anti-TPO auto antibodies are very important to diagnose autoimmune thyroid diseases and also in estimating its clinical course Autoimmune thyroid disease is detected mostly by measuring circulating antibodies to thyroglobulin which is uncommon measurement of antibodies to TPO that gives reliable information about autoimmune thyroid disease. Eighty percent of Grave's disease patients have high levels of anti-TPO antibodies. About 4% of subclinical hypothyroid patients with positive TPO antibodies develop clinical hypothyroidism. TPO antibodies fix complement, and a complex of membrane and complement are formed, these complexes are present in autoimmune thyroid disease patients. Placental passage of these antibodies has no effect on fetal thyroid, which indicates that T-cell damage is required to initiate autoimmune damage to thyroid.[59]

Anti-thyroid antibodies (ATAs) have been suggested to be independent markers of ‘at-risk’ pregnancy. Euthyroid women with recurrent miscarriage have increased levels of autoantibodies either against thyroglobulin or thyroid peroxidase (TPO) while the probability of abortion in women with ATA has been shown to be greater than in controls.[60] However, the pathophysiological role of these antibodies is still unclear. In recurrent pregnancy loss, the association between pregnancy loss and thyroid antibodies may be a result of: (i) a direct effect of ATAs on fetal tissue

or (ii) the thyroid antibodies representing an underlying more generalized defect in autoimmunity. However, the prevalence of ATA has been reported to be 15–20% in normal pregnant women, compared with 20–25% in women with recurrent miscarriages[61] In Marai et al.'s study, anti-TPO antibodies were the only autoantibodies found to have a significant association with recurrent miscarriage. The anti-TPO antibodies were found in 21% of the women with recurrent miscarriages (8/38) as compared with 0% in women with infertility and no miscarriages (0/20).[62] However, in Shoenfeld et al.'s larger study, there was no association with recurrent miscarriage as a whole, but anti-TG antibodies were associated with late pregnancy loss compared with controls, (OR 8.44; 95% CI 1.6, 43.8), PPV=40%. Therefore, the prognostic value of ATA remains uncertain.[63]

It has been shown that 1) TPO-Ab positive women had significantly increase TSH level; 2) some women with normal TSH level were found to be positive for TPO-Ab. Detection of TPO-Ab in euthyroid patients is beneficial for the identification of patients having high risk for hypothyroidism. In particularly, in women preparing for pregnancy and those having high risk for hypothyroidism following pregnancy, detection of TPO-Ab can be used to predict hypothyroidism in the early stage of pregnancy and postpartum thyroid dysfunction. However, in some TPO-Ab positive patients, they have no obvious thyroid lesions. Whether the different antibodies or these auto-antibodies together with other risk factors contribute to this phenomenon is still unclear.[64] A recent study from the Netherlands by Benhadi et al. concluded that pregnancy outcome might be improved by treating women with mildly elevated TSH, or even with normal TSH if TPOAb is present.[65]

There are two pregnancy-specific conditions, hyperemesis gravidarum and trophoblastic disease, that can lead to hyperthyroidism. Vomiting occurs in normal pregnancy during the 1st trimester and ceases usually by the 15th week. Prolonged nausea and severe vomiting in early pregnancy that causes greater than a 5% weight loss, dehydration and ketonuria is defined as Hyperemesis Gravidarum (HG) and occurs in 0.5-10 cases per 1,000 pregnancies.[66] Hyperemesis is associated with high hCG levels occurring at this time, but the exact cause remains uncertain. For unknown reasons, HG is more prevalent in Asian than Caucasian women. Norwegian data from 1967 to 2005 showed a prevalence of 0.9% but it affected 2.2% of Pakistani women; 1.9% of Turkish women and 0.5% of Norwegian women;[67] When the charge-isoforms profiles of circulating hCG were compared in HG women with different ethnic backgrounds (Samoan vs. European). an increase in total serum hCG concentrations as well as an increase in the proportion of acidic hCG variants in the women suffering from HG, compared with matched control subjects was noted.[68] The same study also confirmed the known association between hCG concentrations in early pregnancy and elevations in thyroid hormone levels .While there was no major association between HG and ethnic background, the authors observed a high prevalence of recurrent HG and a familial predisposition for this condition, suggesting that either long-term environmental factors or genetic factors may play a crucial role in the pathogenesis of HG and gestational transient non autoimmune thyrotoxicosis.[69] Thirty to sixty percent of patients with HG have elevations of serum free thyroid hormone concentrations with a suppressed TSH . Women with hyperemesis and elevated thyroid hormone levels most commonly do not have other clinical evidence of primary thyroid disease, such as Graves' disease. A minor proportion of these patients may have

clinical hyperthyroidism, termed 'gestational hyperthyroidism' or 'gestational transient thyrotoxicosis. Graves' disease can also occur coincident with hyperemesis. Many common signs and symptoms of hyperthyroidism may be mimicked by a normal pregnancy. The clinical challenge is therefore to differentiate between these two disorders. The etiology of excessive thyroid stimulation is considered to be hCG itself (or derivatives of hCG) via a direct stimulation of the thyroid cells through binding of hCG to the TSH receptor.[70] In virtually all patients with gestational hyperthyroidism, appropriate fluid replacement will lead to resolution of the clinical symptoms. As gestation proceeds and hCG levels progressively fall, normal thyroid function is resumed. In severe (but rare) cases, antithyroid drug treatment may be required. Several investigators have observed that There may even be more subtle form of hyperthyroidism associated with morning sickness.[17] Severity of emesis was correlated with serum free T4 and hCG levels and inversely with the degree of TSH suppression, suggesting strongly that HG may reflect the extreme end of the spectrum of physiological changes that occur at this time in normal pregnancy (Fig. 5). It is possible that high hCG levels cause both an increased estrogen secretion as well as thyroid hyper-function, and in turn explain the coexistence of nausea and vomiting with hyperthyroidism.[70]

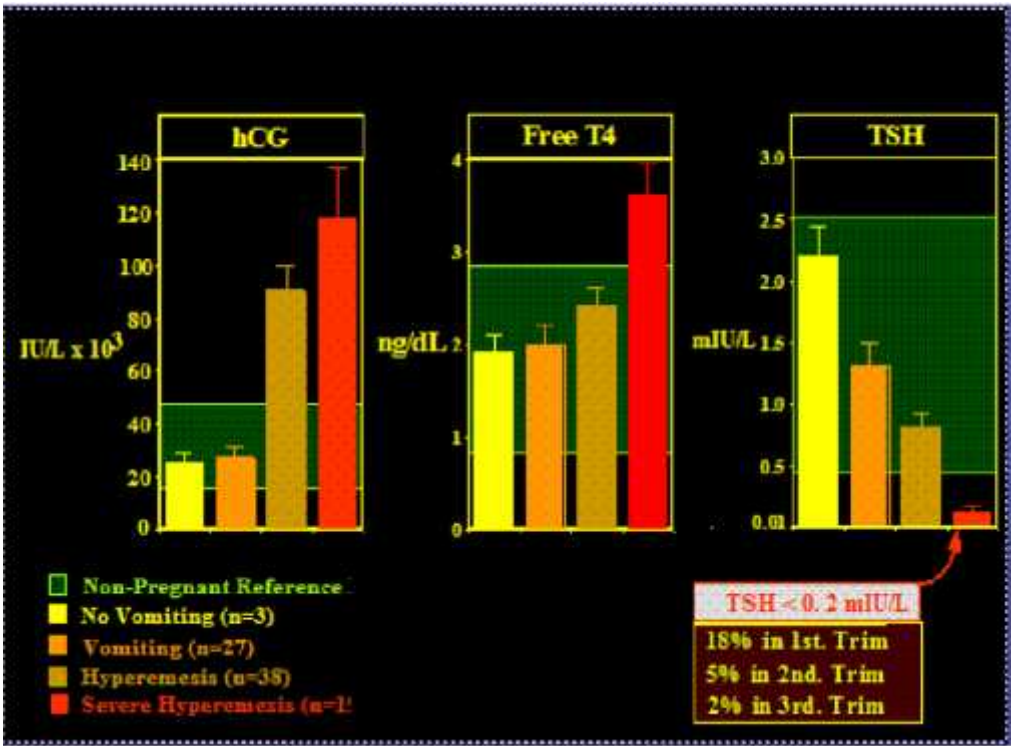


Figure 5. Relation between the severity of vomiting and serum concentrations of TSH, free T4, and hCG (mean + SE). Hormone concentrations differed significantly between each group of patients except as indicated by NS Goodwin et al.[70]

Hyperthyroidism can also occur in women with gestational trophoblastic disease (GTD). GTD is a general term that includes benign and malignant conditions of hydatidiform mole (both complete and partial) as well as choriocarcinomas. The frequency of hydatidiform mole is approximately 1 in 1500–2000 pregnancies and that of choriocarcinoma is 1 in 40–60 000. The frequency of hyperthyroidism in GTD has been estimated as anywhere from 5% to 64%. The etiology of the hyperthyroidism is thought to be related to the increased concentrations of serum hCG in these patients, which can be as high as 1000-fold higher than reference values. As mentioned previously, prolonged increases in serum hCG can clearly cause a significant increase in thyroid function. Hyperthyroidism attributable to GTD should be suspected in patients who

demonstrate increased free T_4 and T_3 concentrations, decreased TSH, and significantly increased hCG. Although free T_4 and T_3 concentrations can be increased with hCG concentrations .50 000 IU/L, in patients with trophoblastic tumors, serum hCG usually exceeds 300 000 IU/L and always exceeds 100 000 IU/L. Thyrotoxic patients also have a higher serum T_4-to-T_3 ratio than patients with Graves hyperthyroidism, a characteristic of hCG-induced thyroid stimulation. The thyroid gland is either not enlarged or only slightly enlarged, rarely to more than twice normal size, and ophthalmopathy is absent. Complete surgical removal of the GTD rapidly cures the hyperthyroidism.[71, 72]

Thyrotoxicosis often improves during pregnancy, especially in the second and third trimesters (as with other autoimmune conditions due to the immunosuppresion in pregnancy). The level of thyroid stimulating antibodies (TSH receptor-stimulating antibodies) may fall with consequent improvement in Graves' disease and a lower requirement for anti-thyroid treatment. However exacerbations may occur in the first trimester, possibly related to human chorionic gonadotrophin (hCG) production and also in the puerperium due to a reversal of the fall in antibody levels seen during pregnancy.[73,74]

Severe and untreated thyrotoxicosis is associated with inhibition of ovulation, menstrual irregularities and infertility. Those who do become pregnant and remain untreated have a significant increased rate of miscarriage, intrauterine growth restriction, premature labour and also higher risk of perinatal mortality.[74] For women with mild hyperthyroidism and those with good control by anti-thyroid drugs, the maternal and foetal outcomes are usually good and unaffected by the disease. Poorly controlled thyrotoxicosis may lead to thyroid crisis (storm) and heart failure, particularly at the time of delivery.[72]

For hyperthyroidism British TFT guidelines recommends following:

- In hyperthyroid women taking anti-thyroid drugs thyroid function tests should be performed prior to conception if possible, and therapy modified if appropriate
- Pregnant women who are hyperthyroid should be seen by a specialist
- The newly diagnosed hyperthyroid patient will require frequent testing during pregnancy (e.g. monthly) until stabilized. fT_4 rather than TSH is the result that will guide therapy.
- Pregnant women receiving anti-thyroid medication should be tested frequently (perhaps monthly) and the dose reduced to the minimum required to maintain a euthyroid state (i.e. fT_4 normal), or discontinued toward term if the patient is euthyroid.
- Women who have been successfully treated previously for hyperthyroidism and are euthyroid at antenatal booking may be checked again once in the second and third trimesters.
- All previously hyperthyroid women should be retested after delivery, as there is a significant chance of relapse at this time.
- Measurement of TRAb at antenatal booking can be useful and if negative or low need not be measured again. A very high titre can predict the chance of intrauterine or neonatal thyrotoxicosis developing.
- The obstetrician must enquire about past history of thyroid disease, as women who have had previous thyroidectomy for Graves' hyperthyroidism and are currently euthyroid or hypothyroid may still have high titres of TRAb with concomitant risk of neonatal Graves' disease.[9]

Serum TSH measurement is the single most reliable test to diagnose all common form of hypothyroidism and hyperthyroidism, particularly in the ambulatory setting. [3]

Prevalence of thyroid dysfunction in pregnancy and recommendation by different guidelines

Concerning the prevalence of thyroid disorders, many studies have shown that 5-15% of pregnant women have thyroid autoantibodies, 2-3% of them have undiagnosed hypothyroidism, and 0.3-0.5% undiagnosed hyperthyroidism.[38] One study in Belgium shows (fig. 6) the different thyroid conditions that were observed in successive prospective cohort studies involving altogether ~2.400 women. The data indicate clearly that the overall prevalence of thyroid disorders associated with pregnancy is sufficiently high to warrant screening.[75]

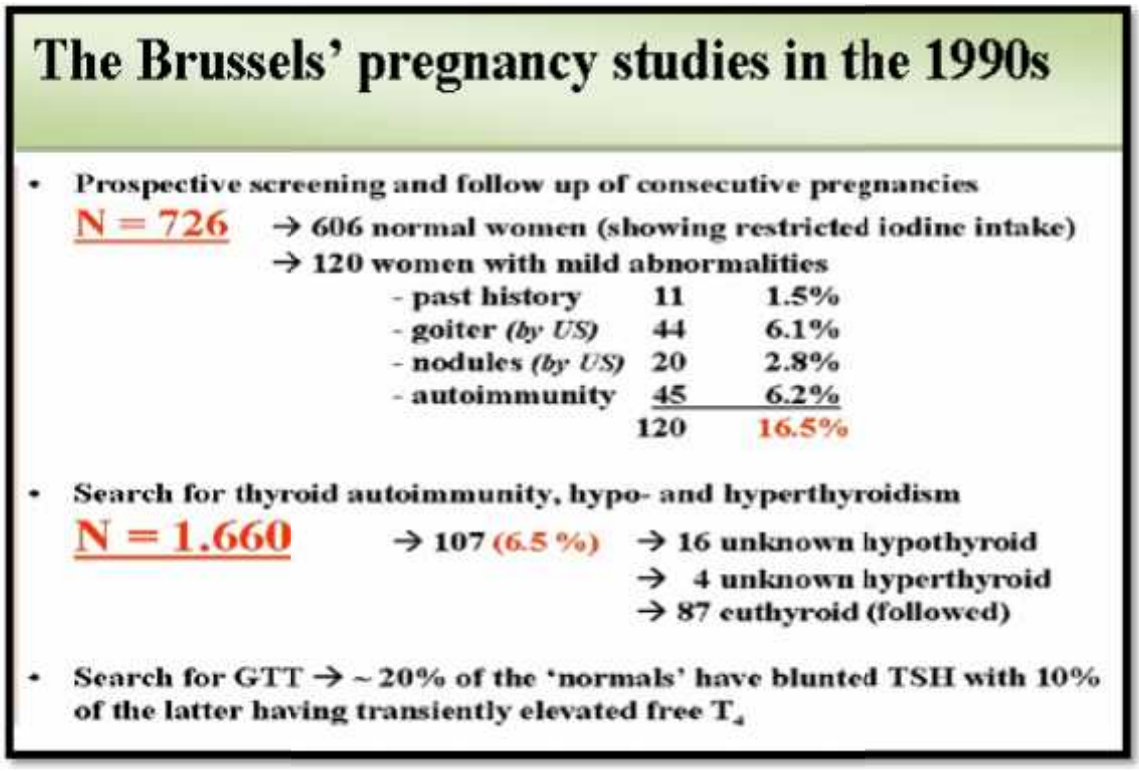


Fig 6: Study N°1 (N=726) showed thyroid abnormalities present in 16.5% of women; study N°2 (N=1.660) showed hitherto unknown thyroid dysfunction and/or thyroid autoimmunity features in 6.5% of women, with 1/5th of healthy pregnant women having a transient blunting in serum TSH. [75]

British Thyroid Association recommendations for screening in pregnancy are as follows:

• Pregnant women in the following categories should have thyroid function assessed either at diagnosis or at antenatal booking, or even before conception if feasible:

- type-1 diabetes
- previous history of thyroid disease
- current thyroid disease
- family history of thyroid disease
- goiter
- symptoms of hypothyroidism

• Thyroid function testing during pregnancy should comprise both TSH and fT_4. TPO-Ab should also be considered as this has predictive value for both post-partum thyroiditis and fetal impairment.[9]

But a single-center cohort study done in united kingdom to assess efficacy of the targeted high-risk case-finding approach in identifying women with thyroid dysfunction during early pregnancy. 40 women (2.6%) had raised TSH (> 4.2 mIU/liter). The prevalence of raised TSH was higher in the high-risk group [6.8 vs. 1% in the low-risk group, relative risk (RR) 6.5, 95% confidence interval (CI) 3.3–12.6, P <0.0001]. However, 12 of 40 women with raised TSH (30%) were in the low-risk group. This study has demonstrated targeted thyroid function testing of only the high-risk group would miss about one third of pregnant women with overt/ subclinical hypothyroidism.[76]

NACB recommends following guidelines for pregnancy:

- Pre-pregnancy or first trimester screening for thyroid dysfunction using serum TSH and TPOAb measurements is important both for detecting mild thyroid insufficiency (TSH > 4.0 mIU/L) and for assessing risk for post-partum thyroiditis (elevated TPOAb).
- Initiation of levothyroxine (L-T_4) therapy should be considered if the serum TSH level is >4.0mIU/L in the first trimester of pregnancy.
- A high serum TPOAb concentration during the first trimester is a risk factor for post-partum thyroiditis.
- Serum TSH should be used to assess thyroid status during each trimester when pregnant patients are taking L-T_4 therapy, with more frequent measurement if L-T_4 dosage is changed.
- **Trimester-specific reference intervals** should be used when reporting thyroid test values for pregnant patients.
- Total T4 and total T3 measurements may be useful during pregnancy if reliable fT4 measurements are not available, as long as the reference ranges are increased by 1.5-fold relative to non-pregnant ranges.
- fT_3 and fT_4 reference ranges in pregnancy are method-dependent and should be established independently for each method.[8]

British TFT guidelines also recommend:

- Both TSH and fT_4 (and fT_3 also when TSH is below the detection limit of a reputable assay) should be used to assess thyroid status and monitor thyroxine therapy in pregnant patients.

- **Trimester- and method-specific** reference intervals should be used when reporting thyroid test values for pregnant patients.[9]

	mIU/L
Prematures, 28-36 wk (1st week of life)	0.7-27.0
Cord blood (>37 wk)	2.3-13.2
Children	
Birth to 4 days	1.0-39.0
2-20 wk	1.7-9.1
21 wk-20 yr	0.7-64.0
Adults	
21-54 yr	0.4-4.2
55-87 yr	0.5-8.9
Pregnancy	
First trimester	0.3-4.5
Second trimester	0.5-4.6
Third trimester	0.8-5.2

Fig.7: Reference range of TSH in different physiologic condition given in Tietz textbook[77]

As per reference range mentioned in Tietz textbook, the pregnancy values are almost equal to non-pregnant value[77], which is contrary to all aforementioned guidelines. Williams Obstetrics also mention that TSH remains normal in pregnancy, [2] these necessitate this study should be performed.

Tietz textbook also mention that different immunoassays (i.e. radioimmunoassay, chemiluminescent assay) and their different generation have different sensitivity and detection limit, [77] that support the need for method-specific reference range.

Reference ranges for TFT are derived from a reference population that comprises a large group of subjects who do not have thyroid disease and are otherwise well. By convention, a reference range usually only comprises 95% of a reference population. Thus 2.5% of 'normal' individuals will fall above the

reference range and 2.5% will fall below the range. For TSH the reference population shows a log normal distribution and has a diurnal variation. For a number of reasons, reference ranges for TFT may show variation between laboratories. Firstly the reference population studied may have included subjects with subclinical hypothyroidism as indicated by the presence of thyroid antibodies. This would have the effect of falsely increasing the calculated upper reference limit for TSH. Secondly assays will have differences in standardization, the matrix of the calibrators and specificity of antibodies. These differences commonly give rise to method-related biases which should be compensated for by the use of method-related reference ranges. Thirdly, the measurement of free thyroid hormones is technically difficult and differences in assay design may lead to significant method related differences in hormone concentration even when measured in identical samples.[8]

Other studies in the same field

A study was conducted in 2159 western-Australian first trimester (9-13 weeks) pregnant women using automated two-step chemiluminescent immunoassay. For each analyte, medians and the 2.5th and 97.5th percentiles were determined by the Harrell–Davis quantile estimator method; for each week within the 9–13-week gestation range and overall for the first trimester. For the purposes of determining reference intervals, women who were positive for TPOAb and/or TgAb were excluded. They had also calculated 95% confidence intervals for the lower and upper limits of the first-trimester reference ranges, using the Harrell–Davis jackknife variance estimator. Three subjects with insufficient sample volume for analysis were excluded, as was one subject with overt hypothyroidism (TSH, 32

mU/L; fT4, 8 pmol/L; negative TPOAb and TgAb). Of the remaining 2155 women, 338 (15.7%) were positive for TPOAb and/or TgAb. In the 1817 antibody-negative women, the median serum TSH concentration increased significantly across the 9–13- week gestation range. Reference range was 0.02 – 2.15 mIU/L in 1817 antibody-negative women. The study shows that applying the conventional TSH reference interval to pregnant women results in misclassification of thyroid status in over 20% of women. Some 16% of women would have been erroneously classified as having subclinical or mild hyperthyroidism, which might lead to inappropriate investigation or treatment, and maternal anxiety. It has been shown that pregnant women with subclinical hyperthyroidism based on TSH at or below the 2.5th percentile are not at increased risk of adverse pregnancy outcomes. In a further 4.5% of the women, TSH was elevated according to the first-trimester-specific reference range; these women would not be identified with the conventional reference interval. More than half of these women (57%) tested positive for thyroid antibodies, suggesting they do have thyroid disease rather than being healthy outliers whose TSH concentrations happen to fall outside the reference range.[6]

Another study was performed in Geneva, Switzerland as a part of routine antenatal care of pregnant women. A total of 2272 samples were included in the study. Exclusion criteria were: miscarriage, currently undergoing treatment for thyroid disease, and any evidence of genetic abnormality in the fetus. Analytes were measured by a high-throughput immunoassay analyzer. Specimens positive for TPO-Ab and/or Tg-Ab were excluded from the reference interval analysis TSH data were log transformed for analysis. TPO-Ab and Tg-Ab were positive in 10.4 and 15.7% of women respectively. For the first trimester, a total of 1014 women were tested. Mean maternal and median gestational age was 30.4 years and 7.6 weeks

respectively. For the second trimester, a total of 661 women were tested. Mean maternal and median gestational ages were 30.5 years and 16 weeks respectively. Overall, TPO-Ab and Tg-Ab status were not related to maternal age (P<0.7394 and P<0.1758 respectively). Mean thyroid hormone values were compared in women with and without thyroid auto-antibodies. TSH and fT_3 were significantly higher in antibody positive women (mean TSH of 1.38 vs 0.98 mIU/l, P<0.0003). Gestational age-specific reference intervals in antibody negative women for TSH assay are shown in figure 8. These reference intervals were used to classify TFT results (e.g. 'high' = above 97.5th confidence limit, 'normal'= within central 95% confidence interval, and 'low'= below 2.5th confidence limit), and were then compared with classifications determined using the non-pregnant assay-specific reference intervals provided by the assay manufacturer. For TSH, a total of 82 (3.6%) women with elevated TSH would not have been identified, and 83 (3.7%) women would have been incorrectly classified as having a low TSH. Potential for misclassification of TSH results was greatest in the first trimester (10.4%).[7]

Table 1 Gestational age-specific reference intervals for thyroid hormones in women without thyroid autoimmunity.

Gestational age (weeks)	N	Mean	Median	2.5th	97.5th
TSH (mIU/l)					
First trimester (<6–12)	783	0.8666	1.0402	0.0878	2.8293
≤6	208	1.1946	1.2401	0.4454	3.2345
>6–12	575	0.7716	0.9467	0.0687	2.8171
Second trimester (>12–24)	528	0.9358	1.0214	0.1998	2.7915
>12–18	335	0.8558	0.9676	0.0932	2.5132
>18–24	193	1.0930	1.1387	0.3319	2.8876

Fig.8: reference range of TSH in first and second trimester of pregnancy in antibody negative women in a study by stricker et.al. Manufacturer's non-pregnant reference interval is 0.35–4.94 mIU/l[9]

Another study has been conducted in United States; following criteria were used to select a reference population for the thyroid measurements among women with viable pregnancies:

- Gestational dates were confirmed by ultrasound
- The first trimester sample was obtained between 8 and 13 weeks of pregnancy
- The second trimester sample was obtained between 15 and 21 weeks of pregnancy (7–8 weeks after the first sample)
- Sufficient sample volume was available for testing
- Pregnancies that satisfied these criteria were taken consecutively.

Figure 9 displays selected centiles of TSH measurements on a week-by-week basis for the late first trimester (8–13 weeks) and the early second trimester (15–21 weeks). Collective measurements are also shown for each of the trimesters. In this study, all of the second trimester TSH measurements were obtained from the same women as those whose measurements are shown in the first trimester. TSH and TPO antibody measurements were performed in 1126 women. Overall, 121 women (10.7% of the total) had a positive TPO antibody measurement (>35 IU/ml) in at least one of their samples. When these women are removed from the reference population, the TSH values defining the selected upper centiles are lower. The availability of both first and second trimester serum samples from this entire cohort of pregnancies allows within-person variability of TSH and TPO antibody levels to be assessed for the first time. The high degree of correlation between TSH measurements in the two trimesters adds support to the validity of interpretation for clinical purposes, especially for measurements above the 98th centile where deficient thyroid function is suspected.[78]

Completed week of gestation	Number of observations	Observed Centile of TSH (mIU/l)					
		5th	25th	50th	75th	95th	98th
8	240	0.13	0.55	1.06	1.67	3.78	5.58
9	312	0.07	0.52	1.03	1.64	4.19	6.61
10	247	0.10	0.53	0.93	1.46	3.06	4.10
11	178	0.11	0.63	1.00	1.59	3.05	4.55
12	110	0.18	0.64	1.10	1.67	3.22	3.69
13	39	0.18	0.68	1.06	1.59	3.61	NC
All First Trimester	1126	0.10	0.56	1.00	1.62	3.40	5.20
TPO Antibody Negative[1]	1005	0.08	0.54	0.94	1.47	2.73	3.61
15	249	0.26	0.87	1.34	1.95	3.28	4.11
16	247	0.37	0.86	1.22	1.80	3.55	4.29
17	260	0.40	0.93	1.34	1.89	2.77	4.43
18	238	0.51	0.88	1.24	1.75	2.67	4.16
19	88	0.33	0.91	1.17	1.68	3.18	3.73
20	32	0.70	0.92	1.45	1.85	3.25	NC
21	12	0.17	0.76	1.33	1.54	2.75	NC
All Second Trimester	1126	0.39	0.89	1.29	1.84	3.17	4.18
TPO Antibody Negative[1]	1005	0.39	0.85	1.22	1.72	2.70	3.71

Fig. 9: Gestational week-specific TSH values in a cohort of 1126 pregnant women with viable pregnancies, sampled in both the first and second trimester[78]

One study has been conducted in Malaysia among 626 antenatal mothers. The distribution of the women based on their 1st:2nd:3rd trimesters were 130:229:267 respectively. The distribution of TSH values in all 3 trimesters is shown in figure 10. The mean TSH levels were decreased during the first trimester and then increased significantly ($p<0.05$) in the second and third trimester. Comparatively, the increase in the mean TSH levels from the second to the third trimester was not statistically significant ($p>0.05$).[79]

Subjects	TSH mIU/L
First Trimester N= 130	1.04 ± 0.08
Second Trimester N= 229	1.82 ± 0.07
Third Trimester N= 267	1.92 ± 0.06
Overall N= 626	1.69 ± 0.04

Fig.10: Reference range of TSH in different trimesters in a study in Malaysia [79]

In a research project in Northern Iran, 120 apparently healthy pregnant women were selected during 2007-08. The exclusion criteria for pregnant women in this study was any history of thyroid disease and if the pregnant women had taken any medicines which were related to thyroid disease at the time of blood sampling. 2 ml of venous blood was taken from each pregnant woman and serum was obtained from each blood sample. Serum TSH levels were determined by the ELISA technique.

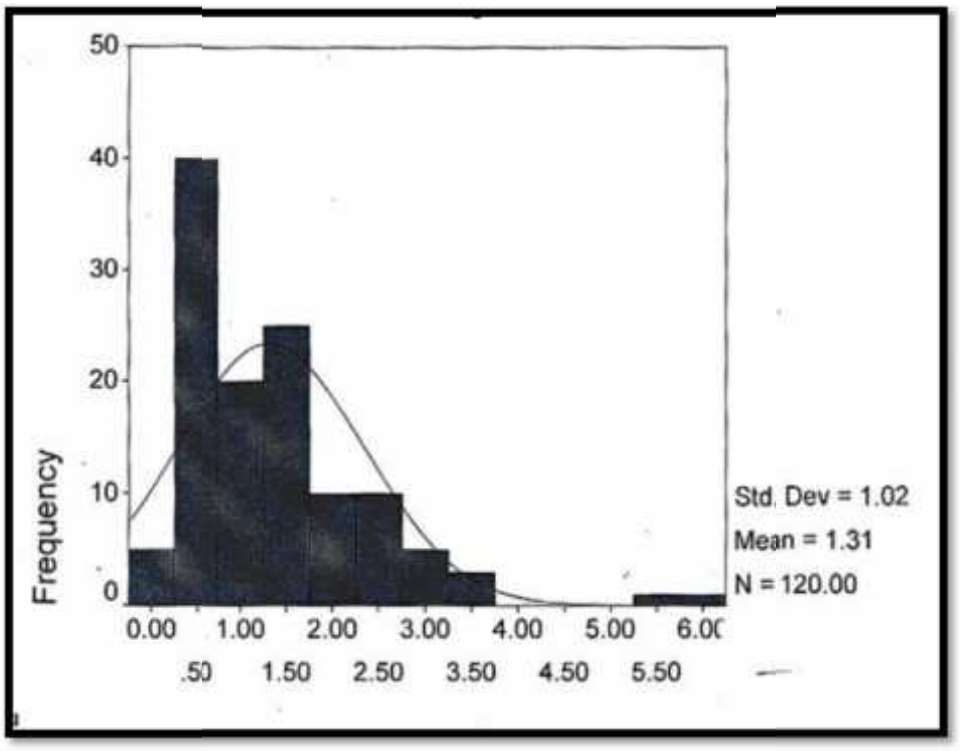


Fig.11. Maternal serum TSH level among pregnant women during first trimester of pregnancy in a study in Northern Iran.[80]

The findings in that research project indicated that according to the method and laboratory kits that they've used and the given reference interval of that method [0.32-5.2 mIU/l], 10% of pregnant women in that region have the serum TSH levels below lower limit of normal values <0.32 mIU/L].[80]

A study, done by Negro et.al is a component of a prospective study of 4657 women who were screened for TSH and thyroid-peroxidase antibody within the first 11 weeks of gestation in southern Italy. To be eligible for the study, women had to have no history of a thyroid disorder and a spontaneous singleton pregnancy. The women were randomly assigned to a universal screening group or a case-finding group and stratified as high risk or low risk for thyroid disease. Both the high- and low-risk women in the universal screening group and the high-risk women in the case-finding group had their serum TSH and thyroid peroxidase antibody levels tested immediately. During pregnancy, the women who were antibody-positive with a TSH >2.5 mIU/L were treated with levothyroxine and the women with a suppressed TSH and an elevated free thyroxine (fT_4) were classified as hyperthyroid and were referred to an endocrinologist. Maternal and neonatal outcomes were assessed. All of the women in the study who were thyroid antibody–negative, not classified as hyperthyroid, and had a serum TSH of ≤5.0 mIU/L were evaluated, but none of these women were treated during pregnancy. Women were stratified into two groups: group A had a serum TSH <2.5 mIU/L and was classified as euthyroid; group B had a serum TSH between 2.5 and 5.0 mIU/L and was classified as having subclinical hypothyroidism. The study outcome was based on the percentage of pregnancy loss, which included miscarriage before 20 weeks, stillbirth after 20 weeks, preterm delivery at 34 to 37 weeks, and very preterm delivery at <34 weeks, in each group.

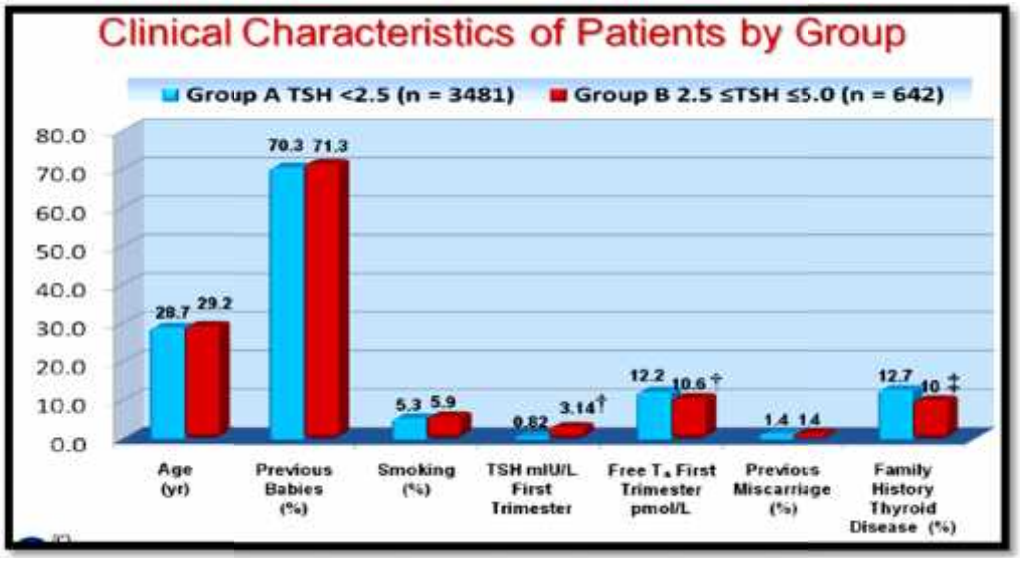


Fig.12: This figure shows the demographic information, pregnancy history, clinical information, and results of thyroid-function tests for women divided in groups in a study by Negro et.al[81]

Group A comprised 3481 women (84.4%), and group B comprised 642 women (15.6%).(fig.5) According to the study design, the median TSH in group A was significantly lower than the TSH in group B (0.82 vs. 3.14 mIU/L; P<0.001). Mean FT4 levels were significantly higher in group A as compared with group B (12.2 vs. 10.6 pmol/L, P<0.01)

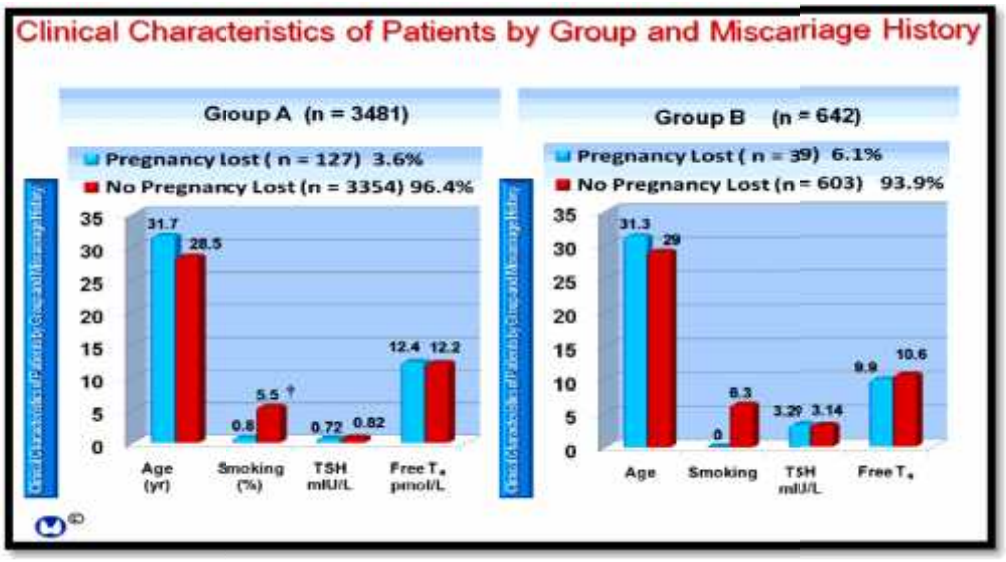


Fig.13: This figure presents age, obstetrical history and thyroid-function tests, and mean gestational age of pregnancy loss in groups A and B broken down by the presence or absence of pregnancy loss in a study by Negro et.al[81]

The rate of spontaneous pregnancy loss in Group A (3.6%, n – 127 of 3481 was significantly lower than the rate of spontaneous pregnancy loss in group B (6.1%, n = 39 of 652 (P = 0.006), shown in fig.13. There was no difference between groups in the rates of preterm delivery in group A (1.85%, vs. 0.93% in group B; P = not significant). To further assess the impact of TSH levels, a simple logistic-regression analysis was performed to predict miscarriage from TSH level and smoking status. The odds ratio (OR) for each point of TSH was 1.157; 95% confidence interval [CI], 1.002 to 1.336; P = 0.047), suggesting a continuous relationship between TSH and miscarriage, controlling for smoking.

So this study concludes that, TSH levels between 2.5 and 5.0 in first-trimester thyroid antibody–negative women is associated with a significant increase in the rate of spontaneous pregnancy loss as compared with the first-trimester thyroid antibody-negative women with TSH levels <2.5 μIU/ml, excluding women with hyperthyroidism.[81]

A case-control study was performed in Coimbatore, India with two groups of women: (1) 75 normal pregnant women randomly selected 25 samples from each trimesters, (They did not have pre-existing thyroid disease, hyperemesis gravidarum, trophoblastic disease, or preeclampsia) and (2) 75 randomly selected non-pregnant healthy female controls. Thyroid function tests were carried out among them. The levels of serum thyroid stimulating hormone (TSH) were measured by a Micro particle Enzyme Immunoassay (MEIA) on the AXSYM System. In each trimester, the mean TSH levels of pregnant women were lower than the mean level of non-pregnant. In the first and third trimesters, pregnant women's

lower TSH levels were not statistically significant, but in the second trimester, pregnant women's mean TSH was significantly lower ($p < 0.05$).[82]

Another study was conducted in New Delhi in which Five hundred and forty-one apparently healthy pregnant women with uncomplicated single intrauterine gestations in any trimester were consecutively recruited. Clinical examination, thyroid ultrasound for echogenicity and nodularity and estimation of fT_3, fT_4, TSH and antithyroid antibodies (antithyroperoxidase [anti-TPO] and antithyroglobulin [anti-Tg]) using electrochemiluminescence technique were carried out. Those with any known factor that could affect thyroid function or those who were being treated for thyroid dysfunction were excluded. 210 women were excluded. The composition of reference population comprising 331 women was 107 in first trimester, 137 in second trimester and 87 in third trimester. The 5th and 95th percentiles values were used to determine the reference ranges of TSH. The reference range of TSH (0.6–5.0, 0.44–5.78 and 0.74–5.7 iu/ml), respectively in 1st, 2nd and 3rd trimester. Analysis of mean, median values for TSH between each trimester showed no significant difference in TSH values (95% CI). Women with antibody positivity and hypoechogenicity of thyroid gland had significantly higher TSH values when compared with women with antibody negativity and normoechogenicity.[83]

Another study was conducted to analyze the prevalence of subclinical hypothyroidism and to identify the prevalence of thyroid autoimmunity in euthyroid pregnant women. Five hundred pregnant women attending two government Obstetrics and Gynecology hospitals in Chennai during a period of 5 months in 2007, were studied. Excluding subjects with known thyroid diseases, 495 subjects

were examined. Detailed physical examination was done and details of pregnancy were recorded. All were subjected to blood tests for Free T4, TSH and TPO antibodies. Exclusion criteria included, women with already known thyroid disease, patients already on levothyroxine therapy, patients with TSH>10 mU/L, and patients with subclinical hyperthyroidism.

Gestational Age	Hypothyroidism		
	Total	n	%
< 13 WKS	50	2	4
13 – 28 WKS	291	8	2.8
>28 WKS	154	4	2.6
Total	495	14	2.8
Trend Chi-square= 0.19, p=0.667			

Fig. 14: Hypothyroidism Vs gestational age in a study by Gayathri et.al[84]

Gestational age	Number of euthyroid women	Number of AB +VE	
		n	%
<13 WKS	48	1	2.1
13-28 WKS	283	20	7.1
>28 WKS	150	13	8.7
Total	481	34	7.1
Trend Chi-square = 1.98, p =0.159			

Fig 15: Anti TPO antibodies Vs gestational age in euthyroid women in a study by Gayathri et.al[84]

Prevalence of subclinical hypothyroidism was 2.8% (14/495) and it was not associated with the gestational age (figure 14). Anti-TPO antibodies were positive in a total of 42(8.5%) of the study group. Among the 14 women with hypothyroidism, 8 (57.1%) were TPO antibody positive, and among the euthyroid women (n=481), 34 (7.1%) had the antibody positivity. The prevalence was

significantly higher in the former group (p=0.012). Figure 15 shows that the antibody positivity in euthyroid women was not dependent on the gestational age (Trend c^2=1.98, p=0.159). It did not show an association with parity also (47.1% in primi and 52.9% in multipara, c^2=0.009, p=0.92). Prevalence of hypothyroidism increased in relation to increasing gestational age (c^2 = 6.02, p=0.014)[84]

Another Indian study was done by Sahu et.al among Six hundred and 33 pregnant women in second trimester. Prevalence of thyroid dysfunction was high in this study, with subclinical hypothyroidism in 6.47% and overt hypothyroidism in 4.58% women. Overt hypothyroids were prone to have pregnancy-induced hypertension (p = 0.04), intrauterine growth restriction (p = 0.01) and intrauterine demise (p = 0.0004) as compared to control. Cesarean section rate for fetal distress was significantly higher among pregnant subclinical hypothyroid women (p = 0.04). Neonatal complications and gestational diabetes were significantly more in overt hyperthyroidism group (p = 0.03 and p = 0.04, respectively).[85]

Another study was conducted by Sakinah et.al among Two hundred and three women (Malay = 85, Chinese = 47 and Indian = 71) in the third trimester and with no known thyroid disease. There was a marked racial disparity in the prevalence of goitre: Indian 61%, Malay 28% and Chinese 29% (p = 0.001). The serum thyrotropic hormone (TSH) was significantly higher in Indians (median: 1.36 uIU/ml) compared to Malays (1.14 uIU/ml, p = 0.009).[86]

Going through the outcomes of aforementioned studies, it is clearly evident that reference ranges of TSH are highly variable, not only in different trimester, also with assay methods and ethnic groups. Therefore all the accepted thyroid guidelines

have preferred reference range specific to each laboratory, than prescribing a universal reference range.[9,87]

Interpretation of thyroid function tests by the population-based reference ranges is difficult, because they depend not only on the composition of the population and the iodine intake but also highly on the laboratory methods used. Therefore, there is a strong need of laboratory-dependent reference ranges, in order not to rely only on the reference range provided by the assay manufacturer. Trimester-specific population-based reference ranges will reduce the global variability of thyroid hormone assessment by approximately 6–18% , but it is important to emphasize that, for this to occur, the use of laboratory and population-specific ranges is crucial, since measurements by different methods in different populations do provide very different ranges.[88]

Hi-tech Laboratory in our hospital also using reference range provided by manufacturer to interpret the result of 'thyroid function test' even in pregnant women (0.55-4.78 mIU/L).[89] Therefore, it is very much necessary to establish a reference range for TSH in pregnancy by chemiluminescent assay, because that will be useful in correct interpretation of 'thyroid function test' in pregnant women of this region, which is catered by this hospital.

MATERIALS AND METHODS

Source of Data: We have screened 147 pregnant women attending antenatal OPD, Department of Obstetrics and Gynaecology in KLES Dr. Prabhakar Kore Hospital and Medical Research Centre, Belgaum in their 1st trimester (Gestational age has been calculated by last menstrual period (LMP) and confirmed by ultrasonography). After excluding as per our exclusion criteria from 117 pregnant women in first trimester and 41 from second trimester, blood samples were collected consecutively from the same subject.

Study design: Cross sectional study with two consecutive samples.

Study period: From December 2011 to September 2013

Statistics: All statistical calculation were done by using MedCalC software version 12.7.2.0 and Microsoft office excel 2007

Consent: A written informed consent was taken from every patient participating in this study. Proforma of consent form is attached in the Annexure-II

Approval from the authorities:

- From institutional ethical Committee
- From Dr. B.R. Desai, Professor and HOD, Department of Obstetrics and Gynaecology, J.N. Medical College, Belgaum
- From MD and CEO, KLES Dr. Prabhakar Kore Hospital and MRC regarding using Hi-tech laboratory for TSH and anti-TPO assay

Selection criteria

Inclusion Criteria

- Spontaneous singleton healthy pregnancy.
- Gestational age has been calculated by LMP and confirmed by ultrasound.
- The first trimester samples have been collected between 8^{th} to 14^{th} weeks of gestation.
- The second trimester samples have been obtained between 15^{th} to 21^{st} weeks of pregnancy (preferably 6-8 weeks after the first sample).
- Sufficient sample volume was collected for each sample.

Exclusion Criteria

- History of thyroid illness or any medication that will affect thyroid hormone status.
- History of abortion.
- History of hyper-emesis gravidarum.
- Family history of thyroid illness.
- Presence of palpable thyroid swelling.
- Presence of antithyroid (anti-TPO) antibody. (>35 IU/L)
- Presence of overt/clinical hypothyroidism or hyperthyroidism.

Study sample: 100 ultrasonographically diagnosed case of pregnancy in first trimester after excluding 17 anti-TPO +ve subjects. Among them 41 subjects came back 6-8 weeks after their 1st visit (in their 15th-21st gestational weeks) from those subjects, we have collected our 2nd trimester samples.

Sample collection: 3-5 ml of venous blood has been collected from the pregnant women under aseptic precautionary measures using disposable syringe into plain containers and allowed to clot adequately before centrifugation. All the samples were analyzed immediately.

Methods of assay:

- Serum:
  - Thyroid-stimulating hormone (TSH): chemiluminescent assay using ADVIA Centaur Analyser.
  - Anti-TPO (autoantibodies against thyroid peroxidise): chemiluminescent assay using Immulite 1000 Analyzer

Estimation of Thyroid stimulating hormone (TSH) by chemiluminescent immunoassay:

Principle and Procedure:

The ADVIA Centaur TSH3-Ultra assay employs anti-FITC (anti-fluorescent isothiocyante) monoclonal antibody covalently bound to paramagnetic particles, an FITC-labeled anti-TSH capture monoclonal antibody, and a tracer consisting of a proprietary acridinium ester and an anti-

TSH mAb antibody conjugated to bovine serum albumin (BSA) for chemiluminescent detection.

The system automatically performs the following actions:

- dispenses 100 µL of sample into a cuvette
- dispenses 50 µL of Ancillary Reagent and 50 µL of Lite Reagent and incubates for 2.75 minutes at 37°C
- dispenses 200 µL of Solid Phase and incubates for 5.5 minutes at 37°C
- separates, aspirates, and washes the cuvettes with Wash 1
- dispenses 300 µL each of Acid Reagent and Base Reagent to initiate the chemiluminescent reaction
- Reports results according to the selected option, as described in the system operating instructions or in the online help system.

A direct relationship exists between the amount of TSH present in the patient sample and the amount of relative light units (RLUs) detected by the system. The functional sensitivity was determined to be 0.008 µIU/mL, which is the lowest analyte concentration at which the total imprecision for the assay does not exceed 20% [90]

Estimation of anti-thyroid per-oxidase antibody (anti-TPO) by chemiluminescent immunoassay:

Principle and procedure:

Immulite 1000 Anti-TPO Ab is a solid-phase, enzyme-labelled, chemiluminescent sequential immunometric assay. [91] This method is based on the oxidation of luminol by H_2O_2 as catalyzed by the peroxidase. Chemiluminescence was measured with a Luminometer 1250 (LKB Wallac, Uppsala, Sweden). The 1-mL reaction mixture contained 1 mol/L glycine–NaOH buffer, pH 9, 1 mmol/L EDTA, 10 μmol/L luminol, and aliquots of TPO. The reaction was initiated by injection of 0.1 mmol/L H_2O_2; the emitted signals were measured at 37 °C. The resulting light output was determined in mV with a chart recorder (LKB 2210). The luminol–peroxidase blank was simultaneously determined in every measurement.[92]

RESULTS

During the time period December 2011 to September 2013 we have enlisted 147 cases of pregnant women in their 1st trimester. Among them 47 pregnant women were excluded as per our exclusion criteria:

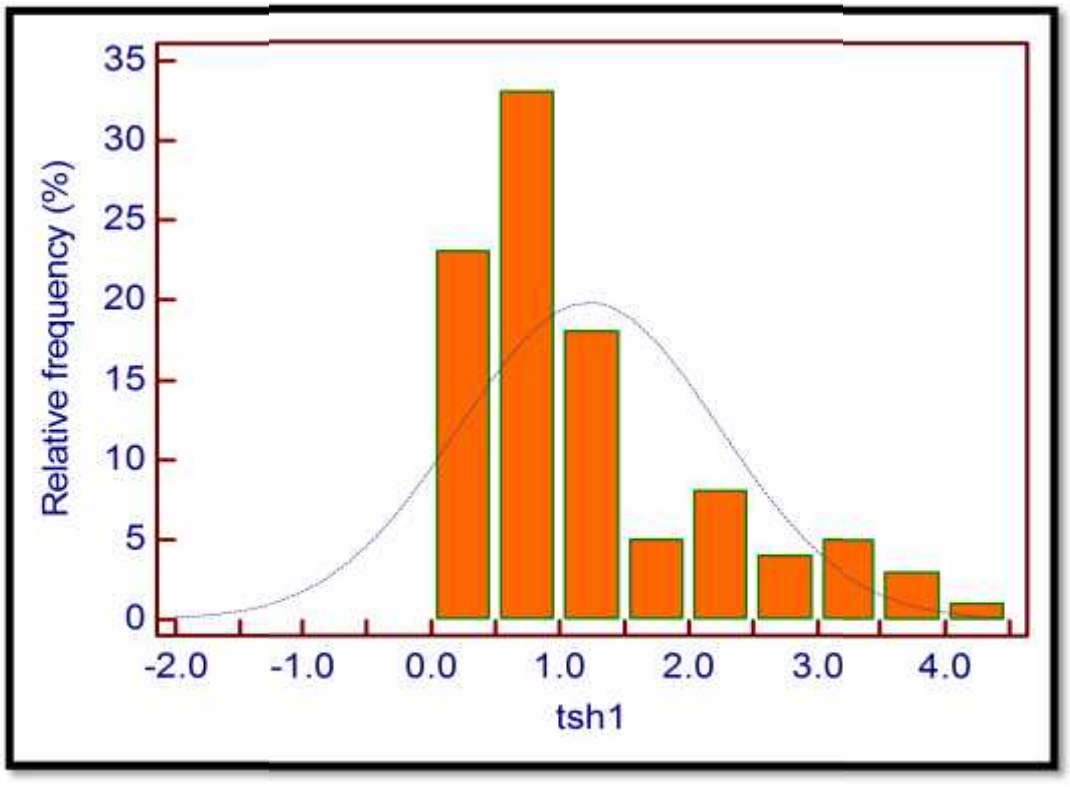
Cause of exclusion	No.
History of abortion in previous pregnancy/pregnancies	21
History of thyroid disease and taking thyroid supplement or anti-thyroid drug	3
Overt hypo/hyperthyroidism	4
Hyperemesis gravidarum	1
Presence of visible and palpable thyroid swelling	1
increased titre (>35 IU/L) of anti-TPO antibody	17
Total no. of exclusion	47

Table 1: Cause of exclusion and number of patients excluded

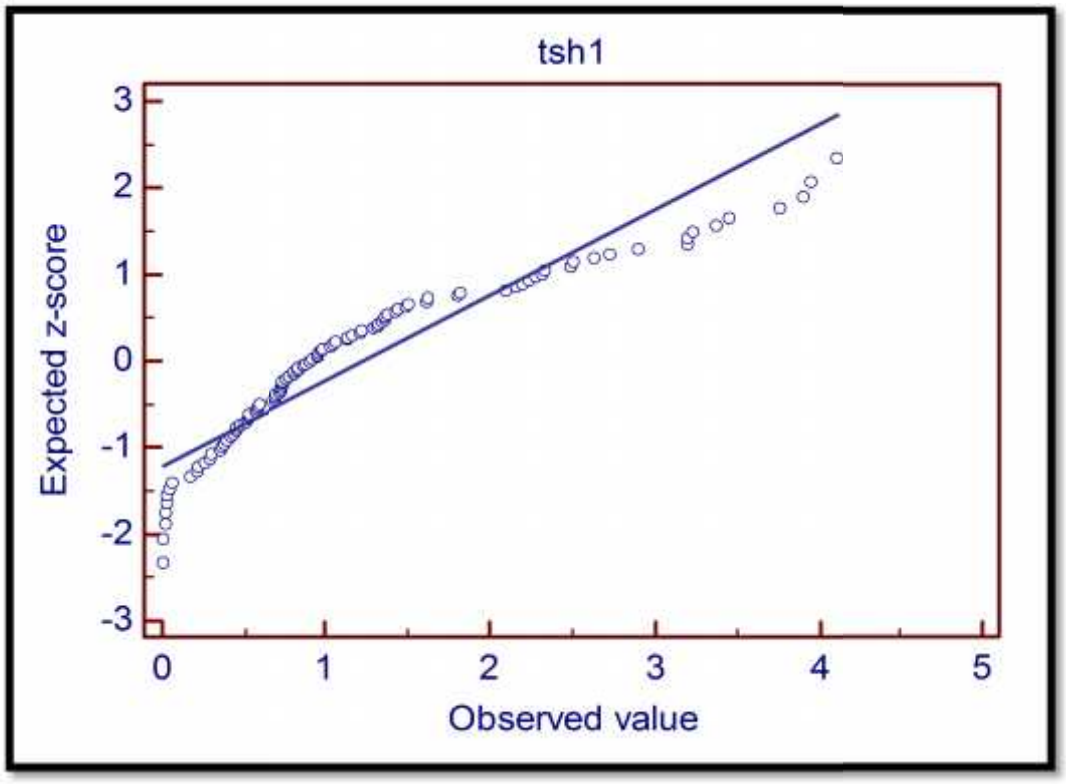
We have calculated 1st trimester reference range from the remaining 100 pregnant women. We have made our 1st trimester data normalized using square-root transformation. Then we have calculated 1st trimester reference range by parametric method which can be applied even with 40 subjects. First we have checked outlier by Generalized ESD (extreme studentized deviate) test and no outlier has been found. It is clearly evident from graph 1 and table 2, distribution of TSH in 1st trimester is a skewed one.

TSH level in mcIU/ml	No. of pregnant women
0 - 0.5	24
>0.5 - 1	32
>1 – 1.5	18
>1.5 - 2	5
>2 – 2.5	8
>2.5 - 3	4
>3 – 3.5	5
>3.5 – 4	3
>4 – 4.5	1

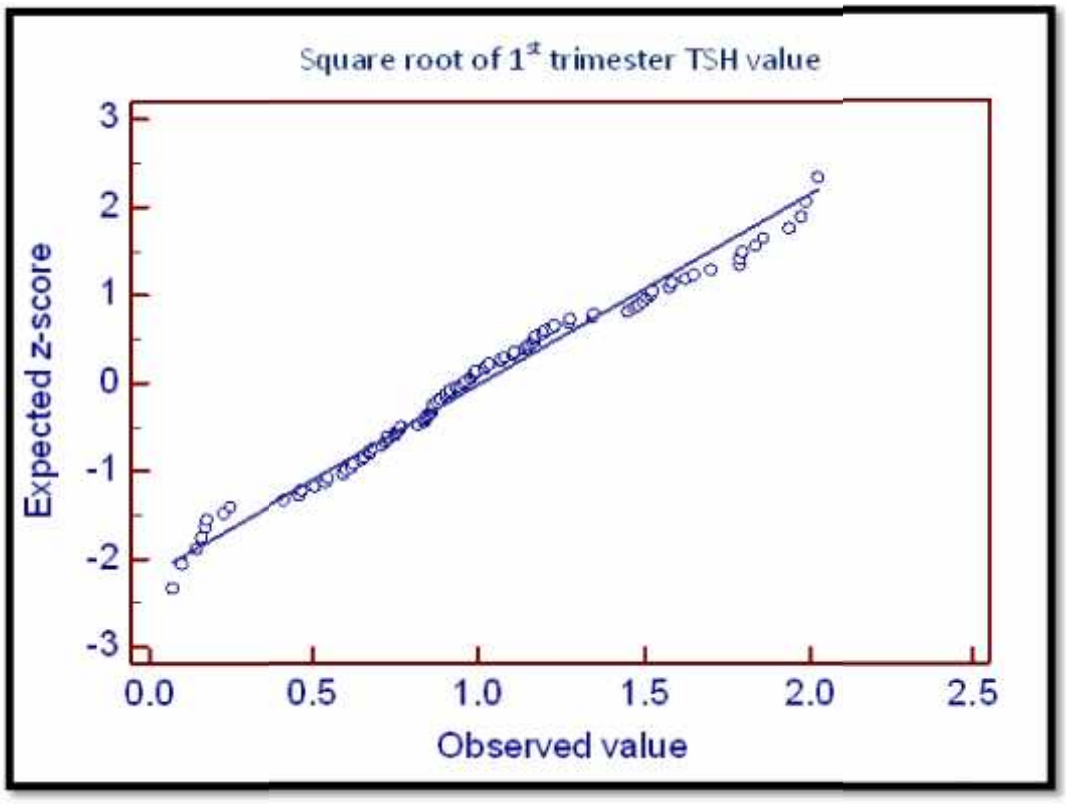
Table 2: Relative frequency distribution of TSH in 1st trimester in tabular form



Graph 1: Relative frequency distribution of TSH in 1st trimester in graphical form



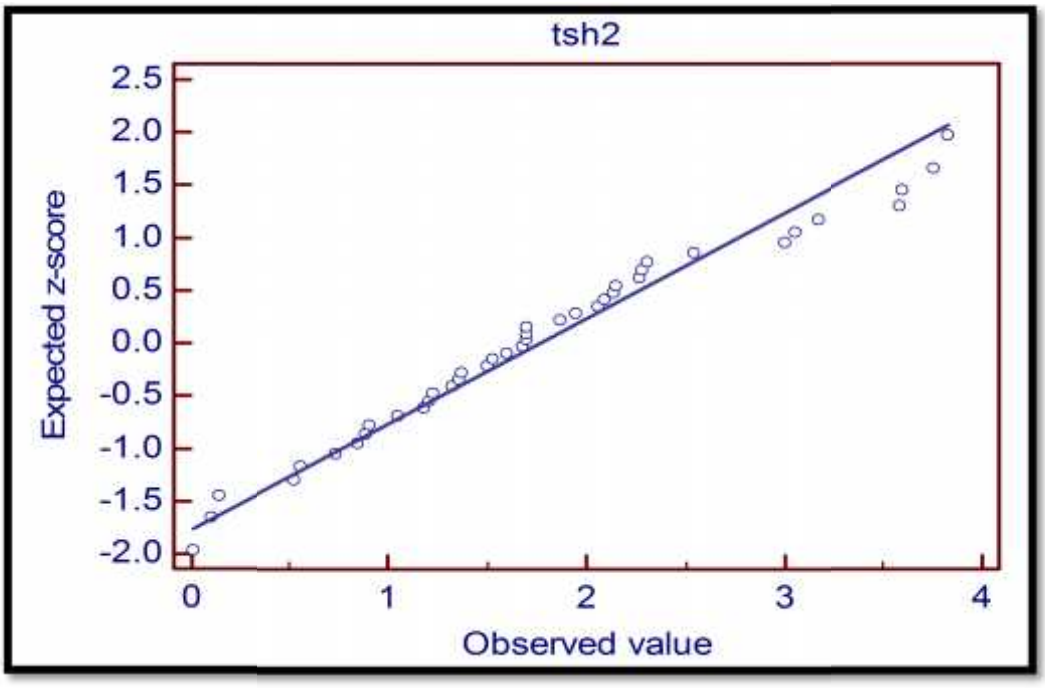
Graph 2: Normalcy curve for TSH values in 1st trimester



Graph 3: Normalcy curve for square root of TSH values in 1st trimester

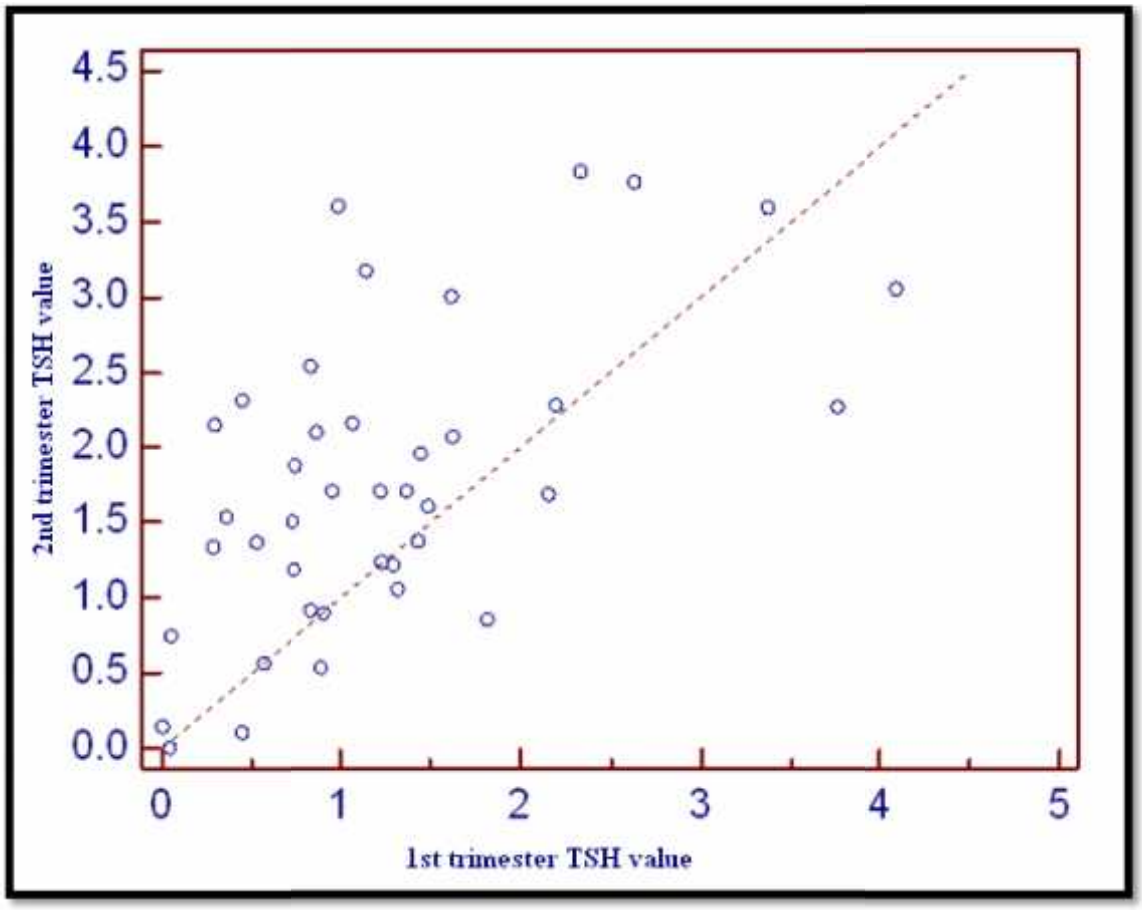
Still, we have checked whether it is normal distribution with Kolmogorov-Smirnov test and it has rejected Normality ($p<0.0001$). (graph2) After doing square-root transformation of all data it accept Normality ($p>0.10$). (graph3) So, we have applied the parametric method to calculate reference range. After back-transformation (again squaring the values) we have got the reference range 0.011 (90% CI 0.00056-0.057) - 3.646 (90% CI 3.162- 4.165). Calculated reference range for late 1st trimester is 0.01-3.65 mIU/L for our study group.

Among them 41 subjects came back 6-8 weeks after their 1st visit (in their 15th-21st gestational weeks) from those subjects, we have collected our 2nd trimester samples.We have checked for outliers and found a far-out value 7.1. So, we have excluded that value. After doing box-cox transformation and back-transformation after that we have done test for normality with Kolmogorov-Smirnov test which accept Normality ($P>0.10$). (Graph 4)



Graph 4: Normalcy curve for 2nd trimester TSH values doing back-transformation after box-cox transformation

As our sample size is too less, we were not able to calculate reference range by non-parametric method, we have used the parametric method and got the reference range 0.117 (90% CI 0.009 to 0.366) - 4.041 (90% CI 3.430 to 4.683). Calculated reference range for early 2nd trimester is 0.12-4.04 mIU/L for our study group.



Graph 5: Scatter diagram showing relation between TSH values in 1st and 2nd trimester. X-axis representing 1st trimester and Y-axis representing 2nd trimester value

Scatter diagram of both trimester TSH values (graph 5) showing correlation coefficient r 0.5935with significance level p=0.0001 (95% Confidence interval for r 0.4066 to 0.8209) Paired samples t-test has shown mean difference 0.6219, standard deviation 0.7709 (95% CI 0.3460 to 0.7638) test statistic t 3.639, two-tailed probability p = 0.0008, means in same patient the difference of TSH level in 1st and 2nd trimesters are statistically significant.

DISCUSSION

Kit reagent literature has provided the reference range 0.55 – 4.78 mcIU/mL.[89] If this reference range was used to interpret their late 1st trimester TSH level, potential misclassification would have occurred. 22% of the pregnant women would have misclassified as having hyperthyroidism whereas 4% of them would have been classified as normal even after having hypothyroidism. We can't comment the same about 2nd trimester as we have found only 1 misclassification has occurred during interpretation of early 2nd trimester TSH level using normal non-pregnant reference value. But we can tell TSH level is significantly different in different trimester justifying the need of trimester-specific reference range of TSH in pregnancy.

We have excluded anti-TPO positive women; their first trimester TSH values are significantly different from anti-TPO negative women. Standard error is 0.2817 (95% CI - 0.05848 to 1.1746), Test statistic t 2.189 with significance level P = 0.0307, it has justified the exclusion of anti-TPO +ve from our samples.

A study conducted in west-Australia in 2006 involving 2159 pregnant women (9–13 weeks' gestation), where TSH was measured by automated two-step chemiluminescent immunoassay on an ARCHITECT analyzer (Abbott Diagnostics, Sydney, NSW).[6] They have excluded Anti-TPO Ab +ve patients like us, but they have also excluded Anti-Tg Ab +ve patients. We have got 14.53% Anti-TPO +ve pregnant women almost similar to their 15.7%. We have got TSH level significantly different in Anti-TPO +ve and Anti-TPO –ve patients like them. They have got reference range 0.02–2.15mU/L by taking values between 2.5th and 97.5th percentiles, which is definitely narrower than reference range in our study, though we are not able to

calculate our reference range by non-parametric method as our sample size is less than 120 till now.

Another cross sectional study was conducted involving 2272 pregnant women from Switzerland. They've got Anti-TPO positivity in 10.7% patients and whereas we have 14.53% patients anti-TPO +ve.[7] We have also collected 2nd trimester sample only from those pregnant women 1st trimester serum samples were collected like their study. Collective median value is higher (1.69 mIU/L) in 2nd trimester than 1st trimester value (0.92 mIU/L) in our study like them. They have measured anti-TPO positivity in both the trimester whereas we have performed this only in 1st trimester due to limited resources. In their study 1st trimester TSH value is correlated with 2nd trimester. In our study also we have r value 0.59

A cross-sectional study was conducted in Malaysia in 2009 recruiting 626 women in different trimesters. They have used immunoassay to measure all the parameters whereas we've used chemiluminescent assay to measure TSH.[79] They have found TSH data normal distribution after doing log transformation whereas we our 1st trimester TSH values were not normally distributed even after log transformation; we had to take inverse of the data to get normal distribution. We have also got 2nd trimester TSH values significantly different from 1st trimester values.

Another study was conducted in Iran in 2007-08 which has measured TSH in 1st trimester pregnant women by ELISA.[80] Their study showed that 10% of women would have misdiagnosed as having hyperthyroidism compared to 22 % in our study. We can assume reason behind this difference is that their exclusion criteria were not proper and they haven't taken consideration of thyroid autoimmunity status whereas we have excluded anti-TPO +ve patients and the sample size is not of much difference

(120 versus 100). Their mean TSH value was 1.31 mIU/L compared to 1.23 mIU/L in 1st trimester in our study. They have collected sample from any 1st trimester pregnant women whereas we have collected between 8-14 weeks.

A cross-sectional study was conducted by Marwaha et.al at armed force clinic, New Delhi in 2008. They have calculated reference interval for fT3, fT4 and TSH in all the 3 trimester by the electro-chemi-luminescence technique.[83] But they haven't taken follow-up in same patient which is the advantage of our study. They have used ultrasound of thyroid gland to exclude goiter patients whereas we have relied upon history and physical examination only. Though they've calculated anti-TPO Ab for all patients, they haven't excluded Anti-TPO +ve (>35IU/L) subjects during reference range calculation. Their TSH value was not following normal distribution so they normalized that using log transformation. But for us we didn't get normal distribution even after log transformation, we have to do box-cox transformation for getting normal distribution. They have got 1st trimester TSH reference range 0.6–5.0 IU/ml whereas their kit provider reference value is 0.7-4.2 IU/ml. Their upper reference limit increased in 1st trimester, which should be decreased due to thyrotropic action β-hCG. Their 1st trimester value is significantly different from our study, which may be due to above mentioned reasons. In their study when the trimester-wise 95th percentile of TSH from the reference population was applied to the total population, the number of women with SCH decreased to36 (6.6%) from78 (14.4%). But almost all other studies have concluded that there will be misclassification (i.e. missing of hypothyroid and wrong identification as hyperthyroid which are actually normal) if non-pregnant reference range would have been applied to pregnant women. Instead of reducing misclassification this study has increased the misclassification.

A case-control study was conducted in Tamil Nadu by P. Pasupathi et.al. They have compared the thyroid function in all 3 trimesters in normal pregnant women with non-pregnant women. They've expressed all the value in mean ± SD for all the parameters. Their 1st trimester TSH values (1.93 ± 1.53 mIU/ml) are not significantly different from non-pregnant women (2.54 ± 1.32 mIU/ml), when the value should come significantly different, as the effect of β-hCG is maximal at the end of first trimester.[82] But they have significantly different ($p<0.05$) TSH level in 2nd trimester. Our study also has decreased mean TSH value in both 1st and 2nd trimester; our TSH level in 1st trimester is significantly different ($p<0.0001$) from normal non-pregnant value. May be the reason for difference is that, they have included any 1st trimester patient, but we have included 8-14th week patients when the effect of hCG is maximum.

Another cross-sectional study also was conducted in Chennai, Tamil Nadu in 2007. They considered the upper limit of TSH 5 mIU/L and classified pregnant women with 5<TSH<10 mIU/L and no clinical features as 'subclinical hypothyroidism'.[84] But actual numbers of pregnant women with subclinical hypothyroidism are much more as the upper limit of TSH for pregnant women should be 3.77 mIU/L as shown by our study. In our study prevalence of thyroid autoimmunity is much higher (14.53%) than their study (8.5%). This difference may be attributed to the difference in sample size. They have taken patients of all trimester and found 2.8% pregnant women having subclinical hypothyroidism whereas we have found 4%. The difference may be due to less sample size in both cases.

CONCLUSION

In our study it is evident that 22% pregnant women in their 1st trimester would have classified as hyperthyroid when they were actually euthyroid and 4% of them would have missed their diagnosis of hypothyroidism if we have applied the normal non-pregnant women reference range of TSH (0.55-4.78 mcIU/ml) to interpret thyroid function test in them. Though we couldn't comment the same about early second trimester, but for that further study are needed involving more samples.

Finally, on the basis of the findings from this study, we conclude that in this region within North Karnataka:

- Late 1st trimester (8th-14th week) reference range is 0.01-3.65 mIU/L and early 2nd trimester (15th-21st week) reference range is 0.12-4.04 mIU/L
- Their 'Thyroid function test' should be interpreted on the basis of this established reference range, otherwise potential misclassification will occur.
- TSH values are significantly different in late 1st and early 2nd trimester.

❖ **<u>Advantage of our study:</u>**

  - Consecutively both trimesters were taken so that we are able to compare 1st and 2nd trimester TSH in same patients.
  - Patients with overt thyroid disease are excluded.
  - Anti-TPO antibody +ve patients are excluded as it is associated with subclinical hypothyroidism in pregnancy and post-partum

thyroiditis and NACB guidelines specifically recommend that specimens used for such studies should not contain thyroid auto-antibodies (TPO-Ab).

- All the samples are analyzed immediately in our hi-tech laboratory.

❖ **Disadvantage of our study:**

- Less sample size (even less than 120), so that we were not able to calculate reference range by non-parametric method
- Problem for follow-up, so that we were able to collect 2nd trimester sample only from 41 women.

❖ **Scope of further study**

- Establishment of second trimester reference range with more sample size to assess the potential misclassification applying non-pregnant reference range.
- Whether case-finding in high-risk patient is enough or universal screening is required to find out thyroid dysfunction in pregnant women.

SUMMARY

Subclinical hypothyroidism is associated with several complications like preterm birth, placental abruption etc. Partial suppression of TSH occurs at late 1st trimester due to thyroid stimulating action of peak level of hCG. There are other changes also which can affect thyroid functions in pregnancy. For that reason NACB recommends there should be gestational age specific reference range during reporting thyroid function test in pregnancy. Some studies already shows applying reference range of normal non-pregnant women to interpret thyroid function test for pregnant women would lead to misclassification i.e. subclinical hypothyroid pregnant women might have missed the diagnosis.

That's why our objective is establishment of gestational age-specific and method specific reference of TSH and applying it to interpret the TFT in pregnant women in late 1st and early 2nd trimester.

Our present study we have enlisted 147 cases of pregnant women attending antenatal OPD of KLES Dr. Prabhakar Kore Hospital and MRC during their 1st trimester of pregnancy between the time period December 2011 to September 2013. We have excluded those pregnant women whose thyroid function may be disturbed due to several reason like thyroid medications, history of abortion and patients with overt thyroid diseases. We have excluded samples with thyroid autoimmunity (anti-TPO Ab +ve samples) as per standard guidelines. For second trimester we have collected from those 41 pregnant women who come 6-8 weeks after 1st sample collection (in their 15th-21st gestational week). For all 1st trimester samples we have done immediate analysis for serum TSH and anti-TPO in our Hi-tech laboratory by

chemiluminescent assay by Advia-Centaur analyzer. For 2nd trimester we have performed only serum TSH. Institutional ethical clearance was taken and written informed consent was taken from every pregnant women.

All the statistical analysis was carried out with MedCalC 12.7.2.0 and Microsoft Office Excel 2007. As our sample size is less than 120 we are not able to calculate reference range by non-parametric method. We have done square-root transformation to normalize the data. Then we were able to calculating reference range by in parametric method back-transforming the data, by parametric method reference range can be calculated from even 40 subjects. In this way we have got 1st trimester reference range of TSH 0.01-3.65 mIU/L. for 2nd trimester we have excluded one sample as outlier by generalized ESD test. Then we have seen other 40 2nd trimester values got normalized doing Box-cox transformation. Then, we have got 2nd trimester reference range 0.12-4.04 mIU/L.

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ANNEXURE-I

INFORMED CONSENT

INFORMED CONSENT (MODEL OF THE CONSENT FORM IS ENCLOSED) DESCRIBING THE FOLLOWING

Mrs.…… you are invited to participate in **"ESTABLISHMENT OF REFERENCE RANGE OF SERUM THYROID STIMULATING HORMONE IN FIRST AND SECOND TRIMESTER OF PREGNANCY IN A TERTIARY CARE HOSPITAL – A CROSS SECTIONAL STUDY"**.

Participation in this study is completely voluntary. All the registered pregnant women will be enrolled in this study at Jawaharlal Nehru Medical College, Belgaum.

PURPOSE OF THE STUDY

Subclinical hypothyroidism is associated with adverse perinatal outcome. For that reason pregnant women should be properly classified according to their thyroid status. So there should be separate reference range of serum TSH for pregnant women of this region.

PROCEDURE

For all pregnant women participating in the study, 3-5 ml of venous blood will be collected in first and second trimester under aseptic precautionary measures using sterile disposable syringe.

RISKS

Since the blood is drawn under aseptic precautionary measures by trained persons there is no scope for any risks. Further only small volume of blood is collected which will be spontaneously replenished in the body. However there may be minor risks associated with having blood drawn that may include bruising, redness, discomfort or bleeding at the puncture site.

BENEFITS

No direct benefit for participants. But this study will help the pregnant women in future for better management of their thyroid status during pregnancy.

OPTIONS

If you decide not to participate in this study, the hospital will provide you the usual standard care and treatment.

NEW INFORMATION

Reference range of serum TSH in this region of India using chemiluminescent assay can be established.

PRIVACY AND CONFIDENTIALITY

All information collected about you during the course of the study will be kept confidential to the extent permitted by law. You will be identified in this research record by the code numbers. Information which identifies you personally will not be revealed without your written permission. However your records may be revealed to the sponsor of the study. Information from this study may be published but your identity will be confidential in any publication.

INSTITUTIONAL POLICY

In the event that you are physically injured as a result of participating in this research emergency care will be available. There is no commitment to provide any

compensation for research related injury. The Jawaharlal Nehru Medical College will provide, within the limitations of the laws of the state of Karnataka, facilities and medical attention to subjects who suffered any harm as the result of your participation in this study. In the event you believe that you have suffered any how as a result of your participation in this study you may contact research guide Dr.ANIL MALLESHAPPA, Associate Professor, Department of Biochemistry, Jawaharlal Nehru Medical College, KLE University, Belgaum.

COST FOR PARTICIPATION

You will not be charged for the test to be carried out on your blood sample.

FINANCIAL INCENTIVE FOR PARTICIPATION

You will not receive any remuneration for participating in this study.

VOLUNTARY PARTICIPATION/WITHDRAWAL

If you decide not to participate in this study, it will not affect the quality of the medical care you receive at this institution.

You may withdraw from the study anytime. The researchers might use the information learned from the study in scientific journal articles or in presentations.

In case you have any questions regarding your rights as a study participant, you may please contact Dr.V.D.Patil, Principal, Jawaharlal Nehru Medical College, KLE University, Belgaum Telephone No. 0831-2471701 and Dr.P.V.Patil, Chairman of Jawaharlal Nehru Medical College Institutional Ethics Committee of Human Subjects Research, Telephone No. 0831-2474025

EMERGENCY PROVISION

If you have questions as a participant in our study, you can contact the Department Of Biochemistry, J. N. Medical College, KLE University, Belgaum – 590 010

CONSENT TO PARTICIPATE IN A RESEARCH TRIAL

I voluntarily agree to take part in this study. If I choose to take part in the study, I may withdraw at anytime. I am not giving any of my legal right by signing this form. My signature below indicates that I have read, or had read to me, this entire consent form including the risks and benefits. I may ask questions at any time.

_____ _____

Signature of participant Date

_____

Participants Name (Printed):

_____ _____

Name and Signature of witness-1 Date

_____ _____

Name and Signature of witness-2 Date

_____ _____

Signature of researchers or Date
Person obtaining consent

ANNEXURE-II

PROFORMA

QUESTIONNAIRE (PROFORMA) USED FOR COLLECTING THE DATA

"ESTABLISHMENT OF REFERENCE RANGE OF SERUM THYROID STIMULATING HORMONE IN FIRST AND SECOND TRIMESTER OF PREGNANCY IN A TERTIARY CARE HOSPITAL – A CROSS SECTIONAL STUDY"

Name :

Age : O.P.D. No. :

Date of visit :

Address :

Past history

- History of thyroid dysfunction in the past.
- History of diabetes or glucose intolerance.

Family history

- Thyroid dysfunction in family members.

Drug history

Any medication that can change thyroid status:

- Antithyroid drugs
- Lithium carbonate

- Levodopa
- Iodine-containing contrast agent

Personal history

- Whether they are taking iodised salt or prepare themselves:

Obstetrics history

- Para :
- Gravida :
- L.M.P. :
- Any past history of miscarriage :
- Weight gain during present pregnancy :
- Vomiting in pregnancy

General Physical Examination

Vitals

Pedal oedema	:	Pulse rate	:
Blood pressure	:	Respiratory rate	:
Icterus	:	Temperature	:
Weight	:		

Examination of thyroid gland

Presence of any palpable thyroid swelling:

Investigations

Blood

Serum TSH :

- First trimester:
- Second trimester:

Serum Anti-TPO :

USG

First trimester for calculating gestational age:

ANNEXURE-III

MASTER CHART

Sl. No.		**Age** (in years)	**Gestational age** (in weeks)	**Anti-TPO** (in IU/L)	**TSH 1st trimester** (in mIU/L)	**TSH 2nd trimester** (in mIU/L)
PRW	1	21	8	0.71	0.74	1.18
PRW	2	21	14	2.5	2.32	
PRW	3	21	11	3	1.17	
PRW	4	26	13	8.6	0.38	
PRW	5	17	10	2.7	0.05	0.004
PRW	6	19	8	3.6	3.45	
PRW	7	22	10	6.6	4.1	3.05
PRW	8	21	14	4.4	3.2	
PRW	9	21	14	3.8	0.91	0.89
PRW	10	24	11	3.3	0.87	2.09
PRW	11	19	13	2.8	2.24	
PRW	12	24	11	3.9	0.3	2.14
PRW	13	25	10	4.2	3.23	7.1
PRW	14	22	8	9.2	0.004	0.14
PRW	15	19	13	4	0.36	1.53
PRW	16	20	10	2.6	1.14	
PRW	17	22	10	4	0.45	2.31
PRW	18	21	12	3.6	1.04	
PRW	19	30	9	12.6	0.84	0.91
PRW	20	23	13	11.6	3.2	
PRW	21	25	14	3.4	0.79	
PRW	22	21	12	9.2	1.63	2.06
PRW	23	28	13	24	1.38	
PRW	24	22	12	24.4	0.39	
PRW	25	26	11	11	1.43	1.37
PRW	26	27	10	10	0.75	1.87
PRW	27	28	11	26	2.16	1.68

Sl. No.		**Age** (in years)	**Gestational age** (in weeks)	**Anti-TPO** (in IU/L)	**TSH 1st trimester** (in mIU/L)	**TSH 2nd trimester** (in mIU/L)
PRW	28	25	8	31.6	1.14	3.17
PRW	29	20	9	10.3	0.58	
PRW	30	21	13	31.3	1.32	1.05
PRW	31	20	11	10	2.2	2.28
PRW	32	23	9	10	3.95	
PRW	33	22	14	10	0.84	2.54
PRW	34	20	14	10	3.37	3.59
PRW	35	30	11	21.6	2.9	
PRW	36	21	13	10.6	0.93	
PRW	37	21	13	40.6	0.96	1.7
PRW	38	21	13	10	1.45	1.95
PRW	39	20	8	10	0.5	
PRW	40	20	11	10	1.62	3
PRW	41	24	10	25.5	0.29	1.33
PRW	42	32	9	29.7	0.45	0.1
PRW	43	19	13	30.9	0.52	
PRW	44	18	8	<10	0.03	
PRW	45	23	11	15.7	1.37	1.7
PRW	46	20	13	<10	0.75	
PRW	47	24	14	32.3	0.73	
PRW	48	25	13	10.5	1.22	1.7
PRW	49	18	10	10	0.75	
PRW	50	23	12	<10	0.96	
PRW	51	17	9	12.6	0.99	3.6
PRW	52	20	11	14.8	1.49	1.6
PRW	53	28	13	9.9	0.566	
PRW	54	23	13	9.7	0.025	
PRW	55	25	8	9.6	1.07	2.15
PRW	56	31	14	<10	0.77	
PRW	57	20	11	<10	0.71	

Sl. No.		**Age** (in years)	**Gestational age** (in weeks)	**Anti-TPO** (in IU/L)	**TSH 1st trimester** (in mIU/L)	**TSH 2nd trimester** (in mIU/L)
PRW	58	20	13	9.2	1.3	1.21
PRW	59	24	11	<10	0.258	
PRW	60	22	9	<10	0.97	
PRW	61	22	9	15.4	2.332	3.83
PRW	62	19	10	16	0.028	
PRW	63	20	8	24.3	1.33	
PRW	64	19	14	14.9	1.81	
PRW	65	21	8	24.4	0.47	
PRW	66	30	13	9.8	2.63	3.76
PRW	67	23	10	15	0.7	
PRW	68	25	10	9.2	0.01	
PRW	69	20	12	18.5	3.9	
PRW	70	19	8	12.4	2.1	
PRW	71	19	9	<10	0.42	
PRW	72	18	9	9.6	0.22	
PRW	73	21	13	9.6	0.06	0.74
PRW	74	19	13	<10	0.53	1.36
PRW	75	21	11	<10	0.35	
PRW	76	20	9	23.8	2.28	
PRW	77	20	12	9.8	2.51	
PRW	78	19	13	9.9	0.57	0.56
PRW	79	21	10	8.9	0.89	0.53
PRW	80	25	12	9.9	1.23	1.23
PRW	81	21	13	<10	0.82	
PRW	82	22	9	<10	0.67	
PRW	83	20	8	11.1	0.43	
PRW	84	28	13	<10	2.73	
PRW	85	19	8	9.2	3.76	2.27
PRW	86	30	13	13.6	1.37	
PRW	87	23	9	16.7	1.05	

Sl. No.		**Age** (in years)	**Gestational age** (in weeks)	**Anti-TPO** (in IU/L)	**TSH 1st trimester** (in mIU/L)	**TSH 2nd trimester** (in mIU/L)
PRW	88	22	8	9.6	0.7	
PRW	89	19	14	<10	1.35	
PRW	90	23	11	<10	1.82	0.85
PRW	91	24	12	33.3	1.51	
PRW	92	24	12	9.9	0.02	
PRW	93	23	8	26.1	0.82	
PRW	94	30	12	<10	0.17	
PRW	95	30	8	26.9	2.49	
PRW	96	21	13	<10	0.21	
PRW	97	23	8	35.8	0.53	
PRW	98	20	14	<10	0.74	
PRW	99	25	13	24.8	0.59	
PRW	100	18	14	11.4	0.98	