Longitudinal Concentrations of Vitamin $B_{12}$ and Vitamin $B_{12}$-binding Proteins during Uncomplicated Pregnancy

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Background: Because reference values for vitamin $B_{12}$ concentrations and vitamin $B_{12}$-binding capacities for pregnant women have not been established, the reference values for nonpregnant women are often applied to assess vitamin $B_{12}$ status. The aim of the present study was to describe ranges of biochemical indices of vitamin $B_{12}$ status, including red blood cell (RBC) vitamin $B_{12}$, saturated and unsaturated cobalamin-binding proteins, and binding capacities in all trimesters of uncomplicated pregnancy.

Methods: A total of 39 healthy pregnant women with long-term daily intake of vitamin $B_{12}$ $>2.6$ μg/day and uncomplicated pregnancies participated in the study throughout their pregnancies. RBCs and serum vitamin $B_{12}$, holo-haptocorrin, unsaturated cobalamin-binding proteins, unsaturated and total vitamin $B_{12}$-binding capacities, total homocysteine (tHcy), and RBC count were assessed in weeks 9–12, 20–22, and 36–38 of gestation.

Results: Significant changes in vitamin $B_{12}$ status occurred in the course of pregnancy. Serum vitamin $B_{12}$ concentrations and percentage of saturation of vitamin $B_{12}$-binding proteins decreased steadily throughout pregnancy. In the third trimester, 35% of the participants had serum vitamin $B_{12}$ concentrations $<150$ pmol/L and 68.6% had $<15\%$ saturation of total vitamin $B_{12}$-binding capacities, but no women had RBC vitamin $B_{12}$ concentrations $<148$ pmol/L. However, the decrease in these indices was not associated with reduced hemoglobin concentrations or RBC count or with increased tHcy concentrations.

Conclusions: Our findings suggest that the reference values for vitamin $B_{12}$ status in nonpregnant women may not be applicable to pregnant women.

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One of the biochemical functions of cobalamin (vitamin $B_{12}$) in mammals is to maintain normal folate metabolism, which is essential for cell multiplication during pregnancy. Because maternal vitamin $B_{12}$ stores in women eating a mixed diet are $\sim3000$ μg and the vitamin $B_{12}$ requirement of the fetus is $\sim50$ μg, it may be assumed that the event of a single pregnancy has minimal impact on maternal stores (1). On the other hand, vitamin $B_{12}$ deficiency, defined as low serum vitamin $B_{12}$ concentrations, occurs in 10–28% of uncomplicated pregnancies (2).

At present little information is available regarding the normal changes in vitamin $B_{12}$ metabolism and concentrations of cobalamin-binding proteins during pregnancy (3). Moreover, much of the reported information (4–6) was collected before the availability of assays for measuring the biologically active form of vitamin $B_{12}$. No reference values are available for most biochemical indices of vitamin $B_{12}$ status in pregnant women, and often the reference values for nonpregnant individuals are used to assess their vitamin $B_{12}$ status. To our knowledge, no longitudinal studies have been performed to validate the applicability of these reference values for pregnant women.
The aim of the present study was to obtain longitudinal information on the biochemical indices of vitamin B₁₂ status throughout pregnancy, especially erythrocyte and serum vitamin B₁₂, holo-haptocorrin, unsaturated cobalamin-binding proteins, unsaturated vitamin B₁₂-binding capacity (UBBC), and total vitamin B₁₂-binding capacity (TBBC) as well as other associated indices, such as total homocysteine (tHcy). In addition, the consequences of decreasing blood concentrations on associated hematologic indices were investigated. The values presented may be used as reference intervals for cobalamin-binding proteins and cobalamin-binding capacities for healthy pregnant women.

**Subjects and Methods**

**STUDY DESIGN, PARTICIPANTS, AND DIETARY ASSESSMENT**

The study was designed as a prospective longitudinal study throughout pregnancy. Healthy pregnant volunteers (n = 39) entered the study and were followed until delivery. Information on dietary intake and blood samples were collected in the first, second, and third trimesters of gestation (weeks 9–12, 20–22, and 36–38). The study was approved by the Ethics Committee of the Division of Human Medicine, University of Giessen, Germany. All participants gave informed consent.

Descriptions of the design, the recruitment methods, and the conduction of the study have been published in detail elsewhere (7). Briefly, the participants were apparently healthy adults. High-risk and twin pregnancies were excluded. A food frequency list was used to assess dietary habits. Interested women eating an average Western diet that corresponded with the average German population, as defined in the results of the German National Consumption Study, were selected by a priori defined selection criteria. Their diet consisted of >300 g of meat and 105 g of meat products per week. Because of missing laboratory data or relocation and birth of the child before the last blood sampling date, the calculation of ratios of vitamin B₁₂ concentrations in the third to the first trimester is based on a subgroup of 29 pregnant women.

Linked to the blood sampling, a 4-day food record, including categories of 152 food items with given portion sizes estimated by typical household measures, was maintained three times throughout the pregnancy by all participants (8). The calculation of food-derived cobalamin intake was based on the German Food Code and Nutrition Data Base BLS II.3 (9). Folate and cobalamin concentrations in multivitamin-fortified juices were taken from producers’ data. In addition, the intake of food supple-

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8 Nonstandard abbreviations: UBBC, unsaturated vitamin B₁₂-binding capacity; TBBC, total vitamin B₁₂-binding capacity; tHcy, total homocysteine; Hb, hemoglobin; RBC, red blood cell; MCV, mean corpuscular volume; BMI, body mass index; and RDA, Recommended Dietary Allowance.
RBC and plasma folate concentrations were determined with a chemiluminescent competitive protein-binding assay (ACS Folate Assay; Ciba Corning Diagnostics GmbH). The between-run CV was 3.9% (n = 15; mean concentration, 412 nmol/L) for RBC folate and 4.1% (n = 13; mean concentration, 16.4 nmol/L) for plasma folate. The tHcy concentration was measured in plasma according to the methods of Ubbink et al. (15) and Araki and Sako (16). For quantification, the samples were analyzed without and with the addition of a standard amount of tHcy; the average response factors for the added tHcy were used to calculate the concentration of endogenous tHcy. Recoveries in individual samples deviating >10% from the mean were rejected, and the measurement was repeated. The between-run CV (n = 20) for tHcy was 4% at a mean concentration of 8.3 µmol/L. Blood smears were prepared on glass slides and stained by the Pappenheim method (17, 18). Segmentation of neutrophil granulocytes was counted twice from two different blood smears of each woman. The blood smears were subjected to a lobe count (100 nuclei) according to the method of Bung et al. (19). Intraindividual counts that deviated >20% were counted two additional times; the mean value of all four counts was used for data analysis. The CV (n = 9; mean, 2.9 lobes) was 14%.

STATISTICAL ANALYSIS
Blood concentrations are presented as arithmetic or geometric means and their 95% confidence intervals. Basic characteristics of participants [e.g., age, body mass index (BMI), and parity] and dietary intake are given as the arithmetic mean ± SD. The relationship between intake of vitamin B₁₂ and vitamin B₁₂ concentrations in blood was described by Pearson correlation coefficients. Low serum B₁₂ concentrations were defined as values <150 pmol/L based on Metz et al. (20). All analyses were repeated using cutoff values of 200 and 250 pmol/L. Low RBC vitamin B₁₂ concentrations were defined as values <148 pmol/L based on Herbert (21). In addition, for the RBC vitamin B₁₂ concentrations, a cutoff of 133 pmol/L, based on the results obtained by Tisman et al. (10), was used. Low haptocorrin saturation and TBBC were defined as <20% and <15%, respectively, based on Herbert (21). Folate deficiency was defined as RBC folate concentrations <320 nmol/L (22).

Plasma folate, serum vitamin B₁₂, apo-haptocorrin, and neutrophil segmentation index were log-transformed to normalize the data. To test the effect of stage of pregnancy (first, second, and third trimester) on biochemical indices during pregnancy, generalized estimating equations were used. Generalized estimating equation models allow appropriate analysis of longitudinal data with repeated measurement and missing values. The effect of potentially confounding variables (i.e., maternal age, BMI, parity, and use of oral contraceptives) was tested. All two-way interactions were tested, but no interactions with P < 0.15 were found. On the basis of the results of these preliminary analyses, final models included maternal age as the only confounding variable. All analyses were performed using SAS 8.2 (SAS Institute Inc.).

Results
DIETARY INTAKE AND OTHER CHARACTERISTICS OF THE STUDY POPULATION
The mean age of the participating pregnant volunteers was 29.1 ± 3.6 years, and the mean BMI before pregnancy was 23.1 ± 4.7 kg/m². The mean parity was 2.0 ± 1.1, and 41% of the participants were primiparous. Four women had more than two previous deliveries, and 12 women took oral vitamin B₁₂ supplements. None of the pregnant women had a twin pregnancy. Eighteen women had taken oral contraceptives within the past 12 months preceding pregnancy. The analysis of covariance showed no effect of BMI, parity, use of oral contraceptives, or cobalamin intake on biochemical indices of vitamin B₁₂ status. The exclusion of women who had more than two previous deliveries and who took oral vitamin B₁₂ supplements did not significantly change any of the reported results.

The mean intake of dietary vitamin B₁₂ from first to third trimester was 5.6 ± 2.0 µg/day. None of the participants had an average vitamin B₁₂ intake below the Recommended Dietary Allowance (RDA) of 2.6 µg/day at any time during pregnancy (22). The intake of vitamin B₁₂ did not correlate with vitamin B₁₂ concentrations in blood. The mean intake of dietary folate from first to third trimester was 273 ± 53 µg/day. Seventeen women (44%) took folate supplements during the current pregnancy. Folate deficiency was observed in 14 women (36%).

RBC AND SERUM VITAMIN B₁₂ CONCENTRATIONS
RBC and serum vitamin B₁₂ concentrations are shown in Table 1. The ratios of selected concentrations of the third relative to the first trimester are shown in Table 2. Serum vitamin B₁₂ concentrations decreased steadily throughout pregnancy. Only one woman showed low serum vitamin B₁₂ concentrations (<150 pmol/L) in the first trimester of pregnancy.

Throughout pregnancy, 3 women (8%) in the second and 12 women (35%) in the third trimester developed low serum vitamin B₁₂ concentrations. In contrast, RBC vitamin B₁₂ concentrations increased significantly during this period. Low RBC vitamin B₁₂ concentrations (<148 pmol/L) were observed in four women in the first trimester and in one woman in the second trimester; RBC vitamin B₁₂ concentrations <133 pmol/L were observed in two women in the first trimester and in one woman in the second trimester. No woman with low serum vitamin B₁₂ concentrations simultaneously showed low RBC vitamin B₁₂ concentrations (<148 pmol/L). Similar to the serum vitamin B₁₂ concentrations, a steady decrease in holo-haptocorrin concentrations was observed.

Serum vitamin B₁₂ concentrations and holo-haptocorrin decreased with increasing maternal age (P = 0.041
and 0.035, respectively). No differences between primiparous and nonprimiparous women were observed. No interactions between maternal age and the decreases in serum vitamin B12 and holo-haptocorrin concentrations between first and third trimester were observed, implying that the degree of decrease was not modified by maternal age.

CONCENTRATIONS OF COBALAMIN-BINDING PROTEINS AND VITAMIN B12-BINDING CAPACITIES

The UBBC and TBBC of the serum increased steadily during pregnancy. TBBC, which also depends on decreasing serum vitamin B12 concentrations, increased less. In the third trimester, 68.6% of women had a TBBC saturation <15%. Whereas the apo-transcobalamin changed only marginally during pregnancy, apo-haptocorrin increased sharply. Consequently, the ratio of apo-transcobalamin and apo-haptocorrin also changed significantly.

CONSEQUENCES OF LOW VITAMIN B12 CONCENTRATIONS

Most of the indicators of vitamin B12 status in the present study, including Hb, MCV, RBC count, neutrophil segmentation, and plasma and RBC folate concentrations, as well as tHcy concentrations changed significantly during pregnancy. However, results of an analysis of variance showed that changes in any of the above indicators were not affected by vitamin B12 status, but by folate deficiency (data not shown). After adjustment for RBC folate as a predictor for folate status, no differences were observed for tHcy concentrations and neutrophil segmentation between women with low vs normal serum vitamin B12 concentrations in the second and third trimesters. After adjustment for serum ferritin concentrations as a predictor for iron status, no differences were observed for Hb, MCV, and RBC count between women with low vs normal serum vitamin B12 concentrations in the second and third trimesters. In addition, no difference was observed in plasma and RBC folate. Different cutoff values for serum vitamin B12 were used to define low serum vitamin B12 concentrations: <150, <200, and 250 pmol/L.

In the second and third trimesters, women with low (<15%) and normal TBBC saturation did not differ with respect to any of the above indicators of vitamin B12 status.

Discussion

Although recent studies have shown that vitamin B12 deficiency can occur during pregnancy, only limited information is available regarding normal changes in the vitamin B12-binding capacities and saturation of the cobalamin-binding proteins during pregnancy (4, 5). More-

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Table 1. Concentrations of RBCs and serum vitamin B12, cobalamin-binding proteins, binding capacities, and vitamin B12-related blood concentrations of pregnant women in all trimesters.

<table>
<thead>
<tr>
<th>Index</th>
<th>First (n = 31)</th>
<th>Second (n = 39)</th>
<th>Third (n = 38)</th>
<th>P&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>RBC vitamin B&lt;sub&gt;12&lt;/sub&gt;, pmol/L</td>
<td>231 (206–256)</td>
<td>254 (235–273)</td>
<td>265 (246–286)</td>
<td>0.002</td>
</tr>
<tr>
<td>Serum vitamin B&lt;sub&gt;12&lt;/sub&gt;,&lt;sup&gt;c&lt;/sup&gt; pmol/L</td>
<td>257 (226–292)</td>
<td>239 (212–268)</td>
<td>178 (161–198)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Holo-haptocorrin, pmol/L</td>
<td>243 (212–275)</td>
<td>227 (194–259)</td>
<td>176 (156–195)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>TBBC, pmol/L</td>
<td>1216 (1152–1279)</td>
<td>1263 (1191–1335)</td>
<td>1329 (1257–1401)</td>
<td>0.003</td>
</tr>
<tr>
<td>UBBC, pmol/L</td>
<td>943 (872–1014)</td>
<td>1009 (940–1078)</td>
<td>1147 (1072–1221)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Apo-transcobalamin, pmol/L</td>
<td>833 (771–895)</td>
<td>878 (817–938)</td>
<td>909 (846–973)</td>
<td>0.018</td>
</tr>
<tr>
<td>Apo-haptocorrin,&lt;sup&gt;c&lt;/sup&gt; pmol/L</td>
<td>98 (79–127)</td>
<td>119 (102–139)</td>
<td>221 (195–251)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Hb, g/L</td>
<td>132 (129–135)</td>
<td>125 (123–127)</td>
<td>124 (122–126)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>MCV, fL</td>
<td>90 (89–92)</td>
<td>91 (90–93)</td>
<td>91 (90–93)</td>
<td>0.004</td>
</tr>
<tr>
<td>RBC count, × 10&lt;sup&gt;12&lt;/sup&gt;/L</td>
<td>4.4 (4.3–4.6)</td>
<td>4.1 (4.0–4.2)</td>
<td>4.1 (3.9–4.4)</td>
<td>0.032</td>
</tr>
<tr>
<td>RBC folate, nmol/L</td>
<td>411 (359–463)</td>
<td>514 (449–579)</td>
<td>476 (420–532)</td>
<td>0.014</td>
</tr>
<tr>
<td>Plasma folate,&lt;sup&gt;c&lt;/sup&gt; nmol/L</td>
<td>16.7 (14.9–21.8)</td>
<td>15.7 (13.8–17.9)</td>
<td>15.5 (13.4–17.9)</td>
<td>0.063</td>
</tr>
<tr>
<td>tHcy, μmol/L</td>
<td>6.9 (6.2–7.6)</td>
<td>5.9 (5.4–6.3)</td>
<td>6.6 (5.9–7.2)</td>
<td>0.050</td>
</tr>
<tr>
<td>Neutrophil segmentation, lobes</td>
<td>2.9 (2.8–2.9)</td>
<td>3.0 (2.9–3.1)</td>
<td>3.2 (3.1–3.3)</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

<sup>a</sup> Values are means and 95% confidence intervals except where indicated.
<sup>b</sup> Significance of time effect in generalized estimating equation model adjusted for maternal age.
<sup>c</sup> Geometric mean and geometric confidence interval.

Table 2. Ratios (third:first trimester) of vitamin B<sub>12</sub> and cobalamin-binding protein concentrations in pregnant women (n = 29).

<table>
<thead>
<tr>
<th>Index</th>
<th>Ratio third:first trimester&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>RBC vitamin B&lt;sub&gt;12&lt;/sub&gt;, pmol/L</td>
<td>1.17 (1.10–1.32)</td>
</tr>
<tr>
<td>Serum vitamin B&lt;sub&gt;12&lt;/sub&gt;,&lt;sup&gt;c&lt;/sup&gt; pmol/L</td>
<td>0.70 (0.62–0.79)</td>
</tr>
<tr>
<td>Holo-haptocorrin, pmol/L</td>
<td>0.74 (0.67–0.83)</td>
</tr>
<tr>
<td>UBBC, pmol/L</td>
<td>1.21 (1.15–1.30)</td>
</tr>
<tr>
<td>Apo-transcobalamin, pmol/L</td>
<td>1.08 (1.02–1.16)</td>
</tr>
<tr>
<td>Apo-haptocorrin, pmol/L</td>
<td>2.65 (2.19–3.01)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Geometric mean of ratio (95% confidence interval).
over, for most of the biochemical indices of vitamin B\textsubscript{12} status, no reference values are available for pregnant women throughout pregnancy.

The aim of the study was to monitor vitamin B\textsubscript{12} status throughout uncomplicated pregnancies in women with an adequate vitamin B\textsubscript{12} intake. The average intake of food-derived vitamin B\textsubscript{12} by the participants was more than twice the RDA, and none of the participants had an average vitamin intake below the RDA for pregnant women (2.6 µg/day) (22). Vitamin B\textsubscript{12} intake was not related to vitamin B\textsubscript{12} concentrations in serum. On the basis of these data, adequate vitamin B\textsubscript{12} intake of all participants can be assumed.

Nevertheless, in the third trimester almost 35% of the participants had serum vitamin B\textsubscript{12} concentrations <150 pmol/L, and ~70% had a TBBC saturation <15%. In nonpregnant women, serum vitamin B\textsubscript{12} concentrations <150 pmol/L and a TBBC saturation <15% are considered indicative of vitamin B\textsubscript{12} deficiency (20–23). However, in the present study in pregnant women, vitamin B\textsubscript{12} concentrations below these reference values in the second and third trimesters did not seem to have any deleterious consequences on Hb concentration, RBC count, or Hcy concentrations. Higher cutoff points for serum vitamin B\textsubscript{12} concentrations did not alter the results. Similar observations were made by Pardo et al. (24). These results suggest that reference values for nonpregnant women are not suitable for assessing vitamin B\textsubscript{12} status during pregnancy, implying the need for reference values related to specific stages of pregnancy.

The present study provides longitudinal values for most biochemical indices of vitamin B\textsubscript{12} status, which may be interpreted as preliminary reference values for healthy women during pregnancy. Because more than one-third of the participants (36%) showed signs of folate deficiency, the presented blood indices affected by both vitamin B\textsubscript{12} and folate are not suitable as indicators of vitamin B\textsubscript{12} deficiency.

Unsaturated concentrations of cobalamin-binding proteins showed a steady increase over the course of the pregnancies. It may be noted that this finding differs from the results of Fernandes-Costa and Metz (4), who observed a moderate decrease in apo-transcobalamin concentrations between the first and second trimesters, followed by a strong increase near delivery. However, the apo-transcobalamin concentrations reported by these authors may be of limited value because they used chicken apo-haptocorrin concentrations reported by these authors.

In conclusion, the present study indicates that reference values established for nonpregnant women are not suitable for assessing vitamin B\textsubscript{12} status in pregnant women. Furthermore, our results suggest that the observed changes in concentrations of cobalamin-binding proteins reflect physiologic changes during pregnancy rather than vitamin B\textsubscript{12} deficiency. Further studies are needed to confirm the observed changes in cobalamin-binding proteins during pregnancy and their physiologic implications.

We gratefully acknowledge the financial support of the Eden Foundation (Bad Soden, Germany). We also thank the women who participated in the study.

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