Longitudinal vitamin and homocysteine levels in normal pregnancy

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Evidence of the impact of maternal nutritional status on pregnancy outcome is increasing. However, reference values for vitamin and homocysteine concentrations in maternal blood during normal pregnancy are scarce, and are lacking for the preconceptional period and early pregnancy. Thus, in a longitudinal study we evaluated vitamin and homocysteine concentrations in blood were determined from the preconceptional period throughout pregnancy until 6 weeks post-partum. The physiological changes in vitamin and homocysteine concentrations in blood in 102 nulliparous women with an uneventful singleton pregnancy and normal outcome not using supplements. The plasma homocysteine concentration was also measured, considering the essential roles of folate, vitamin B₆ and vitamin B₁₂ in homocysteine metabolism. The concentrations of retinol, thiamin, pyridoxal 5'-phosphate serum folate and vitamin B₁₂ decreased during pregnancy. In contrast, the concentrations of riboflavin, α-tocopherol, and folate in erythrocytes increased or showed only minor changes. Homocysteine concentrations also remained approximately constant during pregnancy. These observations emphasize the importance of preconceptional and post-partum concentrations of vitamins in the evaluation of pregnancy-induced changes. These data have provided valuable reference values for vitamins and homocysteine before, during and after pregnancy in order to contribute to better diagnosis of maternal deficiencies and to study further the relationship between maternal vitamin status and adverse course and outcome of pregnancy.

Vitamins: Homocysteine: Pregnancy: Nutritional status

Maternal nutritional status has been shown to contribute significantly to pregnancy outcome (Brown, 1993). Food intake, processing, absorption, metabolism and clearance determine the individual vitamin status. The embryo and fetus are totally dependent on the maternal–fetal transfer of nutrients and the maternal nutrient status. Thus, it can be expected that maternal nutritional deficiencies, especially periconceptionally, may influence the course and outcome of pregnancy. In addition, marginally deficient values or elevated concentrations still within the normal non-pregnant range might have a significant impact on early embryonic development as well as long-term health outcome. With respect to long-term health outcome, a relationship between low birth weight and insulin resistance and cardiovascular disease in adult life has been reported (Barker et al. 1993; Phillips et al. 1994).

The harmful effects of hypervitaminosis A and the beneficial effects of folate supplementation have already received much attention, which further emphasizes the need for reference values for these vitamins before and during normal pregnancy. With this information, diagnosis of maternal vitamin deficiencies and the evaluation of medical treatment can be improved. In addition, future studies on the relationship between maternal vitamin status and the occurrence of an abnormal course and outcome of pregnancy will become feasible.

Data on longitudinally-determined vitamin concentrations in normal pregnancy are scarce, and there is a lack of preconceptional and first trimester values (Bruinse & van den Berg, 1995). Most available data are derived from cross-sectional studies in which vitamin concentrations are determined at various gestational ages (Baker et al. 1975). Moreover, the study populations concerned are mostly heterogeneous with regard to parity and ethnic descent.

The aim of the present study, therefore, was to determine in healthy women who were not using any vitamin or food supplements and had a normal pregnancy outcome, the blood concentrations of retinol, thiamin, riboflavin, pyridoxal 5'-phosphate, folate, vitamin B₁₂, α-tocopherol and the vitamin-dependent homocysteine concentration from

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the preconceptional period throughout pregnancy until 6 weeks after delivery.

**Experimental methods**

**Protocol**

As part of an extensive study on risk factors for adverse pregnancy outcome in epileptic women, a cohort of 225 women including healthy controls was formed in the period 1987–90. The design of this observational study has previously been described in detail (Steegers-Theunissen et al. 1994). The Medical Ethical Committee of the University Medical Centre St Radboud in Nijmegen, The Netherlands, approved the protocol. Women were recruited preconceptionally and were included after informed consent was obtained. Only nulliparous and singleton pregnancies were included in the present study. Before conception a research nurse visited the women at home every 6 months for history taking and blood sampling. Pregnancy was confirmed by a monoclonal antibody-based pregnancy test. Menstrual history, basal body temperature charting and one or two transvaginal ultrasonographic examinations before week 8 of gestation confirmed the duration of the pregnancy. Non-fasting random blood samples were taken within 3 months prior to conception and subsequently at 6, 10, 20 and 32 weeks of gestation and 6 weeks post-partum.

Only women with an uneventful pregnancy course and normal outcome were included in the present study. Inclusion criteria for normal pregnancy were: no epilepsy; no pregnancy-induced hypertension or pre-eclampsia; no diabetes gravida; no hospital admission other than for labour; spontaneous labour between 37 and 42 weeks of gestation; birth weight appropriate for gestational age according to the Dutch birth weight curve (birth weight between the percentiles 2.3 and 97.7) (Kloosterman, 1970). The study population fulfilling the described criteria comprised 102 women.

The following vitamins were evaluated: retinol, thiamin, riboflavin, pyridoxal 5'-phosphate, folate, vitamin B₁₂; α-tocopherol. Homocysteine was also measured, considering the essential role of vitamin B₆, vitamin B₁₂ and folate in homocysteine metabolism.

**Analytical procedures**

We have participated in external quality assurance programmes of The Netherlands Foundation for Quality Assurance in Clinical Laboratories (thiamin, vitamin B₆, vitamin A and vitamin E), the Landelijke workgroup Bindingsanalyse (vitamin B₁₂ and folate) and the German programme. Deutsche Ringversuch of the German Society for Clinical Chemistry.

Serum retinol concentration was determined using HPLC with fluorescence detection, and retinal acetate as the internal standard. Before HPLC, the serum sample was denatured by freezing and serum was deproteinized using perchloric acid. Thiamine pyrophosphate and thiamine monophosphate were hydrolysed to thiamine by acid phosphatase. HPLC was performed using C₁₈-coated silica with an n-octanesulfonate-containing mobile phase methanol–aqueous citrate buffer, pH 4.0 (45.55, v/v). In a post-column reaction K₂Fe(CN)₆ was used to oxidise thiamine to thiochrome, which was detected by fluorometry with excitation and emission wavelengths set at 364 and 462 nm respectively. The minimum detectable amount was 2.3 nmol thiamine/l. At mean concentrations of 123 nmol/l the intra-assay CV was 4.8 % and the inter-assay CV was 6.8 %.

Riboflavin was determined in heparinized plasma using HPLC and fluorescence detection. Before HPLC, heparinized whole-blood samples were denatured with trichloroacetic acid, centrifuged and the supernatant fraction injected onto the HPLC. Fluorescence excitation and emission wavelengths were 470 and 520 nm respectively. The minimum detectable concentration was 10 nmol riboflavin/l, and the precision in terms of intra- and inter-assay CV for means of duplicate determinations were 3.9 and 8.2 % respectively.

The concentration of pyridoxal 5'-phosphate in whole blood was measured using HPLC. Before HPLC 1 ml heparinized whole blood was denatured with perchloric acid, centrifuged and the supernatant fraction was neutralized with KOH and injected onto the HPLC column. Post-column derivatization was performed with semicarbazide reagent to obtain the pyridoxal 5'-phosphate semicarbazone. The fluorescence was determined using wavelengths for excitation and emission of 367 and 478 nm respectively. The minimum detectable concentration was 2 nmol pyridoxal 5'-phosphate/l and the precision in terms of intra- and inter-assay CV was 1-6 % (twenty replicate determinations; mean concentration 52 nmol/l) and 5.5 % (fifteen determinations on several days; mean concentration 53 nmol/l) respectively.

Vitamin B₁₂ and folate concentrations in serum were determined simultaneously with the dualcount solid phase boil radioassay (Diagnostic Products Corporation, Los Angeles, CA, USA). Folate was also determined in erythrocytes (Dunn & Foster, 1973) and 24 h urine samples. The radioassay makes use of a dual-label tracer solution ([³⁵S]cyanocobalamin and [¹²⁵I]-labelled folic acid) and a suspension of the solid-phase binders for vitamin B₁₂ and folate. After incubation and bound–free separation by centrifugation, the radioactivity of the bound fraction was counted and the concentrations of vitamin B₁₂ and folate were calculated from logit–log transformation of the data to obtain linear standard dose–response curves (with the logit being the natural logarithm of binding responses). Assay precision in terms of intra- and inter-assay CV for means of duplicate determinations were <5 and 10 % respectively for both vitamins.

α-Tocopherol concentrations were determined in serum
by HPLC with fluorescence detection. The serum samples were denatured with ethanol, diluted with twice-distilled water and centrifuged after addition of the mobile phase (n-hexane–ethanol 99:5:0.5, v/v). Using an automated sampler, 20 µL supernatant fraction was injected onto a normal phase guard column which was in line with a silica column and equipped with an automatic HPLC liquid solvent pump. The flow rate was 2-0 ml/min and the fluorescence excitation and emission wavelengths for α-tocopherol were 295 and 390 nm respectively. The minimum detectable concentration was 0-40 µmol α-tocopherol/l. The intra- and inter-assay CV were 2-8 % (ten replicate determinations; mean concentration 22-2 µmol/l) and 6-6 % (thirteen simultaneous assay runs; mean concentration 23-7 µmol/l), respectively.

Homocysteine concentrations in plasma were determined using HPLC with reverse-phase separation and fluorescence detection. Total homocysteine determination by this method is based on complete reduction of all homocystine disulfide bonds in plasma by NaBH4 and dithioerythritol. After derivatization by monobromobimane the resulting homocysteine–monobromobimane complex is separated from interfering substances by reverse-phase HPLC. The intra- and inter-assay CV were 2-1 and 5-2 % respectively.

Statistical analysis

To estimate the longitudinal values of percentiles 5, 50 and 95 from the preconceptional period until 10 weeks post-partum a linear mixed model for repeated measurements was used. Three fixed parameters were used in this model: (1) the overall intercept; (2) the duration of gestation at a given time of measurement; (3) the duration squared. To allow for individual variation, three random parameters were added to the model: (1) the individual intercept; (2) the individual coefficient of duration; (3) the individual coefficient of duration squared. The covariance matrix was left completely unspecified. Except for post-partum values, all measurements were used for estimations within these models. For computational purposes, preconceptional values were used with duration set equal to zero.

The 50th percentile was estimated using the fixed coefficients calculated from the model. Variance estimators for the 50th percentile for individual variation and residual variance were added. The resulting standard deviation was used to compute the 5th and 95th percentiles. If on inspection residuals from the model appeared to be non-normally distributed, a log- or square-root transformation was used on the original variables, the centiles were computed and then transformed back. The only exception to this procedure was for homocysteine, which was measured in a small subgroup of sixteen women. After log transformation, the 50th percentile was computed as the mean, the (total) standard deviation was multiplied by 1 + (1/n), where n is the number of measurements available at the given time, and this value was then used to compute the 5th and 95th percentiles; these values were then transformed back.

The analysis used the SAS procedure MIXED (Statistical Analysis Systems Institute Inc., Cary, NC, USA).

Results

Figs. 1–3 show the median values and the upper (95th) and lower (5th) percentiles of the concentrations of vitamins and homocysteine determined from preconception, throughout pregnancy, to 6 weeks post-partum.

Vitamin A (Fig. 1(a)) concentrations during pregnancy showed only a minor decrease. Post-partum concentrations were markedly elevated compared with preconceptional values.

Thiamin (Fig. 1(b)) concentrations showed a gradual decline. At 6 weeks post-partum preconceptional concentrations were reached.

Riboflavin (Fig. 1(c)) concentrations showed only minor changes during pregnancy, with a slight increase in the third trimester.

Pyridoxal 5′-phosphate (Fig. 1(d)) concentrations gradually declined during pregnancy. Post-partum values were similar to those found preconceptionally.

Serum folate (Fig. 2(a)) concentrations showed a slight decrease during pregnancy and remain decreased up to 6 weeks after delivery. Folate concentrations in erythrocytes (Fig. 2(b)) increased slightly during pregnancy. In the post-partum period erythrocyte folate concentrations were lower than those in the third trimester, but similar to preconceptional concentrations. Fig. 2(c) shows the 24 h secretion of folate in urine which does not seem to change throughout pregnancy. Also no differences were observed regarding folate excretions in the puferium.

Vitamin B12 (Fig. 1(e)) concentrations showed a progressive decline during pregnancy reaching marginal or even deficient levels. At 6 weeks after delivery vitamin B12 reached preconceptional values.

α-Tocopherol (Fig. 1(f)) concentrations during pregnancy showed a progressive increase when compared with preconceptional values. After delivery α-tocopherol concentrations returned to the preconceptional values.

Homocysteine (Fig. 3) concentrations declined slightly in the first trimester and remained approximately constant during pregnancy. Post-partum concentrations were slightly elevated compared with preconceptional values.

Discussion

The present study is the first longitudinal study in which vitamin and homocysteine concentrations in blood have been determined from the preconceptional period through

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Fig. 1. Serum concentrations of (a) retinol, (b) thiamin, (c) riboflavin, (d) pyridoxal 5′-phosphate, (e) vitamin B12 and (f) α-tocopherol in 102 nulliparous Dutch women from the preconceptional period, throughout a normal healthy pregnancy, to 6 weeks post-partum. Samples were taken within the 3 months before conception and subsequently at weeks 6, 10, 20 and 32 of gestation and at 6 weeks post-partum, with gestation shown as the duration weeks of amenorrhoea (weeks). Individual values (○) and the 5th (– – –), 50th (——) and 95th (– - -) percentiles are shown. For details of subjects and procedures, see p. 50.
Fig. 1.
Fig. 1. Continued
Fig. 2.
pregnancy, to the post-partum period, in a homogeneous population of nulliparous Dutch women who did not use vitamin supplements or supplemented diets. The present study has provided valuable reference values for these micronutrients, which are representative for healthy women aged 20–40 years with an uncomplicated singleton pregnancy and normal pregnancy outcome.

The minor decrease in vitamin A during pregnancy and the elevated concentrations after delivery are in accordance with previous studies (Baker et al. 1975; Bruinse & van den Berg, 1995). However, other researchers have reported an increase during pregnancy (Gal & Parkinson, 1974). Tissue retention of the fat-soluble vitamin A during pregnancy and weight loss after delivery may account for the observed changes. Vitamin A and its precursors can readily move across the placenta. Hypervitaminosis A is teratogenic (Lammer et al. 1985; Rothman et al. 1995) resulting in congenital abnormalities and a high incidence of spontaneous abortions. Vitamin A is an important antioxidant and significantly lower concentrations have been reported in pregnancies complicated by pre-eclampsia (Ziari et al. 1996).

The thiamin status did not seem to be affected by pregnancy, showing only a gradual decline. Previous studies on transketolase activity in erythrocytes in well-nourished European women showed an insufficient thiamin reserve in one-third of the investigated population (Heller et al. 1974b; Nicols & Nicols, 1983). Insufficient thiamin intake during pregnancy and lactation as well as hyperemesis gravidarum can lead to symptoms of deficiency, eventually leading to beri beri (Dyckner et al. 1985).

Riboflavin concentrations were also relatively unaffected by pregnancy, showing <5% increase in the third trimester. Other researchers have reported similar results (Decker et al. 1977; Bruinse & van den Berg, 1995). Riboflavin deficiency is associated with elevated glutathione reductase activity in erythrocytes after in vitro incubation with riboflavin (Nicols & Nicols, 1983). In a normal pregnancy the activity of this enzyme shows a 20% increase in the presence of riboflavin. On this basis, 20–40% of the pregnant women investigated by Heller et al. (1974a) were reported to be riboflavin deficient.

Several studies have reported a similar gradual decline in vitamin B₆ concentrations (Lumeng et al. 1976). The decrease is suggested to occur mainly before the 16th week of gestation (Bruinse & van den Berg, 1995). In the present study post-partum concentrations were similar to preconceptional values. At 6 months after delivery 25% of the serum vitamin B₆ concentrations were reported to be below the reference values for non-pregnant women (Bruinse & van den Berg, 1995). This finding indicates that there is maternal vitamin B₆ depletion during pregnancy, with preferential tissue store depletion. Vitamin B₆ deficiency may lead to hyperhomocysteinemia, which is an established risk factor for early-onset atherosclerosis and ischaemic vascular pathology (Boers et al. 1985) and adverse pregnancy outcome (see p. 56).

Folate is stored during erythropoiesis in erythrocytes. The folate concentration in erythrocytes is less dependent on dietary fluctuations than serum concentrations and is therefore a better indicator of folate status. Most studies indicate only minor changes or a slight decrease in the

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**Fig. 2.** Concentrations of (a) serum folate, (b) erythrocyte folate and (c) urine folate in 102 nulliparous Dutch women from the preconceptional period, throughout a normal healthy pregnancy, to 6 weeks post-partum. Samples were taken within the 3 months before conception and subsequently at weeks 6, 10, 20 and 32 of gestation and at 6 weeks post-partum, with gestation shown as the duration of amenorrhoea (weeks). Individual values (○) and the 5th (— — —), 50th (---) and 95th (— — —) percentiles are shown. For details of subjects and procedures, see p. 50.

**Fig. 3.** Plasma homocysteine concentrations in sixteen nulliparous Dutch women from the preconceptional period, throughout a normal healthy pregnancy, to 6 weeks post-partum. Samples were taken within the 3 months before conception and subsequently at weeks 6, 10, 20 and 32 of gestation and at 6 weeks post-partum, with gestation shown as the duration of amenorrhoea (weeks). Individual values (○) and the 5th (— — —), 50th (---) and 95th (— — —) percentiles are shown. For details of subjects and procedures, see p. 50.
concentrations of folate in erythrocytes (Rolschau et al. 1979; Bruinse & van den Berg, 1995). In the present study the concentrations of folate in erythrocytes initially increased slightly during pregnancy, but showed a minor decrease in the third trimester. At 6 weeks post-partum the erythrocyte folate concentrations returned to the preconceptional values. Serum folate concentrations showed a slight decrease during pregnancy, with recovery after delivery. Similar findings have been reported in almost all studies (Rolschau et al. 1979; Ek & Magnus, 1981; Bruinse & van den Berg, 1995). Our data confirm that serum folate concentrations remain decreased within 6 weeks after delivery. The results of a previous study indicated a marginal or even deficient state with respect to serum folate concentrations up to 6 months after delivery (Bruinse & van den Berg, 1995). Possible explanations are the physiological haemodilution during pregnancy, lactation, hormonal influences and contraception or elevated urinary excretion (McPartlin et al. 1993). Folate deficiency has long been reported in association with adverse pregnancy outcome (Hibbard, 1964) and congenital abnormalities, especially neural-tube defects (Kirke et al. 1993). More recent data have confirmed the beneficial effect of periconceptional folate supplementation in the prevention of the occurrence and recurrence of neural-tube defects (Steegers-Theunissen et al. 1993). It has been suggested that either decreased folate intake or genetic disturbances in folate metabolism contribute to these conditions (Steegers-Theunissen et al. 1991; van der Put et al. 1995). The crucial role of folate, as a substrate, in homocysteine metabolism has been established.

The progressive decline of vitamin B\textsubscript{12} concentrations by about 100 pmol/l during pregnancy has been reported previously (Bruinse & van den Berg, 1995). This decline leads to marginal or even deficient concentrations, which recover after pregnancy, and return to preconceptional values at 6 weeks post-partum. Compared with folate, possible relationships between vitamin B\textsubscript{12} deficiency and congenital abnormalities are less clear. However, low or deficient serum and amniotic fluid concentrations of vitamin B\textsubscript{12} have been associated with neural-tube defects (Steen et al. 1998). Additional studies are needed to elucidate the role of vitamin B\textsubscript{12} in the prevention of adverse pregnancy outcome. So far, there is increasing evidence of a synergistic role of folate and vitamin B\textsubscript{12} in homocysteine metabolism (Bronstrup et al. 1998). Maternal megaloblastic anaemia, the result of vitamin B\textsubscript{12} or folate deficiency, does not seem to affect the fetus. High cord blood concentrations suggest active transfer of vitamin B\textsubscript{12} and folate across the placenta.

Vitamin E concentrations during pregnancy progressively increased compared with preconceptional values. This finding is in accordance with previous reports (Mooij et al. 1993). The increase in vitamin E might have a compensatory effect on the increase in oxygen radical formation as gestation progresses (Wang et al. 1991). The elevation of vitamin E seems to correspond with the increase in products of membrane damage with advancing duration of gestation. Fetal growth retardation and pre-eclampsia have been correlated with relatively low maternal concentrations of vitamin E (Von Mandach et al. 1994; Ziairi et al. 1996).

A previous study revealed a significantly lower homocysteine concentration at the end of the first trimester compared with subsequent constant concentrations until delivery (Andersson et al. 1992). Our data confirm the slight decline in homocysteine concentrations in the first trimester. The homocysteine level remained approximately constant during the second and third trimester and was slightly lower than that of non-pregnant women. Post-partum values were slightly elevated compared with preconceptional concentrations. The changes in homocysteine metabolism during pregnancy may be explained by hormonal influences, haemodilution and the increased fetal and maternal needs for methionine and homocysteine (Steegers-Theunissen et al. 1997). Deficiencies of folate, vitamin B\textsubscript{12} and vitamin B\textsubscript{6} or genetic defects in their metabolism, may result in mildly elevated homocysteine concentrations in blood and urine. The fact that the homocysteine level fell as pregnancy progressed while folate, vitamin B\textsubscript{12} and pyridoxal 5'-phosphate levels were also falling, might be explained by the strong effect of haemodilution on all these determinants. The decreased vitamin levels would otherwise be accompanied by a rise in the homocysteine concentration. During pregnancy an increased homocysteine level is a risk factor for recurrent spontaneous abortion, intra-uterine death, abruptio placenta and neural-tube defects (Steegers-Theunissen et al. 1991, 1992; Kirke et al. 1993). It is not clear whether hyperhomocysteinaemia is a primary cause of these diseases and abnormal outcomes or is just an epiphenomenon.

Concentrations of the vitamins evaluated were measured in maternal serum or plasma. It is uncertain whether these concentrations represent vitamin functions at a cellular level, especially during pregnancy when an early shift from serum to tissue is proposed (Bruinse & van den Berg, 1995). Early preconceptional data were, however, lacking in Bruinse & van den Berg’s report. Our data do not substantiate such a serum to tissue shift early in pregnancy. Most observed pregnancy-induced vitamin changes occur progressively during pregnancy. The mechanisms for the changes of vitamin concentrations during pregnancy have not yet been resolved. Increased renal excretion based on elevated glomerular filtration rate has been suggested (Bruinse & van den Berg, 1995). Hormonal influences and haemodilution may contribute to the observed changes. In addition, the increased need for vitamins both for mother and fetus may account for the observed decline of some vitamins. However, increased catabolism compensated by supplementation does not always prevent these pregnancy-induced changes (Metcoff, 1978). Increased umbilical cord concentrations (riboflavin, vitamin B\textsubscript{6} and vitamin B\textsubscript{12}) compared with maternal concentrations suggest active transport to the fetus. Additional longitudinal studies should include hormonal analysis, urinary excretion and umbilical cord concentrations to address the mechanisms of vitamin changes during pregnancy.

Until now, preconceptional and first trimester values were lacking for the vitamins evaluated. Results from the present study suggest that preconceptional concentrations...
of vitamins and their recovery after delivery should be included in the evaluation of pregnancy-induced changes.

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