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Original Research Article

# Folate and cobalamin status, indicators, modulators, interactions, and reference ranges from early pregnancy until birth: the Reus–Tarragona birth cohort study

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A B S T R A C T

**Background:** Folate and cobalamin status, although essential for pregnancy, are not routinely monitored in prenatal care.
**Objectives:** To investigate folate and cobalamin status and determinants throughout pregnancy, in the absence of mandatory folic acid (FA) fortification.
**Methods:** In a cohort study of 831 mothers recruited at <12 gestational weeks (GW), plasma folate, total homocysteine (tHcy), cobalamin, holotranscobalamin (holoTC), methylmalonic acid (MMA), red blood cell folate (RBCF), and the combined cobalamin status indicator (cB12) were determined at ≤12, 15, 24–27, 34 GW, labor and in the cord. Single nucleotide polymorphisms affecting folate and cobalamin status were determined. FA, cobalamin, micronutrient supplement use, and dietary folate and cobalamin intake (food frequency questionnaire) were recorded. Folate and cobalamin status predictors were assessed by multiple linear regression analysis.
**Results:** Only 36.1% of the participants took FA preconceptionally and 47.4% and 7.3% had suboptimal RBCF (<906 nmol/L) and plasma cobalamin status (≤221 pmol/L), respectively, at ≤12 GW. RBCF determinants included planned pregnancy, FA supplementation, plasma cobalamin, and methylenetetrahydrofolate (*MTHFR*) 677C>T genotype. Cobalamin supplementation was positively associated with plasma cobalamin and early holoTC. Smoking and BMI were inversely associated with plasma cobalamin and early holoTC, but none were associated with MMA. Only participants with the *MTHFR* 677TT genotype, exceeding FA supplement recommendations, improved their folate status (interaction term: B (95% CI):0.15 (0.01, 0.29), $P = 0.032$). Smoking was inversely associated with plasma cobalamin status in participants with the methionine synthase reductase (*MTRR*) 524CC genotype only (interaction term:0.07 (0.01, 0.04), $P = 0.014$). Mothers with low early pregnancy cobalamin status and also those with bigger newborns, had lower cobalamin status at labor.
**Conclusions:** Suboptimal early pregnancy folate or cobalamin status affected 47.4% and 7.3% of the participants, respectively. The *MTHFR* 677TT genotype predicted folate status throughout pregnancy. Smoking and BMI were negatively associated with cobalamin status throughout pregnancy.
Clinical Trial Registry number and website where it was obtained: NCT01778205. www.clinicaltrials.gov

*Keywords*: cobalamin, cord, folate, homocysteine, holotranscobalamin, methylmalonic acid, pregnancy

## Introduction

Folate and cobalamin are essential for embryogenesis and fetal development. They interact when the folate cycle provides methyl groups to the cobalamin cofactor of methionine synthase for the remethylation of homocysteine to methionine. Deficiency in either of these vitamins is an established risk factor for neural tube defects (NTDs). Mandatory fortification of wheat flour with folic acid first

*Abbreviations:* cB12, combined cobalamin status indicator; CI, confidence interval; CV, coefficient of variation; EDTA-K2, ethylene diamine tetraacetic acid-dipotassium; FA, folic acid; GW, gestational weeks; HoloTC, holotranscobalamin; ICC, intraclass correlation coefficient; LOD, limit of detection; MMA, methylmalonic acid; MTHFR, methylenetetrahydrofolate reductase; MTR, methionine synthase; MTRR, methionine synthase reductase; NTD, neural tube defects; 1C, 1 carbon; OR, odds ratio; RTBC, Reus–Tarragona Birth Cohort; RBCF, red blood cell folate; SLC19A1, solute carrier family 19A member 1; TCN2, transcobalamin II (gene); tHcy, total homocysteine; UHSJR, University Hospital Sant Joan Reus.

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started 26 y ago in the United States, to reduce the prevalence of pregnancies affected by NTDs [1]. This policy is now implemented in over 80 countries worldwide, except for most of Europe [2].

The prevention of NTDs, in unfortified populations, depends on voluntary supplementation by females with 400 μg/d of folic acid for 8–12 wk before conception and until the end of the 12th wk of pregnancy [3]. Folate deficiency (plasma folate <7 nmol/L) was reported in 24.2% of females of reproductive age from a Spanish study [4] and, albeit applying a higher cut off (plasma folate <13 nmol/L), a study in the United Kingdom reported deficiency in 52% and insufficient red blood cell folate (RBCF) status to protect against NTDs (<906 nmol/L) in 89% of females [5]. In the presence of mandatory fortification of flour with folic acid, insufficient RBCF status was still reported in 25% of nonsupplement using Canadians of childbearing age [6]. However, another Canadian study reported very high first trimester plasma folate status (median of 96 nmol/L) due to use of prenatal supplements in excess of 400 μg/d by over 95% of the participants [7].

The global prevalence of cobalamin insufficiency during the first trimester of pregnancy has been estimated to be as high as 20% and occurs even in the absence of vegetarianism [8]. A British National Diet and Nutrition study reported that 12% of females of fertile age were cobalamin deficient (serum cobalamin concentration <150 pmol/L), and over 40% were insufficient (150–258 pmol/L) [9]. In a recent national birth cohort study in Denmark, 1.3% of females were reported to be vegetarians or vegans during pregnancy. When supplement use was considered, their cobalamin intake was similar to that of omnivorous mothers. Nevertheless, preeclampsia and low birth weight were more prevalent among the vegan mothers and it should be considered that cobalamin status was not reported in this study [10].

Folic acid supplementation is recommended in virtually all clinical practice guidelines [3,11,12], but there are no recommendations for cobalamin supplementation. Underlying gene-metabolic causes of anomalies in embryogenesis or fetal development may be masked in the presence of mandatory fortification or folic acid supplement use and status during the critical early weeks of pregnancy may not be reflected in blood samples affected by physiological changes of pregnancy in mid-to-late pregnancy. We hypothesized that real insufficiencies in folate and cobalamin would be detectable in early pregnancy, in a cohort of mothers unexposed to mandatory folic acid fortification. We set out to investigate *1)* the evolution of folate and cobalamin status and their functional biomarkers from early pregnancy, *2)* their determinants and how they interact, *3)* the patterns of folic acid supplement use among mothers in the absence of mandatory folic acid fortification, and *4)* to propose reference ranges throughout pregnancy.

## Methods

Details of the setting of the longitudinal study and participant recruitment have been described previously [13,14]. Participants were recruited between the years 2005 and 2020 and followed up until the end of their pregnancy. Briefly, the study was carried out by the Unit of Preventive Medicine and Biostatistics, Universitat Rovira i Virgili in collaboration with the Areas of Obstetrics and Gynecology in the University Hospitals, Sant Joan Reus and Joan XXIII Tarragona, Spain. These are the 2 biggest public hospitals in Tarragona province. The study was carried out in compliance with the Declaration of Helsinki, ethical approval was obtained from the Institut d'Investigació Sanitària Pere Virgili Ethics Committee (IISPV-CEIm), and signed informed consent was obtained from all participants.

The Reus–Tarragona Birth Cohort (RTBC) study sample size was calculated based on the required number of participants to investigate the association between first trimester elevated fasting plasma total homocysteine (tHcy) and small for gestational age. The study was sufficiently powered to address the current aim. In a population study in the same geographic region, nonpregnant females aged 18–50 y and not taking folic acid supplements, with the methylenetetrahydrofolate reductase (*MTHFR*) 677TT genotype had an odds ratio (OR) of 6.73 for RBCF <906 nmol/L compared with those with the CC genotype [4]. Assuming the prevalence of RBCF <906 nmol/L in females not taking folic acid at preconception is 60% in those with the CC genotype and 90.5% in those with the TT genotype, accepting a 2-sided confidence level of 95%, and power of 80%, 37 pregnant mothers with the CC genotype, and 37 with the TT genotype would be required to study the evolution of folate status during pregnancy. At an estimated prevalence of 18.1% of the *MTHFR* 677TT genotype, 210 participants were required.

Pregnant mothers attending their first prenatal checkup at the high-risk obstetric units of either hospital were screened for invitation to participate in the study. Eligibility criteria included singleton pregnancy of <12 gestational weeks (GW), no major surgery affecting nutritional status, no use of medication affecting folate or cobalamin status. Fetal viability was confirmed by ultrasound scan and the first prenatal blood draw was programmed before 12 GW. All participants were recommended by their obstetrician to take supplements containing 400 μg/d of folic acid and 2 μg/d of cyanocobalamin at their first checkup, until the end of the first trimester. They were also recommended to take iron supplements of 40 mg/d from 12 GW until the end of pregnancy. Detailed information regarding folic acid, iron, and multivitamin supplement use in the 6 mo before pregnancy and throughout pregnancy, as well as lifestyle habits in the 5 y before pregnancy, was collected from the participants at 20 and 32 GW.

Current and previous smoking exposure was assessed by clinical history, lifestyle questionnaire and by plasma cotinine concentrations ≥ 0.881 ng/mL (indicating active smoking) at ≤12 and 24–27 GW and in the cord. Smoking status was categorized as nonsmoker, passive smoker, smoker during the first trimester of pregnancy only, and smoker throughout pregnancy. Habitual food intake in the 9 mo before and during pregnancy was assessed by a validated 45-item, across 16 food groups, food frequency questionnaire [15] at 12 GW and the day after giving birth. We calculated the energy-adjusted daily dietary intake of folate and cobalamin, without including intake derived from vitamin supplements. Folic acid and cobalamin intake from supplements were calculated separately, based on the information reported by the participants regarding brand, dose, frequency, and timing of supplement use, as previously reported [13]. All data were double entered into the study database to detect errors from data input. Any discrepancies, unreasonable or missing data were solved by re-checking the data at source (questionnaires, clinical history, or from the participants) under the supervision of the principal investigators.

Folic acid supplementation was categorized as:

*Reference*: Adherence to current clinical guidelines for folic acid supplementation: 400 μg/d until the end of the first trimester (some of this group had started supplementation with 400 μg/d before pregnancy). *Less than the reference*: no preconception supplement use and <400 μg/d during the first trimester. *More than the reference*: >400 μg/d during the first trimester, followed by no further use or ≥400 μg/d until the end of the first trimester followed by continued or intermittent use of some form of folic acid–containing supplement during the second and third trimesters. *Highest folic acid exposure*: ≥400 μg/

d from before conception and throughout pregnancy. Regarding cyanocobalamin supplement use, participants were classified as no supplement use, first trimester supplement use only, or use throughout pregnancy.

Fasting blood samples were collected at ≤12, 15, 24–27, and 34 GW and nonfasting samples on admission to hospital with confirmed labor. All samples were collected into ethylene diamine tetraacetic acid-dipotassium (EDTA-K2) vacutainers for plasma and untreated vacutainers for serum. A total of 40 mL of umbilical cord venous blood was collected in utero, before expulsion of the placenta, by obstetricians trained in collection according to biobank stem cell protocols. The cord was cleaned with alcohol and iodine disinfectant solution before inserting an intramuscular needle into the umbilical vein and collecting the blood into 10 mL vacutainers (2 EDTA-K2 and 2 untreated).

For all samples, the EDTA-K2 tubes were mixed by inversion 10 times and kept refrigerated until they were processed in the laboratory. In strict adherence to study protocol, plasma was separated in <1 h after blood draw. First an aliquot of whole blood from the EDTA-K2 vacutainers was diluted 1/10 with 1% ascorbic acid solution and incubated at room temperature for 30 min. Subsequently, plasma was separated and removed, the remaining cells were washed in hemolysis solution until a clean leukocyte pellet was obtained. The pellet was resuspended in cell lysis solution (Qiagen) and incubated at room temperature, for ≥1 mo until the solution was transparent. Subsequently the DNA was extracted from this solution using the Gentra puregene blood kit (Qiagen). Following processing, all plasma and diluted whole blood samples were frozen at −70°C until they were transported by courier to Bevital AS, Bergen, Norway, for the following determinations (as described at https://bevital.no/methods/): plasma and RBCF (microbiological assay using *Lactobacillus casei*; limit of detection (LOD): 2 nmol/L; interassay coefficient of variation (CV): 5%; intraclass correlation coefficient (ICC): 0.56), cobalamin (microbiological assay using *Lactobacillus leichmannii*; LOD: 30 pmol/L, CV: 5%; ICC: 0.82), tHcy (LOD: 0.1 μmol/L, CV: 2%; ICC: 0.72), methylmalonic acid (MMA; LOD: 0.03 μmol/L, CV: 3%; ICC: 0.81) (gas chromatography–tandem mass spectrometry), and cotinine (liquid chromatography–tandem mass spectrometry). Plasma creatinine was determined by Jaffé reaction (Química Clinica Aplicada, SA on the COBAS MIRA autoanalyzer) in the Faculty of Medicine & Health Sciences and holotranscobalamin (holoTC; LOD: 0.08 pmol/L, CV: 2%; ICC: 0.95) by the AxSYM microparticle enzyme immunoassay (Abbott Laboratories Inc.) in the University Hospital Sant Joan Reus (UHSJR) clinical laboratory, as previously described [14]. The holoTC determinations were only carried out for the first 420 pregnancies, due to a change of laboratory supplier and replacement of the AxSyM autoanalyzer at that point. The combined cobalamin status indicator (cB12) was calculated using the spreadsheets provided by Fedosov et al. [16] applying the equation: $3cB12 = \log_{10}[B12/(MMA \times tHcy)] - [379/1+ (\text{maternal age}/230)^{2.6}]$ adjusted for plasma folate concentration. The rs1801133 (*MTHFR* 677C>T), rs1051266 (solute carrier family19 (*SLC19A1)* 80G>A), rs1805087 (methionine synthase *MTR* 2756A>G), rs1801394 (methionine synthase reductase (*MTRR)* 66A>G), rs1532268 (*MTRR* 524C>T), rs9606756 (transcobalamin II (*TCN2)* 67A>G), and rs1801198 (*TCN2* 776C>G) single nucleotide polymorphisms were determined in DNA using the AGENA Bioscience iPLEX Gold reaction kit (at Centro Nacional de Genotipado-Fundación Pública Galega, Grupo de Medicina Xenómica (CEGEN-FPGMX), Santiago de Compostela, Spain). Potential interactions among smoking habits, folate and cobalamin dietary intake, folic acid and cobalamin supplement use, and genotypes were explored.

## Statistical analysis

Percentages (95% confidence interval [CI]), means (95% CI) and medians (P25, P75) or (P10, P90) are reported for descriptive variables. The Kolmogorov–Smirnoff test was used to check the distributions of the variables and, when skewed, they were natural log transformed for the application of parametric tests. Categorical variables were compared between groups by Chi-square, continuous variables by Kruskal–Wallace, Mann–Whitney *U* and Wilcoxon tests for nonparametric variables, and analysis of variance (ANOVA) with post hoc Bonferroni correction for multiple comparisons for parametric variables. Longitudinal comparison of medians throughout pregnancy was done using the Friedman test with post hoc Bonferroni correction of *P* values for multiple comparisons. Spearman correlations were used to assess the associations between folate and cobalamin biomarkers throughout pregnancy. Predictors of plasma folate, RBCF, tHcy, cobalamin, holoTC, MMA, and cB12 were determined by multiple linear regression analysis. Maternal age, first trimester BMI, previous pregnancies, socioeconomic status (low vs. mid-high), smoking habit (active compared with nonsmoking), *MTHFR* 677C>T, *SLC19A1* 80G>A, *MTR* 2756A>G, *MTRR* 66A>G, *MTRR* 524C>T, *TCN2* 67A>G, and *TCN2* 776C>G genotypes (homozygote variant vs. homozygote common and heterozygote variant vs. homozygote common) were included as independent variables in the models. Interactions were tested by including the product of the 2 potentially interacting covariables in the model. Subgroup analysis was performed when interactions were detected. Regression diagnostics were applied to confirm model validity and compliance with assumptions, and sensitivity analysis was carried out when necessary to test for the inclusion of outliers in the multiple linear regression models. Total percentage changes in cobalamin status indicators between ≤12 GW and 24–27 GW and 34 GW and labor were compared using the nonparametric Quade Analysis of Covariance (ANCOVA) test and post hoc Bonferroni correction of *P* values was applied to correct for multiple comparisons. This test was also used to compare cobalamin status indicators between birth weight tertiles. GW at the time of blood draw and cobalamin supplement use compared with no use were included as covariables in the analyses. No imputations for missing data were performed at any point. All reported results are based on the available *N* for each variable at each time point. Statistical analysis was carried out with SPSS version 29.0 for Windows.

## Results

Participant recruitment and follow-up details are illustrated in Figure 1. Of the initial cohort of 831 pregnant mothers enrolled in the study, 791 with blood samples collected strictly at ≤12 GW were included for analysis. The median (P25, P75) age of the participants was 32 y (27, 38), BMI was 23.3 kg/m$^2$ (19.6, 29.9), and gestational age at the first blood draw was 9.0 wk (8.0, 11.0). Although 80% of participants took at least the folic acid dose recommended by obstetricians at the first prenatal checkup, only 36.1% had started folic acid supplementation before conception (Figure 2A). More mothers with an interpregnancy interval of <2 y, planned pregnancy, mid-high socioeconomic status, and that were nonsmokers, took folic acid preconceptionally (Supplemental Figure 1). Notably, 38.9% of participants were active smokers when they became pregnant, and

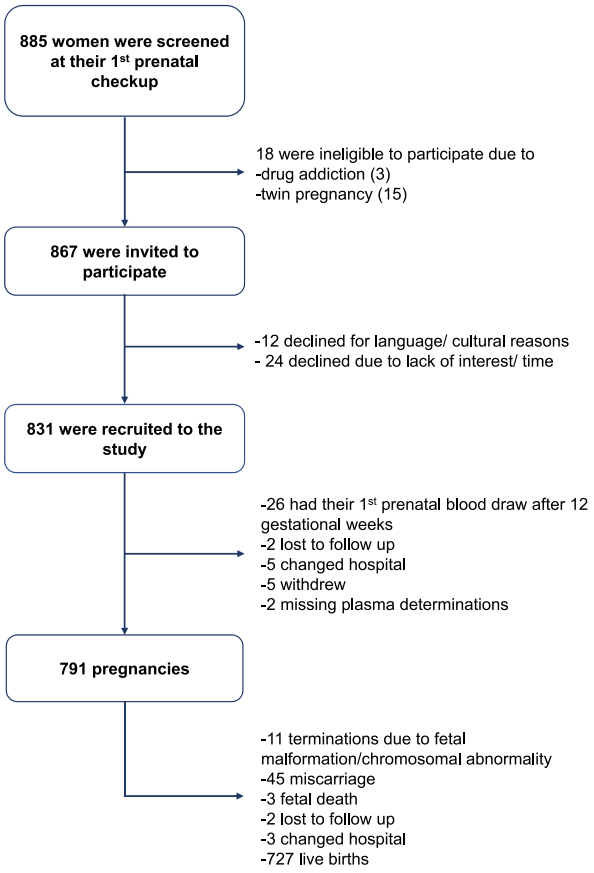


**FIGURE 1.** Flow chart of participant recruitment and follow-up.

24.2% continued smoking throughout pregnancy. All of the reported cobalamin-containing supplements contained cyanocobalamin. None of the participants were vegan, 2 reported being pescatarian, and 1 was lacto-ovo vegetarian. The most prevalent single nucleotide polymorphisms potentially affecting folate transport was *SLC19A1* G80A, and affecting the role of cobalamin in methionine synthesis, was *MTRR* A66G (Supplemental Table 1). The prevalence of the *MTHFR* 677TT genotype was 16.6%.

Participant characteristics according to folate and cobalamin status categories in early pregnancy (<P25, P25–P75, >P75) are reported in Table 1. Compared with the reference category (P25–P75), more mothers in the lowest category for both RBCF and plasma cobalamin, had low socioeconomic status, less of them were nonsmokers and more in the lowest plasma cobalamin quartile had anemia in the third trimester. However, mothers in the highest RBCF category were older, more had an interpregnancy interval of <2 y since their previous pregnancy, and had a history of high-risk pregnancy. Furthermore, more of them had planned their pregnancy and taken folic acid supplements before conception. Participants with the highest tHcy status were in the lowest RBCF or plasma cobalamin tertiles (data not shown). Those with the highest plasma MMA status, the lowest plasma holoTC status and cB12 were all in the lowest plasma cobalamin tertile.

Patterns of folic acid and cyanocobalamin supplement use and the corresponding changes in one carbon (1C) metabolism biomarkers throughout pregnancy are illustrated in Figure 2. In the case of folic acid, 30.2% of the participants adhered to the supplement regime proposed by the obstetric team, 49.8% exceeded it, and 20% did not meet the recommendation. In the case of cyanocobalamin, 20.3% of the participants adhered to the supplement regime proposed by the obstetric team, 51.2% exceeded it and 28.5% did not take any cyanocobalamin-containing supplements (Figure 2B, C). Plasma folate deficiency (<7 nmol/L) in early pregnancy was most prevalent in participants who did not adhere to the recommended folic acid supplement regime (histogram Figure 2D). Throughout pregnancy, the prevalence of plasma folate deficiency increased but was consistently lowest in the participants who exceeded the recommended supplement regime. RBCF deficiency (<340 nmol/L), albeit of lower prevalence than plasma folate deficiency, was most prevalent throughout pregnancy in participants who did not meet the recommended folic acid supplement regime (outlined histogram insert Figure 2F). This was also true for RBCF insufficiency (filled histogram Figure 2F). A U-shaped pattern was observed for RBCF insufficiency and deficiency prevalences. The prevalence of low cobalamin status (plasma cobalamin ≤221 pmol/L), defined outside of pregnancy [17], did not vary according to the pattern of cobalamin supplement use (histogram Figure 2H). Plasma folate gradually declined as pregnancy progressed, reaching its minimum concentration between 15 and 24 GWs (Figure 2D). RBCF was at its highest at 15 GW (Figure 2E) and at this point, plasma tHcy (Figure 2G) was at its lowest concentration. Participants taking >400 μg/d of folic acid had higher plasma folate and RBCF concentrations throughout pregnancy and lower tHcy in mid and late pregnancy, compared with the reference group. Unlike the other categories, tHcy concentrations did not differ from the first trimester until labor, in participants with the highest folic acid intake category.

Plasma cobalamin concentrations gradually fell as pregnancy progressed, plasma holoTC initially declined but then remained stable after 15 GW (Figure 2J). Plasma tHcy (Figure 2G), plasma MMA (Figure 2I), and the holoTC/total B12 ratio (Figure 2K), were higher in late pregnancy than in the first trimester. After 15 GW, cB12 gradually fell until the end of pregnancy (Figure 2L). Globally, low cobalamin status increased from 7% at <12 GW to 44% at labor (Figure 2H), and applying the cB12 cutoff of <−0.5, at labor, its prevalence was 1.7%. Cyanocobalamin supplement users had higher plasma cobalamin and holoTC concentrations throughout pregnancy, with no differences in MMA concentrations compared with those who stopped supplementation after the first trimester.

Umbilical cord venous concentrations of plasma folate, cobalamin, holoTC, and MMA were higher than those in the mother at labor, whereas tHcy was lower. Cord plasma folate concentrations were higher and tHcy lower, in the group of mothers taking >400 μg/d of folic acid compared with the reference group. Furthermore, cord holoTC concentrations were higher in the group of mothers consistently taking cobalamin supplements throughout pregnancy compared with the first trimester only.

Folate and cobalamin status indicator percentiles at each pregnancy time point are reported in Table 2 [16,18]. Limiting the analysis to uncomplicated pregnancies with favorable maternal and child outcomes ($n = 470$), produced similar results (data not shown). P50 for plasma folate, RBCF, and cB12 aligned with adequate nonpregnancy reference values, throughout pregnancy. The nonpregnant reference for folate deficiency of 7 nmol/L for plasma folate concentration [19] aligns with P5 in the first trimester but by labor corresponds with P25. P50 for RBCF in the first trimester aligns with the recommended ≥906 nmol/L to prevent NTDs. The lowest decile of plasma cobalamin concentration in the first trimester approached low cobalamin status, as defined outside of pregnancy, and this was observed at P25 by 24–27 GW and P50 by labor. The proposed nonpregnant reference of metabolic cobalamin deficiency, plasma holoTC ≤32 pmol/L [20] occurred

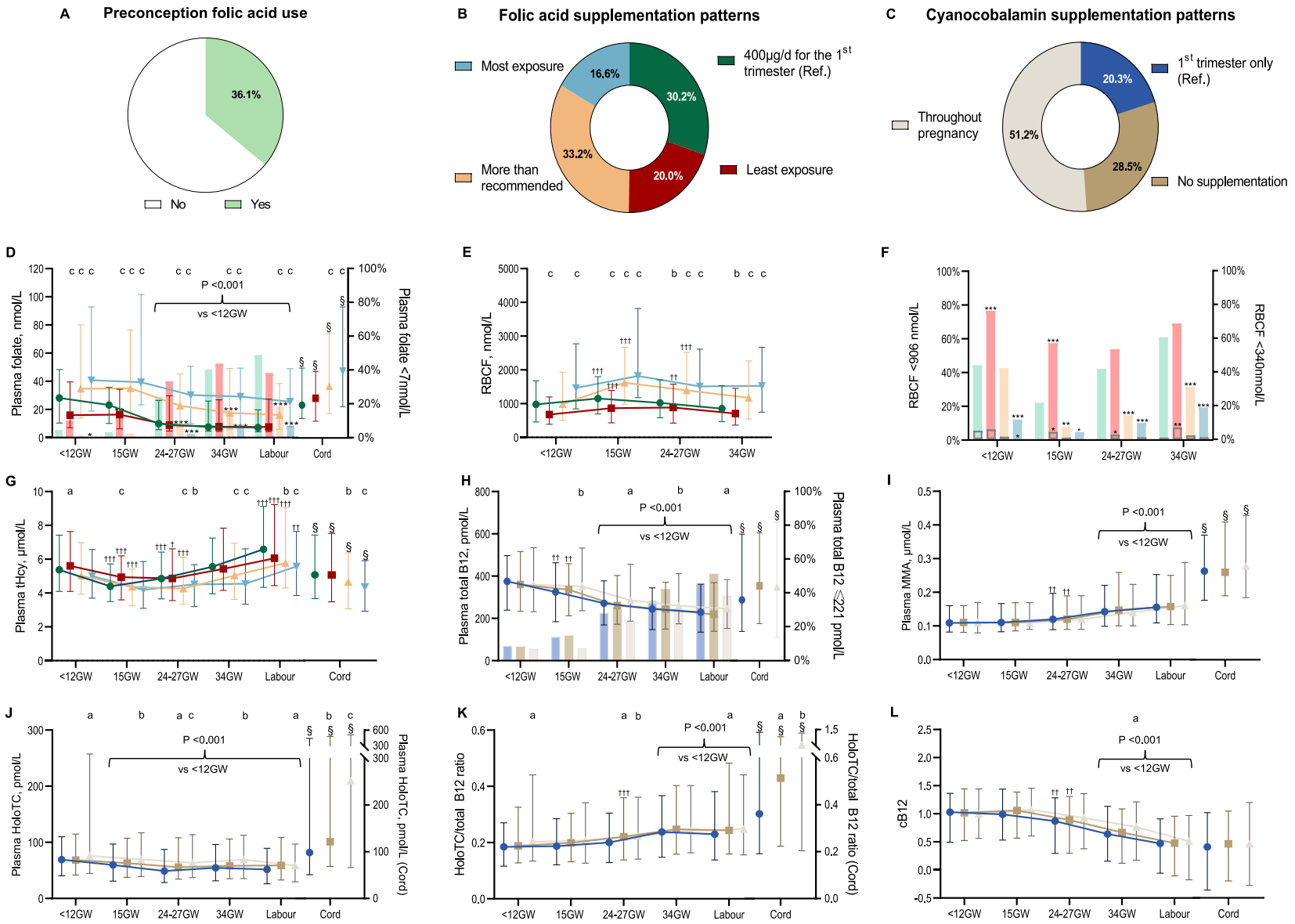


**FIGURE 2.** Changes in one carbon metabolism biomarkers throughout pregnancy. Medians (P10–P90) are reported for biomarker concentrations and percentages for categorical variables. (A) Prevalence of preconception folic acid use ($n = 684$). (B and C) Prevalence of folic acid ($n = 600$) and cyanocobalamin ($n = 694$) supplementation patterns. The curves in panels (D–G) show the changes in biomarkers according to the folic acid supplementation subgroups illustrated in panel (B). The curves in panels (H–L) show the changes in biomarkers according to the cyanocobalamin supplementation subgroups illustrated in panel (C). The colored histograms (panels D and F) correspond with the data reported for the folic acid supplementation subgroups illustrated in panel (B). The histograms in panels (D) and (H) correspond with the prevalences reported on the second *y*-axis of the graphs. The outlined histogram inserts (panel F) correspond with the data reported on the second *y*-axis. Plasma folate, cobalamin, tHcy, and MMA were

determined in 791 (≤12 GW), 438 (15 GW), 670 (24–27 GW), 637 (34 GW), 610 (labor), and 580 (cord) participants. Red blood cell folate was determined in 772 (≤12 GW), 437 (15 GW), 655 (24–27 GW), and 618 (34 GW) participants. Plasma holoTC was determined in 432 (≤12 GW), 368 (15 GW), 386 (24–27 GW), 372 (34 GW), 348 (labor), and 332 (cord) participants. The *n* varies between the models primarily due to reasons stipulated in the flow chart (Figure 1), eg, not showing up at scheduled blood draw, incompliance with sample processing protocol, optional screening blood test at 15 GW, unreturned questionnaires, and COVID-19 pandemic restrictions that prohibited research activities in the hospitals (affecting 11 pregnancies). Chi-square comparing prevalences to the reference group (in green), in panels (D) and (F): $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Friedman test, comparing longitudinally, median concentrations throughout pregnancy with <12 GW with post hoc Bonferroni correction of *P* values for multiple comparisons: $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$, $^{\dagger\dagger\dagger}P < 0.001$ and Mann–Whitney *U* comparing the medians between the different supplementation patterns and the reference group at the same time point: $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$. Wilcoxon test comparing cord and maternal concentrations at labor within each supplementation pattern: $^{\S}P < 0.001$. B12, cobalamin; cB12, combined cobalamin status indicator; GW, gestational weeks; HoloTC, holotranscobalamin; MMA, methylmalonic acid; RBCF, red blood cell folate; tHcy, total homocysteine.

at P3 at ≤12 GW, P5 at 15 GW, P6 at 24–27 GW, P7 at 34 GW, and P12 at labor. tHcy concentration ≥ 10 μmol/L [21] occurred at P99 at ≤12 and 34 GW (not shown) and at P95 at labor. Plasma MMA concentration >0.260 μmol/L [22] occurred at P99 at ≤12 , 15 and 24–27 GW, at P95 at 34 GW and at P90 at labor. A threshold lower than −0.5 of cB12, defined as low cobalamin status in nonpregnant females [16], only occurred at P2 at labor (not shown). To overcome the limitation of comparison with nonpregnancy reference ranges, the prevalence of genetic and functional indicators of folate and cobalamin status is reported for percentile reference ranges in Supplemental Table 2. The highest prevalence of the *MTHFR* 677TT genotype combined with tHcy ≥ P90 occurred below P10–P25 for plasma folate in early pregnancy and at labor. This was true for RBCF below P25-P50 throughout pregnancy. The highest prevalence of plasma MMA ≥ P90 occurred below P25–P50 for plasma cobalamin throughout pregnancy. The highest prevalence of cB12 ≤ P10 occurred below P10–P25 for plasma cobalamin, throughout pregnancy.

Spearman correlation coefficients between plasma folate, RBCF, tHcy, cobalamin, holoTC, MMA concentrations, and cB12 values throughout pregnancy are reported in Supplemental Table 3. The inverse correlation between folate status and tHcy is stronger, throughout pregnancy, than that of any of the indicators of cobalamin status and tHcy. Of these, plasma MMA consistently shows the highest correlation with tHcy. The inverse correlation between holoTC and MMA was consistently stronger than that of plasma cobalamin and MMA throughout pregnancy. Plasma folate and RBCF were positively associated with all indicators of cobalamin status, except MMA, throughout pregnancy. First-trimester RBCF was inversely correlated with plasma MMA from ≤12 GW until 34 GW.

Predictors of folate and cobalamin status throughout pregnancy, determined by multiple lineal regression analysis, are reported in Table 3. All of the multivariate models were statistically significant but only predicted between 13% and 28% of the variability ($R^2$) in plasma folate, RBCF, and tHcy, and less for plasma cobalamin and its biomarkers (9%–14% of plasma cobalamin, 9%–19% of holoTC, and 5%–11% of plasma MMA) or indicators (8%–10% of cB12).

Planned pregnancy was the most influential predictor of plasma folate and RBCF before 12 GW, but was no longer a predictor for the remainder of pregnancy. Folic acid supplement use was the strongest predictor of plasma folate and RBCF throughout pregnancy, of cB12 in mid and late pregnancy, and plasma folate of plasma cobalamin in late pregnancy, of holoTC in mid and late pregnancy, but never of MMA. The *MTHFR* 677 TT compared with CC genotype was inversely associated with RBCF status throughout pregnancy. Dietary folate intake was positively associated with plasma folate concentration in late pregnancy only, but it was positively associated with RBCF throughout pregnancy. Dietary cobalamin intake was positively associated with plasma cobalamin and cB12 in early pregnancy. Cyanocobalamin supplement use was positively associated with plasma cobalamin throughout pregnancy. Maternal BMI was negatively associated with plasma folate and plasma cobalamin throughout pregnancy and positively associated with tHcy in early pregnancy. It was never associated with MMA. In the tHcy models, the explained variability was higher when RBCF was included as a covariate instead of plasma folate. Folate status was consistently a stronger predictor of tHcy than any of the indicators of cobalamin status, throughout pregnancy. Furthermore, the difference in the strength of folate compared to cobalamin status as a predictor of tHcy was greater as pregnancy progressed.

**TABLE 1**
Participant characteristics according to folate and cobalamin status categories at <12 gestational wk.

| | Red blood cell folate (nmol/L) [772] | | | | Plasma total cobalamin, pmol/L [791] | | | |
|---|---|---|---|---|---|---|---|---|
| | <P25 (<640) | P25-75 (640–1386) | >P75 (>1386) | *P* value | <P25 (<286) | P25–P75 (286–443) | >P75 (>443) | *P* value |
| Age[1], y | 32.0 (29.0, 35.0) [*n* = 193] | 32.0 (29.0, 35.0) [*n* = 386] | 33.0 (30.0, 36.0) [*n* = 193] | 0.011[11,12] | 32.0 (29.5, 36.0) [*n* = 198] | 32.0 (30.0, 35.0) [*n* = 395] | 33.0 (29.0, 36.0) [*n* = 197] | 0.695[11] |
| BMI[1], kg/m$^2$ | 23.4 (21.1, 26.2) [*n* = 179] | 23.1 (21.0, 26.4) [*n* = 373] | 23.7 (21.2, 26.6) [*n* = 188] | 0.629[11] | 24.0 (21.4, 27.6) [*n* = 186] | 23.3 (21.3, 26.3) [*n* = 378] | 22.7 (20.9, 25.5) [*n* = 191] | 0.013[11] |
| Low SES[2,4], % | 14.8 (10.4, 20.7) [*n* = 182] | 13.0 (10.0, 16.8) [*n* = 369] | 6.3 (3.7, 10.8) [*n* = 189] | 0.023[13] | 18.8 (13.9, 25.0) [*n* = 186] | 10.9 (8.1, 14.4) [*n* = 377] | 8.4 (5.2, 10.2) [*n* = 191] | 0.004[13] |
| Smoking[2,5], % | | | | | | | | |
| Nonsmoker | 30.7 (24.6, 37.6) | 44.3 (39.4, 49.3) | 59.4 (52.3, 66.1) | <0.001[13] | 38.6 (32.1, 45.5) | 43.2 (38.4, 48.2) | 55.2 (48.1, 62.0) | 0.045[13] |
| Passive/light | 18.0 (13.2, 24.1) | 16.7 (13.3, 20.7) | 12.5 (8.5, 17.9) | | 18.3 (13.5, 24.3) | 17.4 (14.0, 21.5) | 11.3 (7.6, 16.6) | |
| First trimester only | 16.9 (12.3, 22.9) | 14.6 (11.4, 18.5) | 14.1 (9.8, 19.7) | | 16.2 (11.7, 22.0) | 15.3 (12.1, 19.3) | 11.9 (8.0, 17.2) | |
| Throughout pregnancy | 34.4 (28.0, 41.4) | 24.5 (20.4, 29.0) | 14.1 (9.8, 19.7) | | 26.9 (21.2, 33.5) | 24.0 (20.1, 28.5) | 21.6 (16.4, 28.0) | |
| Total | [*n* = 189] | [*n* = 384] | [*n* = 192] | | [*n* = 197] | [*n* = 391] | [*n* = 194] | |
| Anemia[2,6], % | | | | | | | | |
| First trimester | 1.6 (0.6, 4.6) [*n* = 186] | 2.9 (1.6, 5.1) [*n* = 384] | 3.1 (1.4, 6.7) [*n* = 191] | 0.598[13] | 2.1 (0.8, 5.3) [*n* = 190] | 3.4 (2.0, 5.7) [*n* = 385] | 1.5 (0.5, 4.4) [*n* = 194] | 0.377[13] |
| Second trimester | 12.2 (7.9, 18.2) [*n* = 156] | 11.6 (8.6, 15.5) [*n* = 327] | 13.9 (9.5, 19.8) [*n* = 173] | 0.765[13] | 13.4 (9.0, 19.5) [*n* = 164] | 13.7 (10.4, 17.8) [*n* = 329] | 8.9 (5.5, 14.1) [*n* = 169] | 0.275[13] |
| Third trimester | 37.3 (30.3, 44.8) [*n* = 169] | 31.8 (27.1, 37.0) [*n* = 336] | 28.2 (22.0, 35.3) [*n* = 174] | 0.191[13] | 38.2 (31.1, 45.8) [*n* = 165] | 32.2 (27.5, 37.2) [*n* = 354] | 25.0 (19.1, 32.0) [*n* = 172] | 0.034[13] |
| Nulliparous[2], % | 33.5 (27.2, 40.5) [*n* = 191] | 30.7 (26.3, 35.5) [*n* = 384] | 28.5 (22.6, 35.2) [*n* = 193] | 0.567[13] | 25.5 (19.9, 32.0) [*n* = 196] | 32.7 (28.3, 37.5) [*n* = 394] | 32.5 (26.3, 39.3) [*n* = 197] | 0.172[13] |
| Interpregnancy period <2 y[2], % | 19.4 (14.2, 26.0) [*n* = 170] | 26.1 (21.7, 31.1) [*n* = 337] | 34.1 (27.5, 41.4) [*n* = 173] | 0.008[13] | 27.5 (21.3, 34.8) [*n* = 167] | 25.4 (21.2, 30.2) [*n* = 350] | 27.8 (21.7, 34.9) [*n* = 176] | 0.794[13] |
| Previous high risk pregnancy[2,7], % | 79.7 (73.1, 84.9) [*n* = 177] | 73.3 (68.5, 77.6) [*n* = 363] | 84.6 (78.7, 89.0) [*n* = 188] | 0.008[13] | 84.0 (77.9, 88.6) [*n* = 181] | 77.2 (72.6, 81.1) [*n* = 372] | 74.3 (67.6, 80.1) [*n* = 187] | 0.067[13] |
| Preconception folic acid use[2], % | 8.4 (5.0, 13.9) [*n* = 154] | 33.7 (28.9, 38.9) [*n* = 338] | 63.2 (56.0, 69.9) [*n* = 182] | <0.001[13] | 29.3 (23.0, 36.6) [*n* = 167] | 36.5 (31.5, 41.7) [*n* = 340] | 41.8 (34.8, 49.2) [*n* = 177] | 0.054[13] |
| Planned pregnancy[2], % | 64.5 (56.9, 71.3) [*n* = 166] | 81.8 (77.4, 85.5) [*n* = 347] | 93.3 (88.6, 96.1) [*n* = 178] | <0.001[13] | 77.6 (70.8, 83.3) [*n* = 170] | 80.2 (75.7, 84.0) [*n* = 353] | 85.0 (79.1, 89.5) [*n* = 180] | 0.199[13] |
| Regular alcohol intake[2,8], % | | | | | | | | |
| Before pregnancy | 15.2 (10.5, 21.4) [*n* = 165] | 17.1 (13.4, 21.4) [*n* = 340] | 15.7 (11.1, 21.8) [*n* = 178] | 0.842[13] | 14.3 (9.8, 20.4) [*n* = 168] | 18.2 (14.5, 22.5) [*n* = 352] | 15.9 (11.2, 22.0) [*n* = 176] | 0.512[13] |
| During pregnancy | 2.4 (0.9, 6.0) [*n* = 167] | 1.2 (0.5, 2.9) [*n* = 345] | 1.1 (0.3, 4.0) [*n* = 179] | 0.499[13] | 1.2 (0.3, 4.2) [*n* = 171] | 1.7 (0.8, 3.6) [*n* = 355] | 1.1 (0.3, 4.0) [*n* = 177] | 0.832[13] |
| Drug use[2], % | | | | | | | | |
| Before pregnancy | 4.8 (2.5, 9.3) [*n* = 165] | 3.2 (1.8, 5.7) [*n* = 340] | 1.7 (0.6, 4.8) [*n* = 178] | 0.59[13] | 3.6 (1.6, 7.6) [*n* = 168] | 3.7 (2.2, 6.2) [*n* = 352] | 2.3 (0.9, 5.7) [*n* = 176] | 0.228[13] |
| During pregnancy | 0.6 (0.1, 3.3) [*n* = 167] | 0.6 (0.2, 2.1) [*n* = 345] | 0.0 (0.0, 2.1) [*n* = 179] | 0.253[13] | 0.0 (0.0, 2.2) [*n* = 171] | 0.8 (0.3, 2.5) [*n* = 355] | 0.0 (0.0, 2.1) [*n* = 177] | 0.674[13] |
| Plasma folate[3], nmol/L | 13.8 (12.7, 15.0) [*n* = 193] | 28.7 (27.2, 30.4) [*n* = 386] | 47.0 (43.4, 50.9) [*n* = 193] | <0.001[14] | 23.0 (20.6, 25.7) [*n* = 198] | 28.1 (26.3, 30.1) [*n* = 396] | 30.2 (27.5, 33.1) [*n* = 197] | <0.001[14] |
| Red blood cell folate[3], nmol/L | 475 (458, 492) [*n* = 193] | 949 (928, 970) [*n* = 386] | 1864 (1790, 1941) [*n* = 193] | <0.001[14] | 835 (773, 902) [*n* = 194] | 961 (909, 1016) [*n* = 385] | 1034 (964, 1109) [*n* = 193] | <0.001[14] |
| Plasma total cobalamin[3], pmol/L | 330 (313, 348) [*n* = 193] | 354 (342, 367) [*n* = 386] | 379 (363, 396) [*n* = 193] | <0.001[14] | 231 (224, 238) [*n* = 198] | 358 (354, 363) [*n* = 396] | 531 (515, 547) [*n* = 197] | <0.001[14] |
| Plasma holoTC[3,9], pmol/L | 61.6 (57.0, 66.6) [*n* = 107] | 72.4 (67.1, 78.2) [*n* = 200] | 84.7 (76.6, 93.7) [*n* = 112] | <0.001[14] | 47.3 (44.0, 50.7) [*n* = 92] | 70.1 (66.6, 73.8) [*n* = 213] | 108.8 (97.8. 120.9) [*n* = 116] | <0.001[14] |

(*continued on next page*)

**TABLE 1** (*continued*)

| | Red blood cell folate (nmol/L) [772] | | | | Plasma total cobalamin, pmol/L [791] | | | |
|---|---|---|---|---|---|---|---|---|
| | <P25 (<640) | P25-75 (640–1386) | >P75 (>1386) | *P* value | <P25 (<286) | P25–P75 (286–443) | >P75 (>443) | *P* value |
| Plasma MMA[3], μmol/L | 0.126 (0.119, 0.133) [*n* = 191] | 0.113 (0.110, 0.117) [*n* = 385] | 0.111 (0.107, 0.115) [*n* = 193] | <0.001[14] | 0.127 (0.121, 0.134) [*n* = 197] | 0.114 (0.110, 0.117) [*n* = 393] | 0.108 (0.104, 0.111) [*n* = 196] | <0.001[14] |
| Plasma tHcy[3], μmol/L | 6.1 (5.9, 6.4) [*n* = 193] | 5.2 (5.1, 5.3) [*n* = 386] | 4.9 (4.8, 5.1) [*n* = 193] | <0.001[14] | 5.6 (5.5, 5.9) [*n* = 198] | 5.2 (5.1, 5.4) [*n* = 396] | 5.2 (5.0, 5.4) [*n* = 197] | <0.001[14] |
| cB12[1,10] | 0.87 (0.56, 1.0) [*n* = 191] | 1.01 (0.76, 1.24) [*n* = 385] | 1.07 (0.85, 1.28) [*n* = 193] | <0.001[11] | 0.66 (0.46, 0.83) [*n* = 197] | 1.01 (0.83, 1.17) [*n* = 393] | 1.26 (1.11, 1.44) [*n* = 196] | <0.001[11] |
| Birth weight[1], g | 3240 (2940, 3530) [*n* = 175] | 3310 (2950, 3555) [*n* = 357] | 3250 (2905, 3520) [*n* = 184] | 0.337[11] | 3260 (2907, 3560) [*n* = 178] | 3275 (2950, 3560) [*n* = 370] | 3280 (2940, 3560) [*n* = 182] | 0.921[11] |

[*n*] – Variations in *n* between variables are due to incomplete/unreturned questionnaires or giving birth in another hospital. Plasma holoTC determinations were interrupted during the study due to change of the hospital autoanalyzer.

Abbreviations: cB12, combined cobalamin status indicator; holoTC, holotranscobalamin; MMA, methylmalonic acid; P, percentile; SES, socioeconomic status; tHcy, total homocysteine.

Categorical variables were compared between groups by Chi-square and continuous variables by Kruskal–Wallace and Mann–Whitney *U* tests for nonparametric variables, and analysis of variance (ANOVA) with post hoc Bonferroni correction for multiple comparisons for parametric variables. Bonferroni post hoc comparisons between RBCF status categories: plasma folate (<P25 vs. P25–P75: $P < 0.001$; >P75 vs. P25–P75: $P < 0.001$), RBCF (<P25 vs. P25–P75: $P < 0.001$; >P75 vs. P25–P75: $P < 0.001$), plasma total cobalamin (<P25 vs. P25–P75: $P = 0.55$; >P75 vs. P25–P75: $P = 0.78$), HoloTC (<P25 vs. P25–P75: $P = 0.03$; >P75 vs. P25–P75: $P = 0.03$), MMA (<P25 vs. P25–P75: $P < 0.001$; >P75 vs. P25–P75: $P = 1.0$), tHcy (<P25 vs. P25–P75: $P < 0.001$; >P75 vs. P25–P75: $P = 0.02$). Bonferroni post hoc comparisons between plasma total cobalamin status categories: plasma folate (<P25 vs. P25–P75: $P = 0.004$; >P75 vs. P25–P75: $P = 0.733$), RBCF (<P25 vs. P25–P75: $P = 0.009$; >P75 vs. P25–P75: $P = 0.370$), plasma total cobalamin (<P25 vs. P25–P75: $P < 0.001$; >P75 vs. P25–P75: $P < 0.001$), HoloTC (<P25 vs. P25–P75: $P < 0.001$; >P75 vs. P25–P75: $P < 0.001$), MMA (<P25 vs. P25–P75: $P < 0.001$; >P75 vs. P25–P75: $P = 0.152$), tHcy (<P25 vs. P25–P75: $P < 0.001$; >P75 vs. P25–P75: $P = 1.0$).

[1] Values are medians (P25, P75).

[2] percentages (95% CI).

[3] geometric means (95% CI).

[4] Socioeconomic status was classified based on education, employment, and incomes of both parents.

[5] Smoking status was derived from plasma cotinine determinations, questionnaires and clinical records.

[6] Anemia during pregnancy: hemoglobin first trimester (<11 g/dL), second trimester (<10.5 g/dL) and third trimester (<11 g/dL).

[7] Previous medical and or gynecologic high-risk factors.

[8] More than 3 alcoholic drinks/week.

[9] Plasma holoTC in red blood cell folate groups (<P25: $n = 107$; P25–P75: $n = 200$; >P75: $n = 112$) and cobalamin groups (<P25: $n = 92$; P25–P75: $n = 213$; >P75: $n = 116$).

[10] Values of cB12 from 3cB12 equations (16).

[11] Kruskal–Wallace.

[12] Mann–Whitney *U*.

[13] Chi-square.

[14] ANOVA tests comparing the groups in each line within each biomarker.

**TABLE 2**
Percentiles for indicators of folate and cobalamin status throughout pregnancy.

| | | *n* | P5 | P10 | P25 | P50 | P75 | P90 | P95 |
|---|---|---|---|---|---|---|---|---|---|
| Plasma folate, nmol/L[1] | ≤12 GW | 791 | 7.6 | 10.1 | 16.8 | 29.4 | 43.4 | 60.6 | 87.1 |
| | 15 GW | 438 | 7.0 | 10.1 | 16.5 | 26.7 | 38.5 | 53.9 | 73.7 |
| | 24–27 GW | 670 | 5.1 | 5.9 | 8.5 | 14.8 | 27.4 | 39.9 | 48.7 |
| | 34 GW | 637 | 4.2 | 5.0 | 6.8 | 11.2 | 26.2 | 39.5 | 50.4 |
| | Labor | 609 | 4.0 | 4.9 | 6.4 | 10.2 | 23.7 | 36.5 | 45.3 |
| Red blood cell folate, nmol/L[1] | ≤12 GW | 772 | 406 | 467 | 639 | 939 | 1386 | 1829 | 2244 |
| | 15 GW | 437 | 549 | 670 | 914 | 1276 | 1738 | 2208 | 2873 |
| | 24–27 GW | 655 | 472 | 606 | 849 | 1143 | 1581 | 2119 | 2614 |
| | 34 GW | 618 | 387 | 467 | 658 | 960 | 1468 | 2075 | 2575 |
| Plasma total cobalamin, pmol/L[1] | ≤12 GW | 791 | 204 | 233 | 286 | 358 | 442 | 524 | 577 |
| | 15 GW | 438 | 174 | 212 | 264 | 332 | 401 | 469 | 533 |
| | 24–27 GW | 670 | 153 | 179 | 216 | 274 | 343 | 422 | 471 |
| | 34 GW | 637 | 137 | 161 | 196 | 250 | 317 | 387 | 430 |
| | Labor | 604 | 123 | 145 | 181 | 233 | 296 | 370 | 408 |
| Plasma total homocysteine, μmol/L | ≤12 GW | 791 | 3.7 | 4.0 | 4.6 | 5.3 | 6.0 | 7.1 | 7.9 |
| | 15 GW | 438 | 3.2 | 3.5 | 3.9 | 4.5 | 5.2 | 5.9 | 6.1 |
| | 24–27 GW | 670 | 3.1 | 3.5 | 3.9 | 4.6 | 5.4 | 6.3 | 7.0 |
| | 34 GW | 637 | 3.6 | 3.8 | 4.4 | 5.2 | 6.1 | 7.3 | 8.1 |
| | Labor | 610 | 3.8 | 4.3 | 5.1 | 6.0 | 7.3 | 8.6 | 10.0 |
| Plasma holotranscobalamin, pmol/L | ≤12 GW | 421 | 35.0 | 42.5 | 52.3 | 72.1 | 90.1 | 117.8 | 257.5 |
| | 15 GW | 368 | 30.2 | 35.7 | 48.1 | 62.9 | 84.1 | 110.3 | 218.3 |
| | 24–27 GW | 386 | 29.9 | 34.7 | 43.1 | 56.1 | 79.2 | 103.2 | 120.0 |
| | 34 GW | 372 | 29.8 | 33.8 | 43.3 | 61.7 | 83.1 | 105.0 | 120.4 |
| | Labor | 348 | 25.3 | 29.6 | 40.1 | 58.1 | 79.4 | 99.3 | 141.6 |
| Plasma methylmalonic acid, μmol/L | ≤12 GW | 786 | 0.076 | 0.080 | 0.095 | 0.110 | 0.137 | 0.170 | 0.200 |
| | 15 GW | 437 | 0.080 | 0.086 | 0.098 | 0.110 | 0.135 | 0.167 | 0.203 |
| | 24–27 GW | 667 | 0.083 | 0.090 | 0.102 | 0.120 | 0.150 | 0.193 | 0.232 |
| | 34 GW | 635 | 0.091 | 0.100 | 0.120 | 0.142 | 0.180 | 0.238 | 0.280 |
| | Labor | 607 | 0.097 | 0.108 | 0.130 | 0.160 | 0.203 | 0.268 | 0.330 |
| cB12[2] | ≤12 GW | 786 | 0.33 | 0.51 | 0.74 | 0.99 | 1.22 | 1.41 | 1.51 |
| | 15 GW | 437 | 0.43 | 0.56 | 0.80 | 1.04 | 1.25 | 1.42 | 1.49 |
| | 24–27 GW | 667 | 0.14 | 0.36 | 0.64 | 0.89 | 1.11 | 1.33 | 1.44 |
| | 34 GW | 635 | −0.05 | 0.15 | 0.39 | 0.69 | 0.92 | 1.15 | 1.26 |
| | Labor | 601 | −0.29 | −0.12 | 0.24 | 0.47 | 0.74 | 0.94 | 1.06 |

Abbreviations: GW, gestational weeks; P, percentile.

[1] Determined by microbiological assay. For comparison with radioassay, conversion factors should be applied for folate assays [18].

[2] Combined B12 status indicator [16]. The total number of determinations vary between time points due to not showing up for scheduled blood draw and COVID-19 restrictions that prohibited research activities in the hospitals (affecting 11 pregnancies). The smaller *n* at labor is due to omission of sample collection for the study in the busy labor ward, discarding of samples that were not processed in <2 h after delivery, births that occurred in another hospital. Red blood cell folate was not determined at labor. Holotranscobalamin determinations were performed only for the first 420 pregnancies, due to the change of laboratory provider and the replacement of the AxSyM autoanalyzer.

RBCF concentrations were lower throughout pregnancy in mothers who smoked for the duration of pregnancy compared with those who did not (data not shown). Active smoking was consistently negatively associated with plasma cobalamin and cB12 in early and late pregnancy and with plasma holoTC at labor. Birth weight was positively associated with third trimester RBCF. However, it was inversely associated with cobalamin status (borne out by its positive association with MMA and negative association with plasma cobalamin and cB12) in late pregnancy.

Folic acid supplement pattern and the *MTHFR* 677C>T polymorphism interacted in their association with first trimester RBCF (*P* value for interaction term = 0.032). The interaction is illustrated in Supplemental Figure 2. In the CC and CT genotypes, adhering to the recommended folic acid supplement regime of 400 μg/d compared with <400 μg/d was associated with higher RBCF status. However, higher RBCF status compared with the other supplement regimes was only observed in mothers with the TT genotype that exceeded 400 μg/d (Supplemental Figure 2A). Interactions were also observed between cobalamin supplement use and the *TCN2* 776C>G polymorphism in their association with mid and late pregnancy plasma holoTC (*P* values for interaction terms: 0.002 and 0.033, respectively). Plasma holoTC concentrations in the CC and CG genotypes were higher in mothers taking cyanocobalamin supplements compared with those not taking them, but this was not observed for the GG genotype (Supplementary Figure 2B). Cobalamin supplementation also interacted with the *MTRR* 524C>T polymorphism in its association with holoTC in mid-pregnancy (*P* value for interaction term: <0.001). Plasma holoTC concentrations did not differ between supplemented compared with unsupplemented mothers with the CC genotype but were higher in supplemented compared with unsupplemented mothers with the CT and TT genotypes (Supplementary Figure 2C). Smoking interacted with the *MTRR* 524C>T polymorphism in its association with cobalamin in the first and second trimesters of pregnancy (*P* values for interaction terms: 0.014 and 0.017, respectively). Smokers with the CC genotype had lower plasma cobalamin concentrations compared with nonsmokers (Supplementary Figure 2C).

The mean percentage change in cobalamin status indicators during pregnancy according to starting cobalamin status and fetal birth weight is reported in Table 4 [16]. Mothers who began pregnancy in the lowest plasma cobalamin tertile had the smallest decrease in plasma cobalamin

**TABLE 3**
Predictors of folate and cobalamin status at each trimester and at labor.

| | ≤12 GW | *P* value | 24–27 GW | *P* value | 34 GW | *P* value | Labor | *P* value |
|---|---|---|---|---|---|---|---|---|
| Plasma folate[1, 2] | | | | | | | | |
| *n*, $R^2$ | 484, 0.129 | <0.001 | 481, 0.213 | <0.001 | 469, 0.275 | <0.001 | 460, 0.250 | <0.001 |
| Planned pregnancy | 0.166[3] | <0.001 | 0.045 | 0.283 | 0.014 | 0.731 | 0.042 | 0.318 |
| FA supp <400 vs. ≥400 μg/d | −0.158 | <0.001 | −0.343 | <0.001 | −0.345 | <0.001 | −0.385 | <0.001 |
| Folate intake | 0.008 | 0.852 | 0.032 | 0.450 | 0.089 | 0.031 | 0.129 | 0.002 |
| Plasma cobalamin | 0.134 | 0.003 | 0.159 | <0.001 | 0.132 | 0.002 | 0.163 | <0.001 |
| Maternal BMI | −0.126 | 0.006 | −0.090 | 0.044 | −0.080 | 0.064 | −0.094 | 0.033 |
| Anamia | −0.018 | 0.675 | −0.002 | 0.957 | −0.096 | 0.020 | −0.090 | 0.032 |
| Low SES | −0.046 | 0.318 | −0.137 | 0.002 | −0.103 | 0.013 | −0.078 | 0.069 |
| Red blood cell folate[1, 2] | | | | | | | | |
| *n*, $R^2$ | 481, 0.188 | <0.001 | 476, 0.220 | <0.001 | 459, 0.286 | <0.001 | | |
| Planned pregnancy | 0.221 | <0.001 | 0.104 | 0.013 | 0.072 | 0.079 | | |
| FA supp <400 vs. ≥400 μg/d | −0.153 | <0.001 | −0.333 | <0.001 | −0.349 | <0.001 | | |
| Folate intake | 0.117 | 0.007 | 0.102 | 0.017 | 0.108 | 0.009 | | |
| Plasma cobalamin | 0.141 | <0.001 | 0.135 | 0.002 | 0.094 | 0.028 | | |
| *MTHFR* C677T CT vs. CC | −0.076 | 0.104 | −0.095 | 0.038 | −0.060 | 0.186 | | |
| TT vs. CC | −0.138 | 0.003 | −0.134 | 0.004 | −0.132 | 0.003 | | |
| Birth weight | N/A | | −0.005 | 0.905 | 0.094 | 0.022 | | |
| Total homocysteine[1, 2, 4] | | | | | | | | |
| Model I (plasma B12 and RBC folate) | | | | | | | | |
| *n*, $R^2$ | 608, 0.212 | <0.001 | 559, 0.136 | <0.001 | 531, 0.210 | <0.001 | | |
| RBC folate | −0.272 | <0.001 | −0.301 | <0.001 | −0.381 | <0.001 | | |
| Plasma cobalamin | −0.138 | <0.01 | −0.104 | 0.014 | −0.121 | 0.004 | | |
| Maternal BMI | 0.084 | 0.027 | 0.075 | 0.076 | −0.006 | 0.892 | | |
| Model II (plasma holoTC and RBC folate) | | | | | | | | |
| *n*, $R^2$ | 352, 0.279 | <0.001 | 333, 0.179 | <0.001 | 325, 0.232 | <0.001 | | |
| RBC folate | −0.281 | <0.001 | −0.327 | <0.001 | −0.415 | <0.001 | | |
| Plasma holoTC | −0.144 | <0.001 | −0.145 | 0.009 | −0.057 | 0.279 | | |
| Maternal BMI | 0.076 | 0.104 | 0.123 | 0.019 | 0.055 | 0.281 | | |
| Model III (plasma MMA and RBC folate) | | | | | | | | |
| *n*, $R^2$ | 614, 0.202 | <0.001 | 564, 0.180 | <0.001 | 537, 0.236 | <0.001 | | |
| RBC folate | −0.269 | <0.001 | −0.296 | <0.001 | −0.371 | <0.001 | | |
| Plasma MMA | 0.162 | <0.001 | 0.287 | <0.001 | 0.271 | <0.001 | | |
| Maternal BMI | 0.114 | 0.002 | 0.100 | 0.013 | 0.027 | 0.501 | | |
| Plasma total cobalamin[1, 5] | | | | | | | | |
| *n*, $R^2$ | 387, 0.109 | <0.001 | 442, 0.104 | <0.001 | 429, 0.147 | <0.001 | 422, 0.099 | <0.001 |
| Plasma folate | 0.053 | 0.297 | 0.144 | 0.003 | 0.167 | <0.001 | 0.154 | 0.001 |
| Cobalamin supp lementation | 0.158 | 0.002 | 0.058 | 0.211 | 0.116 | 0.012 | 0.125 | 0.009 |
| Maternal BMI | −0.192 | <0.001 | −0.180 | <0.001 | −0.098 | 0.047 | −0.100 | 0.046 |
| Cobalamin intake | 0.160 | 0.001 | 0.072 | 0.121 | 0.067 | 0.150 | 0.026 | 0.586 |
| Anemia | 0.004 | 0.931 | −0.133 | 0.004 | −0.137 | 0.004 | −0.153 | 0.002 |
| Active smoking | −0.111 | 0.029 | −0.051 | 0.760 | −0.147 | 0.002 | −0.054 | 0.260 |
| Birth weight | N/A | | 0.041 | 0.391 | <0.001 | 0.995 | −0.119 | 0.036 |
| Plasma holoTC[1, 5] | | | | | | | | |
| *n*, $R^2$ | 202, 0.165 | <0.001 | 249, 0.192 | <0.001 | 246, 0.168 | <0.001 | 235, 0.094 | 0.005 |
| Plasma folate | 0.033 | 0.632 | 0.340 | <0.001 | 0.281 | <0.001 | 0.185 | 0.005 |
| Cobalamin supplementation | 0.212 | 0.003 | 0.081 | 0.187 | −0.002 | 0.969 | 0.033 | 0.625 |
| Anemia | 0.048 | 0.487 | −0.157 | 0.010 | −0.224 | <0.001 | −0.254 | <0.001 |
| *TCN2* A67G AG vs. AA | −0.079 | 0.248 | −0.120 | 0.050 | −0.106 | 0.094 | −0.158 | 0.018 |
| GG compared with AA | −0.080 | 0.251 | −0.051 | 0.401 | −0.091 | 0.139 | −0.064 | 0.332 |
| Maternal BMI | −0.200 | 0.006 | −0.001 | 0.987 | <0.001 | 0.997 | −0.030 | 0.674 |
| Active smoking | −0.043 | 0.533 | −0.106 | 0.081 | −0.090 | 0.143 | −0.163 | 0.014 |
| Plasma MMA[1, 5] | | | | | | | | |
| *n*, $R^2$ | 617, 0.058 | <0.001 | 569, 0.115 | <0.001 | 547, 0.100 | <0.001 | 524, 0.113 | <0.001 |
| Plasma tHcy | 0.241 | <0.001 | 0.317 | <0.001 | 0.301 | <0.001 | 0.319 | <0.001 |
| Birth weight | N/A | | −0.004 | 0.931 | 0.052 | 0.217 | 0.127 | 0.012 |
| cB12[1] | | | | | | | | |
| *n*, $R^2$ | 391, 0.103 | <0.001 | 457, 0.100 | <0.001 | 444, 0.086 | <0.001 | 436, 0.092 | <0.001 |
| FA supp <400 vs. ≥400 μg/d | −0.048 | 0.363 | −0.179 | <0.001 | −0.181 | <0.001 | −0.173 | <0.001 |
| Cobalamin intake | 0.219 | <0.001 | 0.149 | 0.008 | 0.104 | 0.064 | 0.107 | 0.057 |
| Cobalamin supplementation | 0.077 | 0.135 | −0.024 | 0.596 | 0.060 | 0.196 | 0.039 | 0.540 |
| Active smoking | −0.128 | 0.010 | −0.084 | 0.070 | −0.104 | 0.025 | −0.074 | 0.115 |
| Maternal BMI | −0.143 | 0.005 | −0.082 | 0.088 | −0.019 | 0.696 | 0.026 | 0.599 |
| Anemia | 0.012 | 0.797 | −0.061 | 0.176 | −0.108 | 0.021 | −0.093 | 0.048 |
| Birth weight | N/A | | 0.001 | 0.988 | −0.022 | 0.642 | −0.174 | 0.002 |

Abbreviations: B12, cobalamin; cB12, combined cobalamin status indicator; FA supp, folic acid supplementation; GW, gestational week; HoloTC, holotranscobalamin; MMA, methylmalonic acid; N/A, not applicable; RBC, red blood cell; SES, socioeconomic status; tHcy, total homocysteine.

The *n* varies between the models primarily due to reasons stipulated in the flow chart (Figure 1), not showing up for scheduled blood draw, incompliance with sample processing protocol, unreturned questionnaires, and COVID-19 pandemic restrictions that prohibited research activities in the hospitals (affecting 11 pregnancies). The smaller *n* at labor is due to missed sample collection for the study, on occasion, in the busy labor ward.

[1] All the models were adjusted for: maternal age (y), gestational week at the time of extraction, pregnancy interval ([<2 y vs. nonpregnancy before] and [≥2 y vs. nonpregnancy before]), anemia in each trimester (yes/no), maternal body mass index at the first visit (kg/m$^2$), socioeconomic status (low vs. medium-high), active smoking (vs. none or passive smoking), creatinine (μmol/L), planned pregnancy (yes/no), and previous live birth (yes/no). The 24–27 GW, 34 GW, and at labor models included birth weight (g) variable. Genetic polymorphisms included in the models were *MTHFR*, methylenetetrahydrofolate: C677T (CT/TT) and *SLC19A1*, solute carrier family 19: G80A (GA/AA) for the folate models; *MTR*, methionine synthase: A2756G (AG/GG); *MTRR*, methionine synthase reductase: A66G (AG/GG), C524T (CT/TT); *TCN2*, transcobalamin II: C766G (CG/GG), A67G (AG/GG) for the cobalamin models.

[2] Plasma folate, red blood cell folate and homocysteine models included energy-adjusted folate intake (μg/d), folic acid intake <400 μg/d in each period of time (vs. ≥400 μg/d), plasma total B12 (pmol/L), or other indicated plasma biomarkers.

[3] Standardized beta-coefficients for each of the variables are shown.

[4] The homocysteine models used RBC folate as a predictor instead of plasma folate because the significance of the models was better and the predictors were the same.

[5] In the cobalamin, holoTC, and MMA models these variables were replaced by energy-adjusted B12 intake (μg/d), B12 supplementation (yes/no), plasma folate (nmol/L), and plasma tHcy (μmol/L) in the MMA model.

throughout pregnancy, and the largest increase in MMA. However, mothers of newborns in the lowest birth weight tertile had the highest cobalamin status at 24–27 GW, 34 GW, and labor and the highest cB12 at 24–27 GW and labor. Of the mothers in the lowest plasma B12 tertile at labor, 61% of them were in the lowest plasma B12 tertile at ≤12 GW and 34.6% of them had newborns in the highest birth weight tertile (data not shown).

## Discussion

Although 80.1% of the participants planned their pregnancy, only 36.1% took folic preconceptionally. Preconception folic acid use varies from 16% to 62% across Europe [23–28] but information regarding duration and dose is scant. Other unfortified countries have reported use as low as 4% (Turkey [29]) and 8% (Japan [30]). In agreement with previous reports [31, 32], more prepregnancy supplement users were nonsmokers, of mid-high socioeonomic status, with planned pregnancies, or high-risk gynecologic/ obstetric histories. Insufficient RBCF status for NTD prevention in 47.4% of the participants reflected the low prevalence of preconception folic acid use. Previously, we reported that 24% of females of reproductive age, from the same Mediterranean region, were folate deficient [4]. A high prevalence of insufficient dietary folate intake or folate status for NTD prevention was also reported in other unfortified countries including the United Kingdom [5], Sweden [33], Germany [34], and Switzerland [35] as well as in 25% of nonusers of supplements in a fortified country [6].

Most participants took folic acid supplements during the first trimester. Plasma folate status declined progressively after discontinuation of supplement use, as previously reported for the first completed pregnancies of this study [13,14]. Supplementation had a more lasting association with RBCF than with plasma folate status, even in participants who did not meet the recommended folic acid supplement dose. The median first trimester plasma folate of 29.4 nmol/L contrasted with that of 96 nmol/L, in a Canadian study [7] and the mean of 63.6 nmol/L in a Nepalese study [36].

The observed pregnancy changes in plasma cobalamin, holoTC, MMA, and tHcy are consistent with previous studies [37–40]. Here, we confirm that folate status is consistently a stronger predictor of tHcy than multiple indicators of cobalamin status, throughout pregnancy. Dietary folate and cobalamin intake contributed to folate and cobalamin status to a lesser extent than folic acid and cobalamin supplements. The pregnancy reduction in plasma cobalamin was attenuated in participants in the lowest tertile of plasma cobalamin at ≤12GW and these had greater increases in MMA as the pregnancy progressed, confirming observations from a previous study [38]. This may be due to deteriorating cobalamin status or to prioritization of cobalamin for the methionine synthase over the methyl malonyl-CoA mutase pathway [41]. Mobilization of maternal cobalamin reserves for fetal requirements seems a plausible explanation because mothers of bigger newborns had lower cobalamin status at labor. However, this only explained low cobalamin status in a third of the mothers. Late pregnancy plasma cobalamin was positively associated with birth weight in an Indian study [42]. Mid-to-late pregnancy plasma cobalamin was not associated with birth weight in a meta-analysis of 12 European and Asian studies [43].

Prepregnancy BMI and smoking were inversely associated with plasma cobalamin, BMI with early holoTC and smoking with cB12 and late pregnancy holoTC. BMI was inversely associated with plasma folate. None of these were associated with MMA. Similar associations with plasma folate and cobalamin were reported in a Dutch study [25].

To the best of our knowledge, we report interactions between lifestyle factors and genotypes on pregnancy folate and cobalamin status for the first time. Participants (16.6 %) with the *MTHFR* 677TT genotype apparently required >400 μg/d of folic acid or longer supplementation time, to achieve optimal RBCF status. It is unclear that they would achieve optimal status from mandatory fortification alone. Previously higher folate requirements to protect against NTDs was reported in participants with the *MTHFR* 677TT genotype, compared with the other genotypes [44]. Unlike a previous report in nonpregnant adults, plasma holoTC and MMA did not differ among the different *TCN2* C776G genotypes (data not shown) [45]. However, mid and late pregnancy plasma holoTC concentrations were higher in cyanocobalamin supplement users compared with nonusers for all *TCN2* C677G genotypes except the GG genotype. In the case of the *MTRR* 524C>T polymorphism, plasma holoTC concentrations were higher in supplement users compared with nonusers for all genotypes except the CC genotypes. These findings should be confirmed in a randomized controlled trial. Plasma cobalamin status was lower in smokers compared with nonsmokers in participants with the *MTRR* 524CC genotype. Polymorphisms affecting the *MTRR* gene may alter methionine synthase reductase function [46], but there is scant knowledge to date regarding the consequences of either of these polymorphisms.

To the best of our knowledge, the RTBC is the largest study to date to cover folate and cobalamin status references throughout pregnancy. To help distinguish between real deficiency and low plasma concentrations due to the physiological changes of pregnancy, we cross-referenced status categories with genetic factors, functional markers of folate and cobalamin status, and adherence to folic acid

**TABLE 4**

Changes in cobalamin status indicators in mid-to-late pregnancy according to plasma cobalamin status at <12 GW and according to fetal birth weight.

| | | 24–27 GW | *P* value | 34 GW | *P* value | Labor | *P* value |
|---|---|---|---|---|---|---|---|
| | Plasma cobalamin tertile | | | | | | |
| % Change[1] in plasma cobalamin | Lowest (≤311 pmol/L) | −19.3 (−7.4, −29.6) [*n* = 220] | | −25.6 (−13.1, −36.9) [*n* = 209] | | −28.6 (−16.0, −39.2) [*n* = 194] | |
| | Mid-to-high (>311 pmol/L) | −24.3 (−14.6, −35.9) [*n* = 450] | 0.812[5] | −31.7 (−22.3, −41.7) [*n* = 428] | <0.001[5] | −36.0 (−24.6, −46.8) [*n* = 410] | <0.001[5] |
| % Change[2] in plasma MMA | Lowest[3] | 10.9 (2.2, 30.0) [*n* = 219] | | 34.5 (16.3, 54.5) [*n* = 208] | | 47.0 (23.1, 80.6) [*n* = 195] | |
| | Mid-to-high[4] | 9.1 (8.3, 22.2) [*n* = 448] | 0.398[5] | 27.6 (11.1, 50.0) [*n* = 427] | 0.036[5] | 39.1 (18.4, 66.1) [*n* = 412] | 0.043[5] |
| % Change[2] in cB12 | Lowest[3] | −12.4 (12.1, −42.4) [*n* = 219] | | −42.3 (−17.4, −68.8) [*n* = 208] | | −62.7 (−39.2, −96.9) [*n* = 193] | |
| | Mid-to-high[4] | −10.8 (1.5, −24.7) [*n* = 448] | 0.812[5] | −29.9 (−13.6, −44.2) [*n* = 427] | <0.001[5] | −46.4 (−30.4, −63.8) [*n* = 408] | <0.001[5] |
| | Birth weight tertile | | | | | | |
| Plasma total cobalamin, pmol/L[2] | Lowest[6] | 285 (223, 357) [*n* = 216] | | 260 (215, 321) [*n* = 199] | | 246 (197, 325) [*n* = 186] | |
| | Mid[7] | 251 (208, 328) [*n* = 216] | 0.002[9] | 238 (187, 298) [*n* = 219] | 0.018[9] | 232 (179, 285) [*n* = 224] | 0.001[9] |
| | Highest[8] | 275 (223, 339) [*n* = 223] | 0.009[10] | 248 (196, 318) [*n* = 217] | 0.400[9] | 221 (179, 281) [*n* = 194] | 0.001[9] |
| Plasma MMA, μmol/L[2] | Lowest[6] | 0.12 (0.101, 0.149) [*n* = 216] | | 0.140 (0.118, 0.178) [*n* = 199] | | 0.151 (0.123, 0.190) [*n* = 187] | |
| | Mid[7] | 0.12 (0.107, 0.149) [*n* = 224] | 0.328[9] | 0.142 (0.122, 0.182) [*n* = 218] | 0.227[7] | 0.160 (0.123, 0.209) [*n* = 223] | 0.083[7] |
| | Highest[8] | 0.12 (0.100, 0.150) [*n* = 222] | 0.645[9] | 0.142 (0.119, 0.183) [*n* = 216] | 0.806[7] | 0.160 (0.130, 0.210) [*n* = 197] | 0.272[7] |
| % Change[2] in MMA | Lowest[6] | 11.0 (24.9, −2.6) [*n* = 216] | | 28.6 (47.6, 10.0) [*n* = 199] | | 37.2 (60.0, 13.8) [*n* = 187] | |
| | Mid[7] | 10.1 (24.9, 0.0) [*n* = 224] | 0.523[9] | 30.0 (51.9, 14.9) [*n* = 218] | 0.455[9] | 45.5 (77.0, 22.2) [*n* = 223] | 0.041[9] |
| | Highest[8] | 8.5 (26.8, −2.5) [*n* = 222] | 0.995[9] | 31.5 (51.1, 14.4) [*n* = 216] | 0.375[9] | 42.3 (73.5, 21.8) [*n* = 197] | 0.054[9] |
| cB12 | Lowest[6] | 0.93 (0.71, 1.16) [*n* = 216] | | 0.75 (0.49, 0.98) [*n* = 199] | | 0.55 (0.30, 0.85) [*n* = 186] | |
| | Mid[7] | 0.83 (0.59, 1.04) [*n* = 224] | 0.002[9] | 0.63 (0.36, 0.86) [*n* = 218] | 0.014[9] | 0.41 (0.17, 0.69) [*n* = 222] | <0.001[9] |
| | Highest[8] | 0.93 (0.62, 1.16) [*n* = 222] | 0.014[10] | 0.70 (0.37, 0.93) [*n* = 216] | 0.391[9] | 0.47 (0.16, 0.71) [*n* = 195] | 0.002[9] |

Abbreviations: GW, gestational weeks; MMA, methylmalonic acid;
cB12 combined cobalamin status indicator [16]. Data are reported as Median (P25, P75).

[1] at ≤12 GW;

[2] % change from ≤12 GW;

[3] ≤311 pmol/L.

[4] >311 pmol/L. Quade nonparametric analysis of covariance (ANCOVA) between groups, adjusting for GW at the time of blood draw and cyanocobalamin supplement use vs. nonuse and with post hoc Bonferroni correction for multiple comparisons.

[5] compared with lowest tertile at <12 GW.

[6] <3060 g;

[7] 3060–3440 g;

[8] >3440 g. Quade nonparametric analysis of covariance (ANCOVA) between groups, adjusting for GW at the time of blood draw and cyanocobalamin supplement use vs. nonuse and with post hoc Bonferroni correction for multiple comparisons.

[9] compared with lowest birthweight tertile;

[10] compared with mid birthweight tertile. The *n* varies between time points primarily due to reasons stipulated in the flow chart (Figure 1) and also due to missings for biomarkers (no show at scheduled blood draw, incompliance with sample processing protocol), COVID-19 pandemic restrictions that prohibited research activities in the hospitals (affecting 11 pregnancies).

supplementation recommendations. Danish [47] and Canadian studies [22] from 18 to 39 GW (excluding folic acid and cobalamin supplement users) and median 11 to 16 GW, respectively, also proposed reference ranges for folate and cobalamin status.

For NTD prevention, a nonpregnancy reference of 25.5 nmol/L for plasma folate concentration was proposed to be equivalent to the RBCF cutoff of 906 nmol/L [44], whereas plasma folate < 6.8 nmol/L is indicative of deficiency and <13.4 nmol/L as possible deficiency [19]. Nonpregnant reference ranges do not account for the physiological effects of pregnancy. However, P50 for plasma folate concentration at both ≤12 and 15 GW was relatively close to 25.5 nmol/L. For the remainder of pregnancy, nonpregnancy references corresponded with P75. Using *MTHFR* 677TT genotype and tHcy ≥P90 as indicators of folate deficiency, plasma folate concentrations <P25 (6.4 nmol/L, by labor) appear to reflect real folate deficiency. For RBCF, P50 never falls below 939 nmol/L and the lowest prevalence of combined *MTHFR* TT genotype and elevated tHcy occur at or above P50, throughout pregnancy. This supports the use of the 906 nmol/L reference throughout pregnancy. Regarding plasma cobalamin, ≥P50 throughout pregnancy corresponds with the lowest prevalence of MMA ≥ P90 and cB12 ≤ P10. Plasma MMA does not reach 0.260 μmol/L until P95 at 34 GW and P90 at labor. Based on nonpregnancy plasma cobalamin references, more participants would be classified as having suboptimal (≤221 pmol/L) (7.3%) or deficient (≤148 pmol/L) (1.1%) status at ≤12 GW than by any of the other cobalamin status indicators. Regarding cB12, it declined as pregnancy progressed but low cobalamin status (<-0.5) was observed only at labor, in 1.7% of the participants. To the best of our knowledge only 2 other studies have considered the aforementioned cobalamin status indicators during pregnancy and they observed the same pattern with regard to ranking of classification of cobalamin status by biomarkers tested. A Nepalese study reported that 24.4% of the participants were deficient (plasma cobalamin < 150 pmol/L, at <15 GW) but only 16% had cB12 <−0.5 [36]. A United Kingdom study did not observe a reduction in cB12 before 34 GW and observed low cobalamin status in 5% of the mothers [40]. Proposed reference limits for deficiency from the Canadian [22] and United Kingdom [40] studies were lower than ours for plasma cobalamin and higher for MMA, and lower than ours for holoTC in the Canadian study.

Multiple indicators of folate and cobalamin status were monitored throughout pregnancy in a cohort of 791 pregnant mothers. The influence of dietary intake, supplement use, genetic polymorphisms, and lifestyle characteristics were considered. Plasma holoTC determinations were only available for 420 pregnancies. Nevertheless, all of the models including holoTC were significant, and associations with multiple independent variables were confirmed. Participants were recruited from the 2 principal public hospitals in the region, but those seeking very early prenatal care may not be representative of the population. Microbiota data were not collected and may have improved the overall predictability of our models [48]. cB12 was not originally developed for use in pregnancy, but we observed changes in this indicator that were coherent with other changes in cobalamin status during pregnancy.

In conclusion, almost 50% of the pregnant mothers had suboptimal folate and plasma cobalamin status in early and late pregnancy, respectively. The prevalence of low cobalamin status was lower when alternative biomarkers, or their combination was used. Prenatal practice guidelines should include monitoring of folate and cobalamin status, at least at the first prenatal blood draw. Further investigation is required to elucidate the factors that influence cobalamin status during pregnancy and to determine which is the best biomarker to use.

## Author contributions

The authors' responsibilities were as follows – PC-B, MMM LAS-C: designed research; PC-B, MB, CG, CRR, AR-G, LAS-C, PMU, MMM: conducted research; MMM, LAS-C, CRR: analyzed data; LAS-C, PC-B, CRR, MMM: wrote the paper; MMM, PC-B, LAS-C: primary responsibility for final content; and all authors: read and approved the final manuscript.

## Conflict of interest

The authors have no conflict of interest to disclose.

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## Data availability

Data described in the manuscript can be made available upon reasonable request subject to compliance with the signed ethical agreement by the participants and pending application, review, and approval by the Reus–Tarragona Birth Cohort Steering Committee and IISPV-CEIm. In the event of approval, signed data sharing and access agreement is required.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ajcnut.2024.09.015.

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