Effects of sample handling and analytical procedures on thyroid hormone concentrations in pregnant women's plasma

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Abstract

Background—Maternal thyroid function is a critical mediator of fetal brain development. Pregnancy-related physiologic changes and handling conditions of blood samples may influence thyroid hormone biomarkers. We investigated the reliability of thyroid hormone biomarkers in plasma of pregnant women under various handling conditions.

Methods—We enrolled 17 pregnant women; collected serum and plasma were immediately frozen. Additional plasma aliquots were subjected to different handling conditions before the analysis of thyroid biomarkers: storage at room temperature for 24 or 48 hours before freezing and an extra freeze–thaw cycle. We estimated free thyroid hormone indices in plasma based on T3 uptake.

Results—High correlations between plasma and serum (>0.94) and intra-class correlation coefficients for plasma handling conditions (0.96 to 1.00) indicated excellent reliability for all thyroid hormone biomarkers.

Conclusion—Delayed freezing and freeze–thaw cycles did not affect reliability of biomarkers of thyroid function in plasma during pregnancy.

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Conflicts of Interest:
There is no conflict of interest concerning this study.

INTRODUCTION

Prenatal maternal thyroid function is a critical mediator of fetal brain development[1,2]. The relationship between maternal thyroid hormone concentrations measured in stored blood samples and child neurodevelopmental outcomes is of particular interest in birth cohorts that have adequate statistical power to investigate rarer outcomes such as cerebral palsy, autism, or attention-deficit hyperactivity disorder (e.g. Norwegian Mother and Child Cohort Study and Danish National Birth Cohort)[2–7]. Among maternal thyroid hormone biomarkers, thyroid stimulating hormone (TSH) is of paramount importance[8], but free thyroid hormone levels (free thyroxine, free T4, and free triiodothyronine, free T3) have relevance to the study of offspring neurodevelopmental impairments and disorders[9–13]. In pregnant women, because of changes in plasma binding proteins and circulating thyroid hormone levels, immunoassays for free thyroid hormone levels perform poorly[14,15]. However, the gold standard assay for free T4, incorporating equilibrium dialysis, can only be performed on serum[8]. In the two birth cohorts mentioned above, however, maternal plasma but not serum was stored[4,16]. And as with many large-scale birth cohort studies, including the Norwegian Mother and Child Cohort Study and the Danish National Birth Cohort, the protocol was streamlined and cost-efficient, with unrefrigerated shipment of samples before long-term freezing at a central biorepository, and samples subjected to various processing schedules, transport durations and temperatures (eTable 1). Furthermore, analyses of stored biospecimens will necessarily be performed on thawed samples, with specimens having potentially experienced several freeze–thaw cycles due to aliquoting for various analytical purposes. Although effects of some handling conditions and storage on thyroid hormone measurements in blood have been studied[17–21], few data on pregnant women are available.

Thus, the goals of the present study were to evaluate in blood specimens from pregnant women the reliability of thyroid hormone biomarkers in plasma versus serum, including an alternate estimate of free thyroid hormone levels in plasma, and the effects of storage at ambient temperature and freeze–thaw cycles on plasma biomarkers.

METHODS

We enrolled 17 pregnant women who were having blood drawn as part of their routine prenatal care at the North Carolina Women's Hospital (Chapel Hill, North Carolina, USA), and obtained informed consent. Eligible women were in their third trimester of pregnancy, age 18 or older, spoke English, and had no known thyroid-related condition. All samples were given anonymously. This study was approved by the Institutional Review Board of UNC Chapel Hill.

From each woman, we collected 25 mL of blood and processed it within one hour of collection. Bloods were spun and separated into one 1.5 mL serum and five 1.5 mL plasma aliquots. Serum and one plasma aliquot were stored immediately at −80°C, representing the optimal treatment condition. One aliquot of plasma was left unrefrigerated at room temperature for 24 hours, and one for 48 hours, before being stored at −80°C. After overnight storage at −80°C, one plasma aliquot from each time point was allowed to thaw completely (two hours at 4°C) and re-frozen at −80°C (Figure 1).

Biomarker measurements in serum and plasma were conducted at ARUP Laboratories (Salt Lake City, Utah, USA) using immunoassays (see eAppendix 1): total T3, total T4, TSH, T3 uptake, and thyroid peroxidase antibody. Additionally, free T4 was measured by equilibrium dialysis followed by high performance liquid chromatography-tandem mass spectrometry in serum only. T3 uptake, which is an indirect measure of thyroxine binding globulin binding capacity[22], was used to calculate the indices of free T4 and free T3 (eAppendix 2)[29].

We used Spearman's rank correlation to examine the strength of association between serum and plasma biomarker levels, and Wilcoxon signed-rank test to investigate potential differences between serum and optimally treated plasma. For parametric analysis, variables were natural log-transformed if necessary to achieve normal distributions. The 95% confidence intervals (CI) were computed from the bootstrap distribution.

As a measure of reliability across the five plasma handling conditions (Figure 1), we calculated intra-class correlation coefficients (two-way random effects model) for each thyroid hormone variable (except free T4). The intra-class correlation coefficient reflects the proportion of the intra-individual variance attributed to sample handling. A value close to one indicates a high reliability across sample handling categories.

To investigate the effect of time spent at ambient temperature and an additional freeze–thaw cycle on plasma thyroid hormones, we fitted a linear mixed-effect model (random slope model) to the data for each measured variable using R and lme4[23,24]. Consistent with other studies[17,25] we assumed a linear relationship between time and freeze–thaw with hormone levels. We performed analyses on a long file containing thyroid hormone levels as the dependent variable ($N_{cases}=17 \times 5$). The fixed effects were *time* (delay before freezing: 0, 24 and 48 hours), *freeze* (1 vs 2 freeze–thaw cycles), and the interaction between time and freeze.

RESULTS

Spearman's rho was > 0.94 for all measured biomarkers comparing plasma to serum (Table 1). The average value of T3 uptake was somewhat higher in serum as compared to optimally treated plasma (Table 1; eFigure 1). Across the five plasma handling conditions (see Figure 1) intra-class correlation coefficients were high; ranging from 0.98 to 1.00 for total T4, total T3, T3 uptake, thyroid peroxidase antibody, and TSH (Table 1). We found no fixed effects of time or an additional freeze–thaw cycle on T3 uptake, total T4, TSH or thyroid peroxidase antibody in plasma (Table 2). There was, however, a positive effect of time on total T3 levels, increasing levels by about 1.35 ng/dL for each 24 hours (95% CI 0.01–2.66). No interactions between time and freeze–thaw cycles were found.

Free T4 levels in serum measured using the equilibrium dialysis approach showed strong, positive associations with T3 uptake and the calculated free T4 index in optimally treated plasma (Figures 2a–b). There was a weaker association between serum free T4 with total T4 in optimally treated plasma (Figure 2c). Free T4 showed a similar pattern of association with serum levels of free T3 index, T3 uptake and total T4 (eFigure 2). Furthermore, intra-class correlation coefficients for free T4 and free T3 indices across plasma handling categories

were high (> 0.96; Table 1). Free T3 index was higher in serum versus optimally treated plasma, following the pattern of T3 uptake (Table 1).

DISCUSSION

The high correlation of all measured biomarkers between serum and optimally treated plasma, and high intra-class correlation coefficients across the plasma handling conditions, indicates that plasma collected from large-scale birth cohort studies subjected to variable handling conditions are likely to provide reliable measures of total T4, total T3, TSH, T3 uptake, and thyroid peroxidase antibody. Although a small, positive fixed effect of time at ambient temperature was found for total T3 levels, even with this increase for 48 hours the change was < 2% of the median.

To our knowledge, no other studies have investigated the effect of time spent at ambient temperature and freeze–thaw cycles on total T3 levels in plasma or serum, although other thyroid function biomarkers have been examined. Various handling conditions in repeated samples from the same healthy, non-pregnant individuals (n=10) showed no effect on free T3, free T4, or TSH in plasma kept 0, 2, 4, 6, 24 or up to 72-hours at room temperature[17]. No differences were found in measured levels of TSH, free T4, thyroid peroxidase antibody, or thyroglobulin antibody in sera samples from non-pregnant women (n=8) stored up to 6 days at 4°C[25]. Additionally, comparisons of frozen (n=50) versus fresh sera (n=50) from pregnant subjects showed that a freeze–thaw cycle resulted in only a minor increase in free T3[25]. In a study of pooled serum samples from various patients, TSH appeared stable in serum stored up to 4 days at room temperature[20].

It may be important to distinguish between the effects of time delays both pre- and post-blood separation. Our study specifically addressed post-separation time delays, as was typical for plasma in the Norwegian Mother and Child Cohort Study[16,26] (eTable 1). Although pre-separation delay may be more relevant for the Danish National Birth Cohort [4,27], a previous study has indicated that thyroid hormone biomarkers in plasma and sera are similarly stable in room temperature with up to 72 hours delay in blood separation[17].

Accurate assessment of free T4 and free T3 during pregnancy is of great importance to the study of offspring neurodevelopmental outcomes[9–13]. However, pregnancy-induced elevations in thyroxine binding globulin and total thyroid hormones and decreases in blood albumin interfere with immunoassays of free thyroid hormone levels and the equilibrium dialysis approach in serum is recommended[8,22]. Maternal plasma, and not serum, is stored in birth cohorts such as the Norwegian Mother and Child Cohort Study and the Danish National Birth Cohort. We investigated a surrogate biomarker, T3 uptake, that measures the relative amount of unbound thyroxine binding globulin[20] and can be used to calculate free T4 and free T3 indices [28]. These indices may provide a more accurate estimate of free thyroid hormones than immunoassays since they mathematically account for the increased thyroxin binding globulin levels during pregnancy[14,15,22]. We found that the calculated free T4 index in serum and plasma, and plasma free T3 index, were within normal values for non-pregnant[29] and pregnant women[14,30], and the free T4 index showed a high, positive

correlation with serum free T4. Additionally, the high intra-class correlation coefficients across plasma handling conditions suggest these calculated indices provide a reliable estimate of free T4 and free T3 during pregnancy.

In conclusion, plasma levels of total T4, total T3, TSH, T3 uptake, and thyroid peroxidase antibody are reliable biomarkers even when subjected to extended time at ambient temperature and an additional freeze–thaw cycle.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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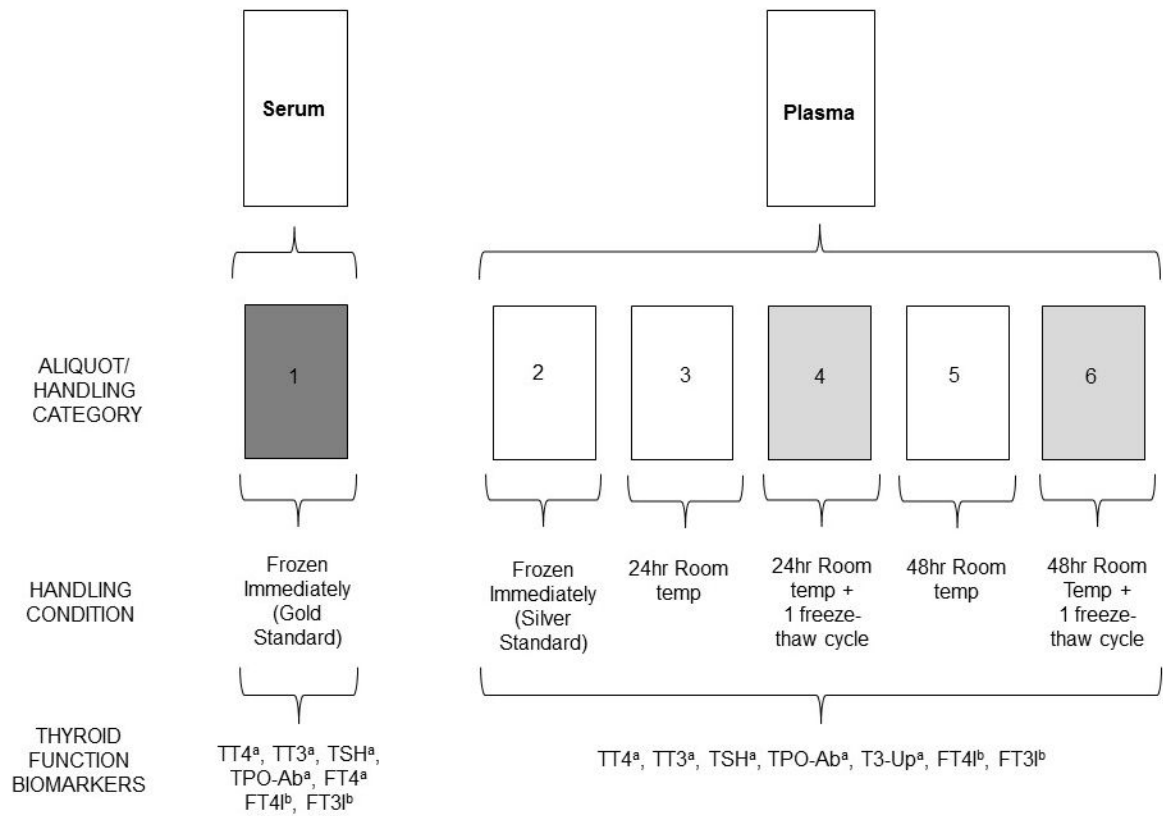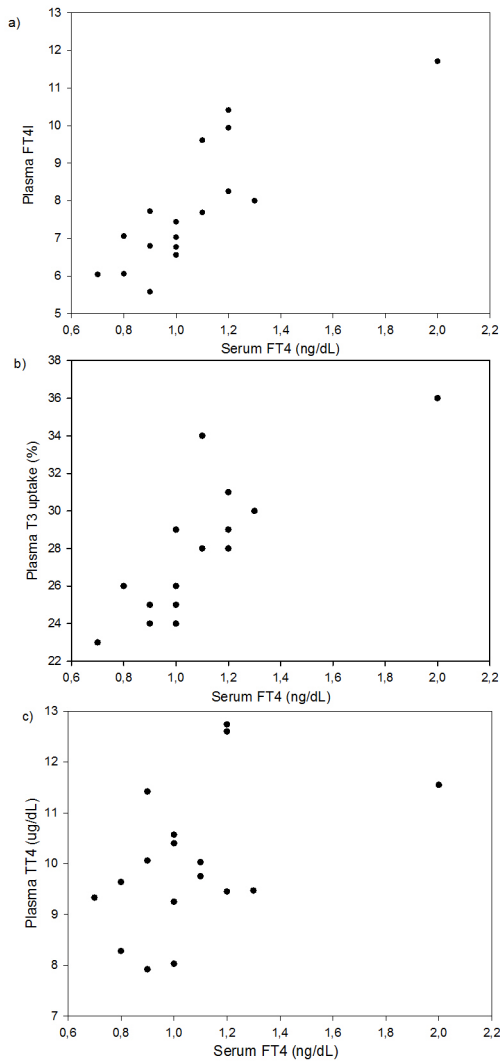
Figure 1.
Schematic overview of handling conditions of serum and plasma from 17 pregnant women and the thyroid function biomarkers that were analyzed.

TT4=Total thyroxine, TT3=Total triiodothyronine, FT4=free thyroxine, FT4I= free thyroxine index, FT3I=free triiodothyronine index, TSH=Thyroid stimulating hormone (thyrotropin), TPO-Ab = Thyroxine peroxidase antibody, T3-Up=T3 uptake

[a]TT4, TT3, and T3-Up were measured by electrochemiluminescent immunoassay (ECLIA). TSH and TPO-Ab were measured by chemiluminescent immunoassay (CLIA). FT4 in serum was measured by equilibrium dialysis with high-performance liquid chromatography-tandem mass spectrometry (ED-HPLC-MS/MS).

[b]FT4I and FT3I in plasma were calculated from T3 uptake.

Figure 2.
Scatter-plots of free thyroxine (free T4, FT4) in serum of 17 pregnant women measured by "gold standard" method equilibrium dialysis with high-performance liquid chromatography–tandem mass spectrometry in relation to **a)** Free T4 index (FT4I) in optimally treated plasma (Spearman's rho, r_s =0.81, CI 0.52–0.94), **b)** T3 uptake (T3-Up) in optimally treated plasma (r_s=0.79; CI 0.49–0.92)[1] and c) Total thyroxine (total T4, TT4) in optimally treated plasma (r_s=0.45, CI −0.02–0.73).

[1] Only 15 points are showing in plot b) due to overlapping points for two samples on two occasions.

Table 1

Intra-class correlation coefficient of thyroid hormones variables across all five plasma handling categories (see Figure 1), and comparing serum and optimal treated plasma from 17 pregnant women (only 14 women had thyroxine peroxidase antibody above detection) using Spearman's rank correlation and Wilcoxon signed-rank test.

Thyroid variable	Serum vs. plasma	Plasma (handling category 2–5)
	Spearman's rho (r_s) (95% CI lower, upper)	Intra-class correlation coefficient (95% CI lower, upper)
Total thyroxine, total T4	0.97 (0.84, 1.00)	0.98 (0.95, 0.99)
Total triiodothyronine, total T3	0.99 (0.93, 1.00)	0.98 (0.95, 0.99)
Thyroid stimulating hormone, TSH	1.00 (0.96, 1.00)	1.00 (0.999, 1.000)
Thyroxine peroxidase antibody, TPO-Ab	0.94 (0.78, 0.99)	0.98 (0.96, 0.99)[a]
T3 uptake	0.98 (0.95, 1.00)[b]	0.98 (0.95, 0.99)
Free T4 index	0.97 (0.84, 1.00)	0.97 (0.95, 0.99)[a]
Free T3 index	0.99 (0.95, 1.00)[c]	0.96 (0.93, 0.99)[a]

[a] variable was ln-transformed to approximate normal distribution before analyses

[b] Difference plasma vs. serum (Wilcoxon signed-rank test; 95% CI −1.0000, −0.9999)

[c] Difference plasma vs. serum (Wilcoxon signed-rank test; 95% CI −7.0, −1.5)

Table 2

Fixed effects of time left unrefrigerated and freeze–thaw cycle(s) on measured thyroid hormone biomarkers in plasma of pregnant women

	Time Left Unrefrigerated[a]		Freeze–Thaw[a]		Interaction between time and freeze–thaw[b]	
	Fixed effect	95% Confidence Interval	Fixed effect	95% Confidence Interval	Fixed effect	95% Confidence Interval
TT3	1.35	0.01, 2.66	0.41	−2.07, 2.72	−0.88	−4.08, 2.17
T3 uptake	0.12	−0.36, 0.26	−0.06	−0.06, 0.28	0.29	−0.13, 0.77
TT4	0.03	−0.04, 0.10	−0.05	−0.16, 0.05	0.05	−0.10, 0.21
TSH	0.01	−0.01, 0.02	−0.01	−0.02, 0.00	−0.01	−0.03, 0.02
TPO-Ab[c]	0.001	−0.01, 0.02	−0.02	−0.02, −0.01	0.04	−0.01, 0.10

[a]Model includes only fixed effect of time left unrefrigerated (ordinal categorical) or freeze–thaw (dichotomous) respectively.

[b]Model includes fixed effects of time left unrefrigerated, freeze-thaw, and time*freeze thaw interaction term.

[c]The variable was natural log-transformed to approximate normal distribution