Low-Normal Concentrations of Free Thyroxin in Serum in Late Pregnancy: Physiological Fact, Not Technical Artefact

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Free thyroxin (FT₄) concentrations, total thyroxin/thyroxin-binding globulin (T₄/TBG) ratios, and thyrotropin (TSH) and albumin concentrations were measured in serum in a longitudinal study in each of the three trimesters of 25 normal pregnancies. In late pregnancy, FT₄ estimates by assays reputedly either affected or unaffected by albumin were in the lower half of the reference range for nonpregnant subjects. T₄/TBG ratios and albumin concentrations were similarly lower. FT₄ overall was significantly (P < 0.001) correlated with these latter two values. Serum TSH concentrations increased as FT₄ declined in late pregnancy. Nonesterified fatty acid (NEFA) concentrations were too low to displace T₄ from its binding proteins and were not correlated with other measurements. Within any one of the trimesters, FT₄ and T₄/TBG were independent of variations in TBG or albumin concentrations. This implies that lower FT₄ concentrations in late pregnancy are real, merely coinciding with parallel decreases in albumin. They are not artefacts of albumin-affected assays.

Additional Keyphrases: thyroid function in pregnancy • albumin • nonesterified fatty acids • thyroxin-binding globulin

During the last decade, especially since the use of free thyroxin (FT₄) analog assays, the thyroid status of the normal pregnant woman has been a controversial subject.4 Because both thyroxin (T₄) and thyroxin-binding globulin (TBG) are increased in pregnancy (1-4), FT₄ concentrations reflect thyroid functional status more accurately (5, 6). This was first measured routinely by thyroid hormone binding tests (THBR; e.g., T₃ uptake, T₃U) combined with T₄ assays (7, 8). Correction of increased T₄ by the results of THBR, empirically approximated to an estimation of FT₄ (free thyroxin index, FTI). FTI values for pregnant women usually lay within the normal nonpregnant reference interval. High-TBG sera were approximately normalized (9, 10). However, FTI was less successful for nonpregnant subjects with TBG excesses as great as or greater than those of late pregnancy (1, 9-11).

Direct equilibrium dialysis (12) indicated that FT₄ for normal late pregnancy was in the low end of the reference interval. Indirect methods such as the Sterling-Brenner technique (13) gave less consistent results (14-16). Later studies involving direct dialysis or ultrafiltration techniques (17-19) confirmed the earlier findings (12). T₄/TBG ratios in late pregnancy were also in the low part of the reference range (3, 4, 20). Such ratios largely define FT₄ (3, 4, 20) so that lower estimates should result.

When one-step analog techniques for direct estimation of FT₄ emerged (21), values during late pregnancy were again clustered near the lower border of the assay's reference interval (21-23). However, the validity of these assays was queried when they were found to be affected by the albumin concentration in serum (24-27). Because albumin concentrations are about 25% lower in late pregnancy (28), the low values for analog FT₄ assays were claimed to be falsely low through assay interference (28). Nevertheless, several other “unaffected” methods also gave low-normal values (29-31).

To resolve this uncertainty, we re-examined the relationship between FT₄ (or T₄/TBG) estimates and albumin in a longitudinal study of 25 normal women through pregnancy. In each trimester, FT₄ was measured by three direct one-step methods (all from Amersham International plc, Amersham, Bucks., U.K.): an analog radioimmunoassay (Amerlex-M™ FT₄), which is affected by tracer binding to serum albumin (24-28); a nonradioactive luminescence analog method (Amerlite™ FT₄), which is unaffected by albumin or other proteins, except for thyroid hormone auto-antibodies (32, 33), and a “labeled antibody” assay (Amerlex-MAB™ FT₄) that is claimed to be insensitive to protein interference (including that from auto-antibodies). T₄, TBG, thyrotropin (TSH), nonesterified fatty acids (NEFA), and albumin were also assayed. We could then examine whether (a) lower FT₄ was a technical artefact, or merely a physiological phenomenon linked coincidentally with parallel changes in albumin concentrations during gestation and (b) NEFA concentrations were normally high enough to displace T₄ from its binding proteins, hence increasing the concentration of FT₄.

Materials and Methods

Subjects and Samples

We selected 25 women in good health (ages 19-39, mean 27 years) without history or symptoms of thyroid dysfunction and taking medication only for minor complaints unconnected with thyroid disease. All were negative for rhesus factor. The only medication given during pregnancy was anti-anemia therapy with folate/B₁₂. Pregnancy was uncomplicated, with normal outcomes and recovery. Two subjects did not present in the third trimester.

For each patient in each trimester, 10-mL blood samples were taken by venipuncture into syringes with no additives. Samples were clotted in stasis at room temperature (20 °C) for 1 h, then separated with use of a bench centrifuge. The serum obtained was immediately divided into aliquots and stored at −20 °C until analyzed.

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**Assays**

$F_{T4}$. To measure $F_{T4}$, we used an analog radioimmunoassay (Amerlex-M $F_{T4}$) (A) in which the tracer is a $^{125}$I-labeled derivative of $T_4$ (thyroxin-ethylene diaminediacectate, $T_4$-EDTA) (34); an analog immunoassay (B) (Amerlite) based on the use of enhanced luminescence, the tracer being a conjugate of $T_4$ and horseradish peroxidase (EC 1.11.1.7); and a "labeled antibody" immunoradiometric assay (Amerlex-MAB) (C), with $^{125}$I-labeled monoclonal anti-$T_4$ antibodies, in which serum $F_{T4}$ and an accepting solid phase compete for binding. All these assays were commercially available from Amer sham International plc.

$T_4$, TSH, and TBG. These were measured by commercially available enhanced-luminescence immunoassays (Amerlite $T_4$, TSH, and TBG). The reference intervals (central 95th percentiles) recommended by the manufacturer are $T_4$, 65-165 (mean 104) nmol/L; TSH, 0.15-3.2 (mean 1.16) milli-int. units/L; TBG, 13.8-30.5 (mean 20.8) mg/L; and $T_4$/TBG, 3.31-7.21 (mean 4.71) nmol/mg. The TSH assay was unaffected by chorionic gonadotropin concentrations $>10^8$ int. units/L. Its sensitivity was 0.04 milli-int. unit/L. The interassay CVs quoted for each assay are 7.9-9.1% for $T_4$, 6.5-7.8% for TSH, and 6.9-9.0% for TBG.

**Albumin.** Serum albumin was measured by the bromocresol green method in an Olympus AU5000 analyzer (BDH Diagnostics Ltd., Poole, U.K.). The central 95% reference interval was 38-52 g/L, with an interassay CV of 4.3%.

**NEFA.** We used a commercially available kit (NEFAC™; Wako Chemicals GmbH) obtained from Alpha Laboratories, Eastleigh, U.K. The central 95% reference interval for nonpregnant subjects was 0.1-0.6 mmol/L, and the interassay CV was 1.1-2.7%.

Estimates in all assays were made in duplicate, according to the manufacturers' assay protocols. Results were analyzed with a standard statistical package and a desktop computer.

**Results**

**$F_{T4}$ Estimations**

Figure 1 shows values for serum $F_{T4}$ in each trimester by assays A–C. First-trimester values were scattered throughout the nonpregnant 95% central reference interval (Figure 1, a, d, g). In the last two trimesters, values were clustered near the bottom of the range (Figure 1, b, c, e, f, h, i) whether the assays were allegedly responsive (24-27) or not (32, 33) to variations in $T_4$-binding protein concentrations (primarily albumin). Assay results were strongly correlated. Linear-regression equations and correlation coefficients (n = 73) were respectively:

1) $[F_{T4}]_{A}(\text{Amerlex-MAB}) = 0.94 [F_{T4}]_{A}(\text{Amerlex-M}) + 0.43$. SE = 0.033, r = 0.93.

2) $[F_{T4}]_{A}(\text{Amerlex-MAB}) = 0.92 [F_{T4}]_{A}(\text{Amerlite}) + 0.79$. SE = 0.056, r = 0.93.

3) $[F_{T4}]_{A}(\text{Amerlite}) = 1.01 [F_{T4}]_{A}(\text{Amerlex-M}) - 0.27$. SE = 0.395, r = 0.99.

In the last two trimesters, mean $F_{T4}$ estimates were 72-78% of the 95% central reference-interval mean, but about 100% in the first trimester (Table 1). Values were more narrowly spread in later trimesters. "Albumin-sensitive" assay A mimicked the "insensitive" assays B and C. Differences were significant ($P < 0.001$, paired $t$-test) between the first and later trimesters but not ($P > 0.05$) between the two later ones.

It is very unlikely that the above results could emanate from artefacts arising from the choice of antibodies in these assays, particularly as the polyclonal antibodies used in the Amerlex-M and Amerlite assays are quite different in both origin and detailed interactivity with $T_4$ from the monoclonal antibody used in the Amerlex-MAB assay. In addition, assays from other sources also show similar trends (29-31).

**Estimation of $T_4$, TBG, and $T_4$/TBG Ratios**

Mean $T_4$ concentrations were (1st trimester) 174 (SD 49.5); (2nd) 157 (SD 31.8); (3rd) 170 (SD 27.9) nmol/L,
167%, 151%, and 163% of the nonpregnant 95% central reference-interval mean. For TBG, the means were (1st trimester) 35.1 (range 21–50); (2nd) 42.0 (range 27–59); (3rd) 43.0 (range 36–56) mg/L, 169%, 202%, and 207% of the 95% central reference-interval mean.

First-trimester \( \frac{T_4}{TBG} \) ratios were distributed in the reference interval similarly to \( FT_4 \) (Figure 2). The mean (4.94) was 105% of the nonpregnant euthyroid 95% central reference-interval mean (4.71), and the 95% range was 3.28–6.60. Values in the first trimester were significantly larger (\( P < 0.001 \), paired t-test) than in either of the later ones. In the latter, ratios were similarly (\( P > 0.05 \)) lower in the range or slightly below it. For these trimesters, the mean was 3.79 (95% range 2.36–5.22) for the second and 3.95 (95% range 3.00–4.90) for the third. Mean values, expressed as percentages of the nonpregnant 95% central reference-interval mean, were 80% and 84% in the last two trimesters. These compare closely with all of the \( FT_4 \) results. \( \frac{T_4}{TBG} \) was correlated with results of these assays (\( P < 0.001 \), \( r = 0.72–0.80 \), \( n = 72 \)).

**Albumin Concentrations**

Serum albumin ranges and means (g/L) were 36–49, mean 41.3 (1st trimester); 33–38, mean 35.8 (2nd); 34–41, mean 35.2 (3rd). Mean concentrations in later trimesters were significantly (\( P < 0.001 \), paired t-test) lower than in the first (85% and 80% of the first trimester and 95% central reference-interval mean, respectively). Late trimesters did not differ (\( P > 0.05 \)).

**Correlation between Serum Albumin and \( FT_4 \) Estimations**

For all sera (\( n = 73 \)), \( FT_4 \) and albumin estimates were strongly (\( P < 0.001 \)) correlated. Figure 3 depicts the Amerlite assay. Regression equations and correlation coefficients for \( FT_4 \) and \( \frac{T_4}{TBG} \) against albumin were:

- **Amerlex-M**: \( \left[ FT_4 \right] (\text{pmol/L}) = 0.715 \text{[albumin]} (\text{g/L}) - 13.4 \), \( SE = 2.127 \), \( r = 0.63 \).
- **Amerlite**: \( \left[ FT_4 \right] = 0.74 \text{[albumin]} - 14.5 \), \( SE = 2.127 \), \( r = 0.65 \).
- **Amerlex-MA.B**: \( \left[ FT_4 \right] = 0.59 \text{[albumin]} - 9.14 \), \( SE = 2.233 \), \( r = 0.50 \).
- **\( T_4/TBG \)**: \( \frac{T_4}{TBG} = 0.28 \text{[albumin]} + 0.33 \), \( SE = 0.802 \), \( r = 0.38 \), \( P < 0.001 \).

Sera were grouped by their trimester. Subgroups were compared, spanning limited ranges of albumin concentrations, both within a trimester (having different mean albumin concentrations) and between trimesters (having similar concentrations). If albumin interference caused lower \( FT_4 \) estimates (28) then, in a given trimester, \( FT_4 \) values in sera with more albumin should be higher on average than in those with less. Conversely, in sera with similar albumin concentrations compared between trimesters, \( FT_4 \) should likewise be similar. If true lower \( FT_4 \) concentrations in serum arose physiologically, and were not the result of a technical artefact, estimates should be independent of albumin within a trimester, but significantly different between trimesters, regardless of albumin concentrations.

On using these subdivisions, \( FT_4 \) estimates (all methods) were similar (\( P > 0.05 \), Student's unpaired t-test) within a trimester, in subgroups where mean albumin differed by 4.3 g/L. Differences were significant (\( P < 0.001 \)) between first trimester and other subgroups with means differing by only 2.1 g of albumin per liter (Table 2).

Thus, the decrease in \( FT_4 \) in late pregnancy is physiologically based. Significant correlations, based on all sera (Figure 3), merely reflect parallel declines in \( FT_4 \) and albumin concentrations during gestation.

**TSH Concentrations**

Mean TSH in the first trimester was 0.86 milli-int. unit/L (SD 0.47, range <0.06–2.1), significantly lower (\( P < 0.001 \), paired t-test) than in the second (mean = 1.50, SD 0.73, range 0.7–3.6) or third (mean = 1.49, SD 0.54, range 0.6–2.5). The first-trimester mean was 74% of the 95% central reference mean (1.16 milli-int. units/L) and in later trimesters, 129%. One first-trimester estimate (<0.06) was below the 95% central reference interval's lower limit (\( FT_4 \) 21 pmol/L) and one in the second exceeded its upper limit (\( FT_4 \) 11 pmol/L). TSH was uncorrelated with TBG or albumin (\( P > 0.05 \)) but was inversely correlated with \( \frac{T_4}{TBG} \) and \( FT_4 \) (\( P < 0.001 \), \( r = 0.32–0.38 \)).
Table 2. Mean Serum FT4 Concentrations in Pregnancy, Subgrouped According to Albumin Concentration

<table>
<thead>
<tr>
<th>Trimester</th>
<th>Albumin concentration ranges (g/L)</th>
<th>Mean albumin concentration ( \text{FT4} ) (mmol/L)</th>
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<tbody>
<tr>
<td>1st</td>
<td>33-35</td>
<td>39.1 (12)</td>
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<tr>
<td>2nd</td>
<td>34.4 (11)</td>
<td>37.0 (14)</td>
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<tr>
<td>3rd</td>
<td>34.9 (10)</td>
<td>37.6 (13)</td>
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Amerlex-M

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<td>3rd</td>
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<td>3rd</td>
<td>11.7</td>
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Amerlex-MAB

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<th>Mean albumin concentration ( \text{FT4} ) (mmol/L)</th>
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<tbody>
<tr>
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<td>11.9</td>
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<td>3rd</td>
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NEFA Concentrations

Mean concentrations (mmol/L) were: 1st trimester, 0.48, SD 0.20, range 0.17-0.85; 2nd, 0.51, SD 0.27, range 0.25-1.26; 3rd, 0.73, SD 0.30, range 0.35-1.54. These were too low to saturate albumin binding sites (35-37). Concentrations were different at low significance in the first two trimesters \( (P < 0.02) \) but in both cases were much lower \( (P < 0.001) \) than those in the third. NEFA estimates were independent of measurements of thyroid function or of albumin.

Molar NEFA/albumin ratios were: 1st trimester, mean 0.70 (range 0.24-1.21); 2nd, 0.85 (range 0.41-2.04); 3rd, 1.21 (range 0.60-2.64). Differences were significant \( (P < 0.01) \) but not between the third and earlier trimesters, but not between the first and second \( (P > 0.05) \) (Table 3). These ratios were too small to effect displacement of protein-bound \( \text{T}_{4} \) (36). Also, concentrations of unoccupied TBG binding sites are much higher in pregnancy. \( \text{T}_{4} \) displacement is more likely when sera have both less TBG and higher relative hormonal occupancy of binding sites.

Summary of Findings

Figure 4 links together the changes observed in the concentrations of the various analytes measured throughout gestation in the serum of each subject used in this study. Although for a minority of subjects the values occasionally resist the trends observed overall, the vast majority form a coherent whole in this respect. Table 3 also summarizes the changes in the mean concentration for each analyte studied.

Discussion

Our study of thyroid function in normal pregnant women has shown:

(a) First-trimester FT4 estimates were scattered within the normal reference interval, with a mean and spread of values typical of the nonpregnant reference group. Albumin concentrations in serum were typical of nonpregnant subjects. Increased FT4 was not found \( (38, 39) \). Higher frequencies of increased FT4 with low TSH were noted with severe morning sickness of early pregnancy \( (39) \), but TSH was subnormal in only one of 25 of our first-trimester group, with high-normal FT4.

(b) Mean serum FT4 was consistently 20 to 25% lower in the last two trimesters, compared with early pregnancy or nonpregnant subjects. This was unconnected with assay dependency on albumin. Allegations of errors in Amerlex-M FT4 \( (28, 37) \) were unsupported. FT4 values during late pregnancy were tightly clustered within the 95% reference interval for nonpregnant subjects, close to its lower border \( (3, 4, 9, 21) \).

(c) \( \text{T}_{4}/\text{TBG} \) ratios and FT4 changed similarly \( (3, 4, 20) \). This was expected because, especially in high-TBG pregnant women, such ratios largely determine FT4 \( (3, 4, 9, 20, 40, 41) \). Albumin concentrations also declined in pregnancy, but the connection with FT4 was physiological, and was not explained by technical artefacts as others have claimed \( (28, 37, 39) \).

(d) TSH changed reciprocally with FT4 and albumin. Its concentration in the first trimester was usually low in the 95% reference interval \( (42) \). High chorionicadotropin concentrations in early pregnancy could augment TSH activity in vivo \( (43) \). Hence, to maintain normal FT4, hypothalamus-pituitary-thyroid feedback control may require less TSH. Blunted responses to stimulation by thyrogblerin in early pregnancy \( (38) \) need not require increased FT4 \( (38, 39) \). In later trimesters, chorionicadotropin concentrations decline, allowing TSH to resume its usual regulatory potency. FT4 placement in the low-normal region of the 95%
reference interval then has as its corollary a small but significant increase in TSH to higher regions of its corresponding range (37, 42).

(c) Low NEFA concentrations in pregnancy serum could not increase FT₄ by displacing bound T₄ from TBG or albumin (36, 37). Indeed, NEFA concentrations did not change significantly within the first two trimesters at a time when FT₄ and albumin concentrations had already declined to those typical of late pregnancy. Hence, FT₄ assays were probably not compromised by NEFA effects, as proposed elsewhere (28).

Serum FT₄ in late pregnancy thus seems to mimic "mild compensated hypothyroidism" (44). As well as hormonal changes, cellular thyroid hormone receptors are increased (44, 45), compensating for lower concentrations of FT₄ in blood and promoting normal uptake rates (46).

Rejection of these conclusions (28), which encouraged opposition to FT₄ analog assays, stemmed largely from overemphasis of "albumin effects" in earlier methods (28) and historical reliance on empirical, inaccurate FTI results. FTI methods generally compensate incompletely for high TBG in pregnant or nonpregnant subjects (9, 41, 47) but perform better with moderately increased TBG typical of oral contraception therapy (9).

Prospective studies have advantages over those in which sera from each trimester are randomly selected. Longitudinal studies allow direct comparisons of parameters within changing physiologies unique to each patient, avoiding the problems of population variability. Our findings confirm and extend other studies on thyroid function in pregnancy (48) in which other FT₄ assays allegedly lacking "albumin artefacts" were used.

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