Folate, vitamin B_{12} and vitamin B_6 status in pregnant Albertan women

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Abstract

Folic acid supplementation and food fortification policies have improved folate status in North American women of child bearing age. Recent studies have reported the possible inadequacy of vitamin B_{12} and B_6 in the etiology of neural tube defects in folate fortified populations. The aims of this study were to describe folate status and its relationship to supplementation and to assess vitamin B_{12} and B_6 status in a cohort of pregnant women. Supplement intake data was collected in each trimester from the first cohort (n=599) of the APrON (Alberta Pregnancy Outcomes and Nutrition) study. Red blood cell folate (RBCF) and plasma folate, holotranscobalamin (holoTC) and pyridoxal 5-phosphate (PLP) were measured. Overt folate deficiency was rare (3%) but 24% of women in their first trimester had sub-optimal RBCF concentration (< 906 nmol.L^{-1}). The proportion of the cohort in this category declined substantially in second (9%) and third (7%) trimesters. High RBCF (>1360 nmol.L^{-1}) was observed in approximately half of the women during each pregnancy trimester. Vitamin B_{12} and B_6 deficiency was rare (<1% of the cohort). Women consuming folic acid supplements above the upper limit had significantly higher RBCF and plasma folate concentrations. These data suggest that approximately one quarter of women had sub-optimal folate status in the first trimester of pregnancy that improved over time, while the prevalence of vitamin B_{12} and B_6 deficiency was very low. The considerable prevalence of abnormally high RBCF raises concerns regarding the possibility of over-supplementation of folate during pregnancy and the consequences of high RBCF require investigation.

Key words: folate, folic acid, vitamin B_6, vitamin B_{12}, pregnancy, holotranscobalamin, pyridoxal 5-phosphate

Introduction

Folate, vitamin B_{12} and B_6 are essential for early embryonic development and impact health outcomes later in life. In Canada, folic acid fortification of cereal grains became mandatory in 1998. This policy resulted in a 46% reduction in the prevalence of neural tube defects (NTD) in Canada (De Wals et al. 2007) and an improvement in the folate status of the general population (Colapinto et al. 2012; Shakur et al. 2010). Folate deficiency is now rare in the Canadian population (< 1%); 40% of Canadians have high folate status (Colapinto et al. 2011). The implications of high folate status on health are not well understood; however, high daily intakes of folic acid (above 1000 μg/day) have been reported to negatively impact birth outcomes, particularly birth height (Pastor-Valero et al. 2011). Offspring of pregnant rodents fed folic acid at 20-fold the recommended intake exhibited embryonic delays, growth retardation and reduced fetal body weight and length (Achon et al. 2000; Pickell et al. 2011). Supplemental intakes of folic acid result in the appearance of unmetabolized folate in blood, presumably due to the limited capacity of hepatic dihydrofolate reductase to convert folate to dihydrofolate (Kalmbach et al. 2008; Sweeney et al. 2009). It has been suggested that the presence of unmetabolized folic acid could inhibit normal folate metabolism by competing with coenzymatic form of folic acid for transporters and binding proteins (Bailey and Ayling 2009; Kamen et al. 1985; Qiu et al. 2006). Although the rates of folate deficiency is low, only 25% of Canadian women of childbearing age (n = 1162) reported taking a folic acid supplement and 22% were not achieving folate status sufficient to minimize NTD risk (Colapinto et al. 2011) . A significant proportion of Canadian women may have suboptimal folate status during the critical period of neural tube closure, a period where many women are unaware of being pregnant.

Other concerns associated with high folic acid intakes included negative impact on masking vitamin B_{12} deficiency and the resultant neurological disruption (Morris et al. 2007; Reynolds 2002; Dickinson 1995), progression and development of cancers (Cole et al. 2007; Figueiredo et al. 2009), immune function (Troen et al. 2006) and epigenetic regulation disruption (Sie et al. 2013; Zeisel 2009). Because of the interaction between folic acid and vitamin B_{12} (Scott and Weir 1981), it has been suggested that 34% of all NTD occurring post-folate fortification may be caused by low vitamin B_{12} status (Ray et al. 2003; Ray and Blom 2003; Ray et al. 2007; Ray et al. 2008). Vitamin B_6 status during pregnancy is also of potential concern, as a low plasma pyridoxal 5-phosphate (PLP) has been associated with increased risk of spontaneous abortion and preterm birth (Ronnenberg et al. 2002; Ronnenberg et al. 2007).

Adequate folate, vitamin B_{12} and vitamin B_6 intake is essential for maternal health and fetal development. Status of these nutrients during early pregnancy in Canadian women is not known. The objectives of the current study were (*i*) to evaluate folate status and its relationship to supplementation and (*ii*) to assess the relationship between folate status and the status of vitamin B_{12} and B_6 in a cohort of pregnant women.

Materials and Methods

Study design and subjects

The present study employed the first cohort (n=599) of the Alberta Pregnancy Outcomes and Nutrition (APrON) study. The recruitment and methods of the APrON study have been described in detail (Kaplan et al. 2014). Subjects were enrolled in the APrON study between June 2009 and June 2010 from Edmonton and Calgary and were ≥ 16 years old, able to read and write in

English, and ≤ 27 weeks gestation. Written consent was obtained from all women prior to enrolment, and ethical approval for the study was obtained from the Health Research Ethics Boards at the University of Alberta (Pro 00002954) and the University of Calgary (E22101).

Women recruited at ≤ 13 week of gestation (first trimester, n = 138) were assessed during three trimesters; those recruited between 14-26 weeks of gestation (second trimester, n = 581) were assessed during their second and third trimesters (27-40 weeks, n = 533). Pre-pregnancy information (mental/medical history, physical activity and socio-demographics) was gathered during the first visit. Maternal characteristics including maternal age at study entry (17-30 years or 31-45 years), pre-pregnancy body mass index (BMI; underweight, normal weight, overweight or obese), household income/year (less than 20 000, 20 000 to 39 000, 40 000 to 69 000, 70 000 to 99 000 or ≥ 100 000), education level (≤ high school/diploma/certificate or ≥ high school/university study), ethnicity (Caucasian or other; Native/Asian/Latin American/African American), smoking (never or ever), previous pregnancy (yes or no), marital status (married/common law partner or other; single/divorced) and planned pregnancy (yes or no) were also obtained from women during their first visit and were considered as covariates. Information regarding folate intake from foods and supplementation was obtained from 24-hour recall and supplement intake questionnaires (SIQ), which were completed at each visit under the supervision of trained personnel. Participants' consumption of folic acid-containing multivitamins (type and quantity) during the previous 24 hour period was recorded (Gomez et al. 2013).

Folate, B_{12} and B_6 status

Biochemical analyses were carried out as previously described (Kaplan et al. 2014). Briefly, non-fasting blood samples were taken at each visit, processed for serum, plasma, buffy coat and red blood cells, aliquotted, and stored at -80 °C. For red blood cell folate (RBCF) analysis, a hemolysate was prepared directly after blood sampling. The ion-capture method of analysis confers a number of analytical benefits over the traditional microbiological assay including ease of automation and small sample size requirement. The accuracy and reproducibility of blood folate analyses was assessed by repeated measurements of a whole blood standard reference material with a certified value (29.5 $nmol.L^{-1}$) (Whole blood 95/528; National Institute of Biological Standards and Control, Hertfordshire, United Kingdom). Repeated analysis of this standard in our laboratory yielded an interassay CV of < 10%.

Folate deficiency was defined as RBCF concentration ≤ 305 $nmol.L^{-1}$ (Institute of Medicine 1998). Due to lack of internationally recognized value of suboptimal folate status for NTD risk reduction, we used the cutoff of 305 to < 906 $nmol.L^{-1}$ for suboptimal folate status based on the findings of a nested case-control study in a prospective cohort of pregnant women (Daly et al. 1995). This study (n = 81 cases, 266 controls) conducted between 1986-1990 demonstrated a continuous inverse dose-response relationship of NTD risks with maternal RBCF concentration with its highest incidence at RBCF concentration below 340 $nmol.L^{-1}$ (6.6 per1000 live births) to lowest risk at 906 $nmol.L^{-1}$ (0.8 per 1000 live births). For high RBCF status we used a cutoff of >1360 $nmol.L^{-1}$, reflecting the 97th percentile from NHANES 1999-2004 (Pfeiffer et al. 2007). Plasma folate and plasma holotranscobalamin (holoTC) concentrations were determined using the AXSYM analyzer as per manufacturer's instructions. For plasma folate status, we used a standard cutoff of < 7 $nmol.L^{-1}$ for deficiency (Institute of Medicine 1998) and > 46 $nmol.L^{-1}$ for above the normal range which was previously defined for non-pregnant women (Pfeiffer et al.

2007). The reference value used for normal holoTC was 35 to 140 pmol.L^{-1} (Herrmann et al. 2003; Refsum et al. 2006) previously defined for non-pregnant women. Plasma pyridoxal 5-phosphate (PLP) was determined using an HPLC assay kit (Eagle Biosciences Inc, Nashua, NH, USA). Final plasma concentrations were determined using the calibrator as a reference. The reference range for normal vitamin B_6 status was 20 to 220nmol.L^{-1} as previously defined for non-pregnant women (Institute of Medicine 1998).

Statistics

All statistical analyses were conducted using SPSS version 20.0 (IBM SPSS for Windows, version 20.0, Chicago, IL); $p < 0.05$ were considered significant. Due to data not being normally distributed, median and 95% confidence interval (CI) were used to characterize RBCF, plasma folate, plasma holoTC and plasma PLP. The Mann-Whitney U tests were used to compare means of continuous variables and the Chi-square test was used for categorical variables. Friedman's test was used for data grouped by RBCF status categories to determine longitudinal changes in RBCF status. RBCF and plasma folate concentrations were expressed as mean ± SD grouped by folic acid supplementation above or below 1000 µg/d. Multiple linear regression analysis was used to determine the independent association between folic acid supplements intake and RBCF and plasma folate concentrations. First trimester RBCF and plasma folate values were not included in this analysis because of the very small number of samples available for comparison. The data were adjusted for folate intake from diet in all cases and also for maternal covariates which were found to be significantly associated with blood folate values differ in the bivariate analysis (Table S2).

Results

Subjects

Study participants were predominantly Caucasian, married, held university or post-graduate degrees, and had high family annual income ($100 000+). The majority of participants were multiparous and had a planned pregnancy. Full details of cohort demographics are available in a supplementary table (Table S1).

Folate, vitamin B_{12} and vitamin B_6 status of women

Blood samples were available from 122, 520 and 446 women in their first, second and third trimesters, respectively for determination of folate status (Fig. 1). Median RBCF concentration was significantly higher in the second and third trimesters (1504 nmol.L^{-1} and 1462 nmol.L^{-1}, respectively) compared with the first trimester (1280 nmol.L^{-1}) ($P < 0.05$, Table 1). Only 3 of 122 women in their first trimester, and no women in their second and third trimesters had a RBCF concentration corresponding to overt folate deficiency (< 305 nmol.L^{-1}) (Table 1). However, in the first trimester, 24% of women had an RBCF concentration below the value which has been suggested to minimize risk of NTD (< 906 nmol.L^{-1}, Table 1). Approximately 45%, 62% and 59% of women had RBCF concentrations above the normal range (> 1360 nmol.L^{-1}) during their first, second and third trimesters, respectively (Table 1). All women fell within the normal range (7-46 nmol.L^{-1}) for plasma folate in all trimesters.

Change in folate status during pregnancy

The proportion of women with an RBCF concentration < 906 nmol.L^{-1} decreased, and the proportion of women with a RBCF concentration > 1360 nmol.L^{-1} increased over time compared to 1st trimester ($P < 0.001$, Fig. 2). In the group of women who were recruited in their first trimester and provided samples in all three trimesters, there was a significant increase in RBCF concentration over time (data not shown). Using the women enrolled in their second trimester into the cohort, RBCF concentration were found to increase in the third trimester for the women who identified as having suboptimal status (305 to < 906 nmol.L^{-1}) or status in normal range (906 to 1306 nmol.L^{-1}) in the second trimester ($P < 0.05$, Fig. 3). For women who were classified as having RBCF above the normal range in second trimester, there was a 10% decrease in RBCF concentration in the third trimester. However, all of these women remained with RBCF concentration above the normal range (Fig. 3).

Impact of folic acid supplementation on folate status

Mean RBCF and plasma folate concentration were both significantly higher in women who supplemented with folic acid above the Upper Limit (UL; 1000 µg/d) compared to women who reported taking a daily supplement below the UL ($P < 0.05$, Table 2). After adjusting for folate intake from diet and maternal covariates significantly differ (Table S2), the effect of supplemental folic acid dose on RBCF or plasma folate remained significant (Table 2).

B_{12} and vitamin B_6 status

HoloTC concentrations were within the normal range (35 to140 pmol.L^{-1}) in 88 - 91% of the women (depending on the trimester). Seven women (one in her first trimester and six in their second trimester) were found to have holoTC concentrations that would classify them vitamin

B_{12} deficient (<35 pmol.L^{-1}). More than 80% of the women had plasma PLP concentrations in the reference range during their first and second trimesters of pregnancy, and approximately 17% in the first trimester and 13% in the second trimester had plasma PLP concentrations above 220 nmol.L^{-1}. Due to high proportion of women with plasma folate, holoTC and PLP in the reference range in the first and second trimesters, these biomarkers were not measured in samples collected during third trimester.

Discussion

Although folate deficiency was rare (3%) in the APrON cohort, 24% of women in their first trimester had RBCF concentrations below the concentrations considered to minimize the risk of NTD (Daly et al. 1995; Tam et al. 2009). The proportion of women in this category declined substantially in the second and third trimesters. High RBCF was observed in 45-62% of the cohort (depending on trimester); the significance of this is currently unknown. We observed a very low prevalence of vitamin B_{12} and B_6 deficiency (less than 1% of the cohort).

Our results are in contrast to a relatively recent Canadian study. Ray et al. (2008) investigated vitamin B_{12} status of 10,622 Ontarian women (of child bearing age or pregnant) and reported that 7% of the women of child bearing age and 5% of women <28 days of gestation had a serum vitamin B_{12} concentration below 125 pmol.L^{-1} during critical period of neural tube closure. They suggested that this was the result of women not preparing nutritionally for pregnancy by taking prenatal multivitamins. The incongruous findings between the studies may be partly explained by the differences in demographics between the cohorts; most of the pregnancies in the APrON cohort-1 were planned and the majority of women were in a very high socio-economic status group. The retrospective, cross-sectional study of Ray et al. (2008) was

likely more varied in its demographics since cases were recruited based on concomitant analysis of human beta-gonadotropin compare to the current study where the women recruited were volunteers. The discrepancies among the two studies may also be explained by the difference in the status biomarker selected; where serum B_{12} concentration was chosen in the previous study, the current study employed holoTC as a measure of vitamin B_{12} status. HoloTC is the biologically active fraction of vitamin B_{12} (representing 30% of total plasma B12) and both holoTC and methylmalonic acid (a functional biomarker of vitamin B_{12}) have been reported to provide a better index of true cobalamin status than the measurement of total vitamin B_{12} (Herrmann et al. 2003).

During pregnancy total cobalamin (B_{12}) concentration decreases up to 50% over the gestation period; however, concentration of holoTC remained unchanged (Koebnick et al. 2002; Morkbak et al. 2007). Therefore, total B_{12} may overestimate the proportion of individuals with low B_{12} status and holoTC may be a better indicator of vitamin B_{12} deficiency during pregnancy.

It is recommended that all women of child-bearing age consume a daily supplement containing 400 μg of folic acid (Morin et al. 2002; Health Canada 2009) as demands for folate during pregnancy do not appear to be met in the vast majority of women through their self-selected diets (Houghton et al. 2007). We observed that 24% of the cohort had suboptimal RBCF status during the first trimester. This is most likely due to insufficient preconception folate intake, as folate concentration in RBC during this trimester would be influenced by pre-pregnancy status. This finding was also observed in the Canadian Health Measure Survey which reported that 22% of women of child-bearing age had sub-optimal folate status (Colapinto et al. 2011). In another study, 36% of pregnant Ontarian women had a RBCF concentration below 906 $nmol.L^{-1}$ (Bar-Oz et al. 2008). In the present study, women who had low or suboptimal

250 folate status during early pregnancy had significantly higher RBCF at the second and third
251 trimester (Fig. 3). This is most likely due to supplemental intake of folic acid throughout
252 pregnancy; the women in APrON cohort reported taking a folic acid-containing supplement at
253 94, 97 and 94% during the first, second and third trimesters respectively (Gomez et al. 2013). In
254 the present study, women with less than optimal RBCF status had non-fasting plasma folate in
255 the reference range, suggesting that women were indeed taking folic acid supplements. In
256 support of this, a small but significant difference in plasma folate concentration was observed in
257 women taking a supplement that contained folic acid at levels above the UL compared to those
258 taking a supplement below this. In the current study plasma folate concentrations in a non-fasting
259 blood sample do not appear to be a good indicator for assessing folate status (Table 1) especially
260 during early pregnancy when women may have lower status but have begun to take maternal
261 supplements.
262 Approximately, 45% of the APrON women had RBCF concentration above 1360 $nmol.L^{-1}$
263 during the first trimester and the proportion of women in this category increased over time (62%
264 and 59% during the second and third trimesters, respectively). The impact of very high folate
265 status on fetal development and maternal health is not well elucidated; however, animal studies
266 have demonstrated risks associated with very high intakes of folate during gestation. Feeding
267 pregnant dams twenty times higher folic acid than recommended resulted in reductions in birth
268 weight and fetal length (Achon et al. 2000) and increased the risk of embryonic delay and growth
269 retardation (Pickell et al. 2011). It has been suggested that the detrimental effects of high folic
270 acid intake may be due to a disruption in normal folate metabolism by the presence of
271 unmetabolized folic acid in the plasma (Lucock 2004). The level of supplementation used in
272 these animal studies was undoubtedly much higher than that of the women in the APrON study.

The physiological implications of high RBCF concentrations during pregnancy in women are not known. Recently, it was reported that women who ingested high doses of folic acid supplements (above the UL) during early pregnancy were at significantly higher risk of delivering a baby with small for gestational age-height (OR 5.33, CI 2.08, 13.7) (Pastor-Valero et al. 2011). Folate in the form of tetrahydrofolate is also an essential cofactor for DNA methylation and plays an important role in regulating gene expressions. Sie et al. (Sie et al. 2013) reported that maternal folic acid supplementation 2.5 times higher than the dietary requirements significantly decrease global and site-specific hepatic DNA methylation in weanling rat off-springs. Collectively, these suggest that consuming high levels of folic acid through supplements can have profound impact on fetal development through epigenetic changes. The functional outcomes of these changes warrant further research, particularly in light of the high prevalence of high folic acid supplementation in a folic-acid fortified food environment.

In summary, there was virtually no evidence of deficiency of vitamin B_{12}, B_6 and folate in the first APrON cohort of pregnant women. Despite a lack of deficiency, approximately 24% of the APrON women did not achieve the folate status recommended to minimize the risk of NTD during early pregnancy, suggesting that recommendation of prenatal supplement are not being met by a subgroup of Canadian women. However, a large proportion of the cohort had high RBCF concentrations above the normal range, which has been shown to also exert detrimental effects on fetal outcomes, in animal models. Caution should be taken in generalizing our findings to the entire population as most of the women who volunteered for this study were of high socio-economic status and 90% of them reported taking a multivitamin containing folic acid once they became pregnant (Gomez et al. 2013). Future studies are required to examine potential health risks associated with marginally suboptimal and very high folate status,

296 particularly later in gestation when women appear to be taking high amounts of supplemental
297 folic acid.

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Table 1. Folate, vitamin B_{12} and vitamin B_6 status among pregnant women enrolled in the Alberta Pregnancy Outcomes and Nutrition (APrON) study cohort-1.

	First trimester		Second trimester		Third trimester	
	N	Median (95% CI)	N	Median (95% CI)	N	Median (95% CI)
RBCF (nmol.L^{-1})						
<305	3	250 (175,353)	0	-	0	-
305 to <906	29	573 (547,681)	44	745 (713,782)	29	811 (757,825)
906 to1360	35	1114 (1069,1169)	156	1191 (1143,1187)	152	1186 (1156,1195)
>1360	55	1740 (1735,1992)	320	1740 (1723,1777)	265	1730 (1714,1778)
Total	122	1280 (1199,1428)[a]	520	1504 (1455,1525)[b]	446	1462 (1453,1526)[b]
Plasma folate (nmol.L^{-1})						
<7	0	-	0	-		ND
7 to 46	128	36 (35,36)	534	36 (35,36)		ND
>46	0	-	0	-		ND
Total	128	36 (35,36)[a]	534	36 (35,36)[b]		ND
Plasma holoTC (pmol.L^{-1})						
<35	1	26	6	31 (22,35)		ND
35 to140	108	87 (84,93)	472	81 (80,84)		ND
>140	14	256 (202,290)	43	232 (224,269)		ND
Total	123	92 (95,117)[a]	521	83 (90,99)[b]		ND
Plasma PLP (nmol.L^{-1})						
< 20	0	-	1	14		ND
20 to 220	99	84 (88,106)	457	67 (79,88)		ND
>220	20	359 (332,451)	70	302 (297,334)		ND
Total	119	94 (123,170)[a]	528	76 (106,122)[b]		ND

Note: RBCF, red blood cell folate; ND, not determined. $P < 0.05$ obtained from Wilcoxon Signed Ranks test and letters not similar are significantly different.

Table 2. Folate status in pregnant women according to supplemental intake of folate.

Folic acid supplement (µg/d)	≤1000		>1000			β		
	N	mean ± SD	N	mean ± SD	*P**	Coefficient	95% CI	*P*
RBCF (nmol.L^{-1})								
Second trimester	387	1404 ± 1	115	1536 ± 1	0.006	101.25	11.62, 190.87	0.027†
Third trimester	347	1409 ± 1	86	1557 ± 1	0.003	150.82	57.53, 244.11	0.002
Plasma folate (nmol.L^{-1})								
Second trimester	400	35 ± 1	116	36 ± 1	0.001	1.20	0.24, 2.17	0.015

Note: *$P < 0.05$ was significant, obtained from Mann-Whitney tests for pairwise comparisons. Multiple linear regression analysis was conducted to adjust the data for folate intake from diet and further for maternal covariates significantly different. † adjusted for maternal age, income and ethnicity.

Figures captions

Fig. 1. Women recruitment in APrON cohort-1 during each pregnancy visit for biochemical analyses

Fig. 2. Proportion of women divided by RBCF status. The percentage of women in the <305 nmol.L^{-1} and 305 to < 906 nmol.L^{-1} status ranges decreased and for >1360 nmol.L^{-1} range increased significantly over time ($P < 0.001$ from chi-square analysis)

Fig. 3. Changes in mean RBCF concentration between trimester 2 and 3. Bars are mean ± SD for the women who were recruited in their 2nd trimester and provided samples in their second and third trimesters. The x-axis represents the reference range for RBCF in which women were classified in second trimester. Within each RBCF reference range, bars that do not share a letter are significantly different ($P < 0.05$, obtained from Friedman's test).

Fig. 1.

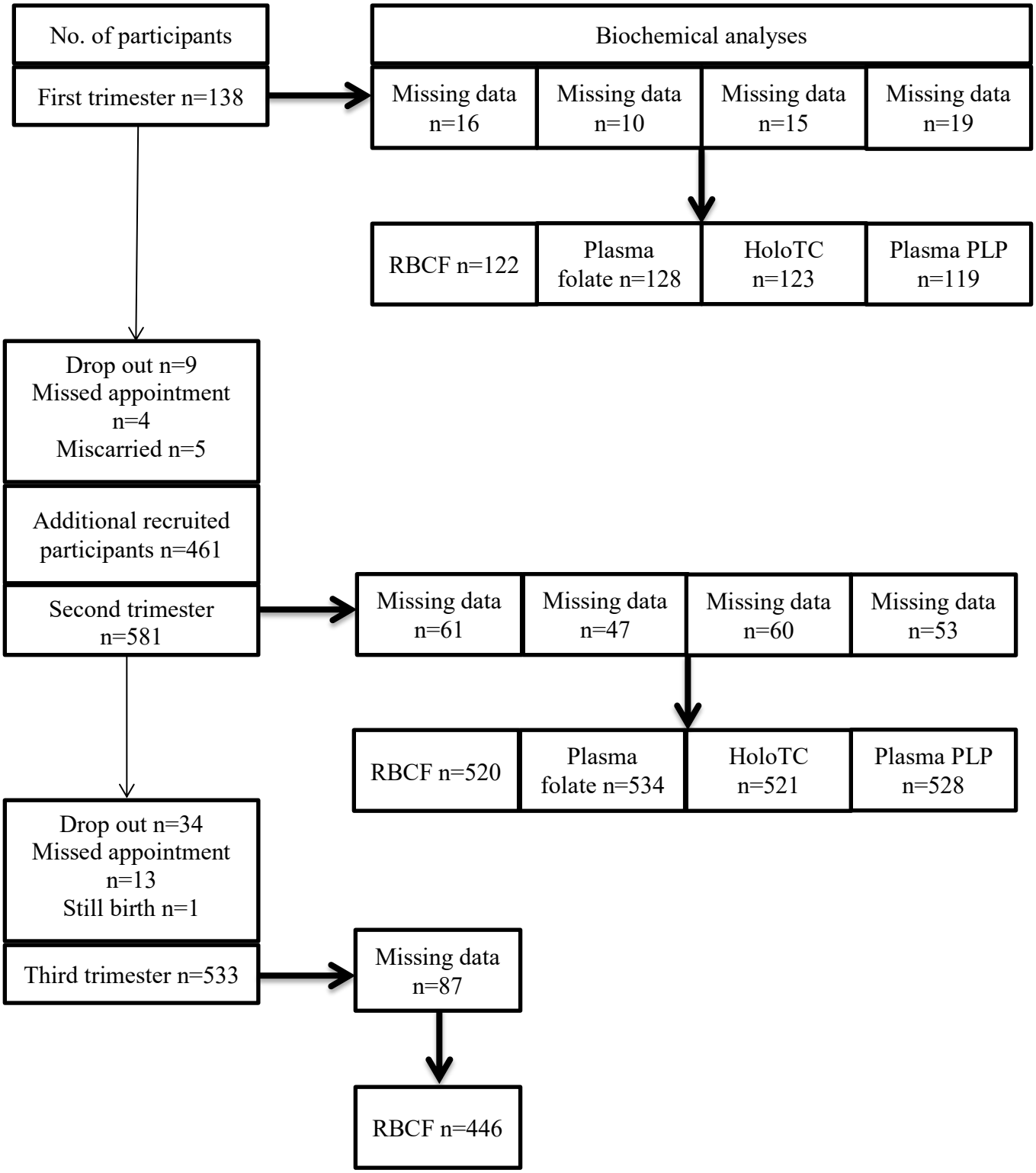

No. of participants
First trimester n=138
Biochemical analyses
Missing data n=16
Missing data n=10
Missing data n=15
Missing data n=19
RBCF n=122
Plasma folate n=128
HoloTC n=123
Plasma PLP n=119
Drop out n=9
Missed appointment n=4
Miscarried n=5
Additional recruited participants n=461
Second trimester n=581
Missing data n=61
Missing data n=47
Missing data n=60
Missing data n=53
RBCF n=520
Plasma folate n=534
HoloTC n=521
Plasma PLP n=528
Drop out n=34
Missed appointment n=13
Still birth n=1
Third trimester n=533
Missing data n=87
RBCF n=446

Fig. 2.

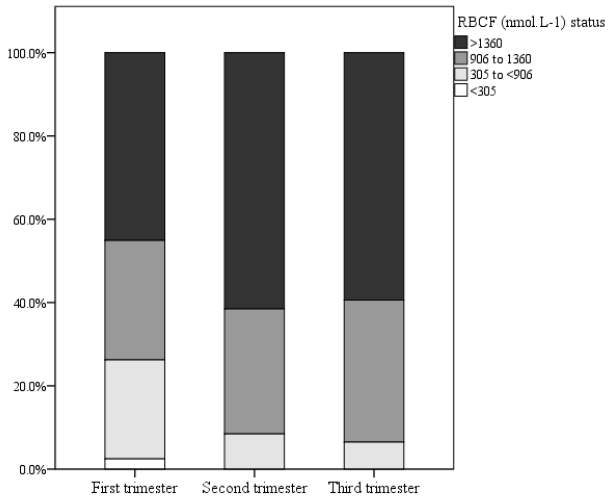
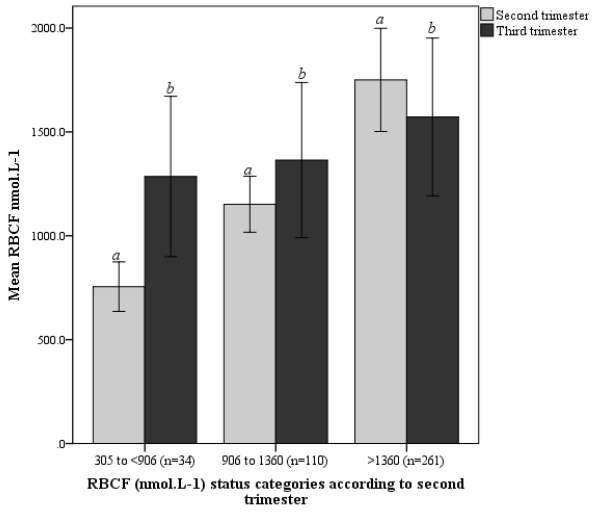

RBCF (nmol.L-1) status
>1360
906 to 1360
305 to <906
<305
100.0%
80.0%
60.0%
40.0%
20.0%
0.0%
First trimester
Second trimester
Third trimester

Fig. 3.


Second trimester
Third trimester
2000.0
1500.0
1000.0
500.0
.0
Mean RBCF nmol.L-1
a
b
a
b
a
b
305 to <906 (n=34)
906 to 1360 (n=110)
>1360 (n=261)
RBCF (nmol.L-1) status categories according to second trimester