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Original Article

A prospective study of maternal fatty acids, micronutrients and homocysteine and their association with birth outcome

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Abstract

Our earlier studies both in animals and in humans have indicated that micronutrients (folic acid, vitamin B12) and long-chain polyunsaturated fatty acids, especially docosahexaenoic acid (DHA), are interlinked in the one-carbon cycle, which plays an important role in fetal 'programming' of adult diseases. The present study examines the levels of maternal and cord plasma fatty acids, maternal folate, vitamin B12 and homocysteine in healthy mothers at various time points during pregnancy and also examine an association between them. A longitudinal study of 106 normal pregnant women was carried out, and maternal blood was collected at three time points, viz., T1 = 16–20th week, T2 = 26–30th week and T3 = at delivery. Cord blood was collected at delivery. Fatty acids were estimated using a gas chromatograph. Levels of folate, vitamin B12 and homocysteine were estimated by the chemiluminescent microparticle immunoassay (CMIA) technology. Maternal plasma folate ($P < 0.05$), vitamin B12 ($P < 0.01$) and DHA ($P < 0.05$) levels were lowest, while maternal homocysteine levels were highest ($P < 0.01$) at T3. There was a negative association between maternal DHA and homocysteine at T2 ($P < 0.05$) and T3 ($P < 0.01$). There was a positive association between plasma DHA in maternal blood at T3 and cord blood. Furthermore, there was a positive association between maternal folate and vitamin B12 at T3 and baby weight, whereas maternal homocysteine at T1 were inversely associated with baby weight at delivery. Our study provides evidence for the associations of folic acid, vitamin B12, homocysteine with DHA and baby weight, suggesting that a balanced dietary supplementation of folate–vitamin B12–DHA during pregnancy may be beneficial.

Keywords: DHA, folate, homocysteine, LCPUFA, vitamin B12.

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Introduction

Long-chain polyunsaturated fatty acids (LCPUFAs), especially *n*-3 fatty acids, play a vital role in enhancing fetal growth (Olsen *et al*. 1986, 1990, 1991, 1993; Allen & Harris 2001; Olsen & Secher 2002; Rogers *et al*. 2004; Guldner *et al*. 2007). A series of studies in humans carried out at our department also confirms the well-established association of LCPUFA, especially docosahexaenoic acid (DHA), with poor pregnancy outcome, leading to low-birthweight (LBW) babies (Kulkarni *et al*. 2010; Dhobale *et al*. 2011; Kilari *et al*. 2011). During pregnancy, fatty acids of both the *n*-3 and the *n*-6 families play an important role in the fetal growth and development (Innis 2007). During the last trimester of pregnancy, it becomes

essential that the fetus receives adequate amount of LCPUFA, especially arachidonic acid (AA) and DHA, due to the rapid synthesis of brain tissue (Clandinin *et al*. 1980; Martinez 1992). Adequate intake of micronutrients has also been reported to reduce the risk of LBW infants (Cetin *et al*. 2010).

We have extensively demonstrated in animals that alterations in maternal levels of folate and vitamin B12 affects the levels of its plasma, liver, brain, milk and placental DHA (Rao *et al*. 2006; Dangat *et al*. 2011; Kulkarni *et al*. 2011a; Roy *et al*. 2012; Sable *et al*. 2012; Wadhwani *et al*. 2012). We have discussed through a series of studies that micronutrients such as folic acid, vitamin B12 and DHA are interlinked in the one-carbon cycle (Kale *et al*. 2010; Chavan-Gautam *et al*. 2011; Sundrani *et al*. 2011; Kulkarni *et al*. 2011b; Dhobale & Joshi 2012). Deficiencies of folate, riboflavin, vitamin B6 or vitamin B12 have been consistently shown to be associated with elevated plasma homocysteine concentrations (Allen 2005). Elevated levels of total plasma homocysteine are observed in preeclampsia, placental abruption, premature delivery, LBW and intrauterine growth retardation (Vollset *et al*. 2000; Murphy *et al*. 2004; Lindblad *et al*. 2005).

Our earlier cross-sectional studies in women with pregnancy complications, such as preeclampsia and preterm pregnancy, indicate altered levels of LCPUFA and micronutrients (folate and vitamin B12) at delivery, which are associated with poor birth outcome (Kilari *et al*. 2010; Dhobale *et al*. 2011, 2012; Kulkarni *et al*. 2011b). Furthermore, we have also reported a negative association between erythrocyte DHA and plasma homocysteine concentrations in preeclampsia (Kulkarni *et al*. 2011b).

Although there are a number of reports that have examined the fatty acid composition of maternal and cord plasma/erythrocyte throughout pregnancy (van Houwelingen *et al*. 1992; Hoving *et al*. 1994; Al *et al*. 1995; Otto *et al*. 1997, 2001; Zeijdner *et al*. 1997; Wijendran *et al*. 1999; De Vriese *et al*. 2001, 2003; Matorras *et al*. 2001; Herrera *et al*. 2004; Stewart *et al*. 2007; Dwarkanath *et al*. 2009), the evidence of human studies examining systematically the association between folate, homocysteine and plasma or blood cell (*n*-3) LCPUFA is limited (Crowe *et al*. 2008). There is therefore a need to undertake longitudinal studies to examine these associations during pregnancy as they are important determinants of the one-carbon cycle, which play an important role in fetal programming and increase the risk of developing non-communicable diseases such as type 2 diabetes (Yajnik & Deshmukh 2008) and cardiovascular disease (CVD) (Erkkilä *et al*. 2008; Martinelli *et al*. 2009) in later life.

It would be very useful to analyse these levels in early pregnancy to examine changes over time, to understand their role in various pregnancy complications. But before such studies are initiated, it is crucial to examine the changes in these micronutrients and their association with each other in a prospective longitudinal study in a normotensive pregnancy/during an uncomplicated pregnancy. This may open new avenues for supplementation of these nutrients to prevent pregnancy complications and optimise fetal development.

We hypothesise that maternal folate, vitamin B12 and DHA are interlinked in the one-carbon cycle and influence pregnancy outcome. The role of fatty acids in pregnancy and fetal growth has been well established. But our data for the first time, in addition, link these through one-carbon cycle to influence pregnancy outcome.

In this study, we report the maternal fatty acids and micronutrient levels at three time points during pregnancy (T1 = 16–20th week, T2 = 26–30th week and T3 = at delivery) in normotensive women delivering at term with baby weight ≥2.5 kg. Association

Key messages

- Our data suggest an association of maternal *n*-3 fatty acids, especially DHA and homocysteine concentrations. These further provide the translational mechanistic basis for improving the health of the mother and subsequently improving birth outcome by balanced dietary supplementation of folate–vitamin B12–DHA.

of maternal fatty acids with cord fatty acids is also examined.

Materials and methods

Subjects

This longitudinal study was conducted at the Department of Obstetrics and Gynaecology, Bharati Hospital, Pune, India. This study was approved by the Bharati Vidyapeeth Medical College Institutional Ethical Committee and a written consent was taken from each subject. This study is part of a large ongoing departmental study, which recruits all healthy women at 16–20 weeks of gestation and follows them throughout pregnancy. The current study includes only those women with singleton pregnancy, delivering at term (total gestation ≥37 weeks and baby weight ≥2.5 kg) and having no medical or obstetrical complications. Women were excluded from the study if there was an evidence of other pregnancy complications, such as multiple gestation, chronic hypertension, type I or type II diabetes mellitus, gestational diabetes, seizure disorder, and renal or liver disease. Pregnant women with alcohol or drug abuse were also excluded from the study.

Fasting plasma samples were obtained at the time of each prenatal visit, scheduled at 4-week intervals until delivery. The first sample was obtained between 16 and 20 weeks of gestation (T1), the second between 26 and 30 weeks of gestation (T2) and the third sample was taken just before going to the labour room (T3). Umbilical cord was also collected just after delivery. One hundred nine pregnant women were enrolled at 16–20 weeks of gestation. First time point blood sample was obtained from 106 women, second time point blood sample was obtained from 90 women and at delivery sample was obtained from 98 women. Umbilical cord blood sample was obtained from 98 women (Fig. 1).

All women were routinely given iron (100 mg per tablet) and folic acid (1 mg per tablet) tablets during the first trimester of pregnancy as per the National Prophylaxis programme. Gestational age was calculated by last menstrual period and then confirmed by ultrasound. All women were administered a food frequency questionnaire during these three time points to estimate the frequency of intake of foods rich in folic acid, vitamin B12 and DHA, and the details of the questionnaire have been reported by us earlier (Kulkarni *et al*. 2011b).

Sample collection and processing

The study visits were scheduled in the morning after an overnight fast. At each visit, maternal fasting blood

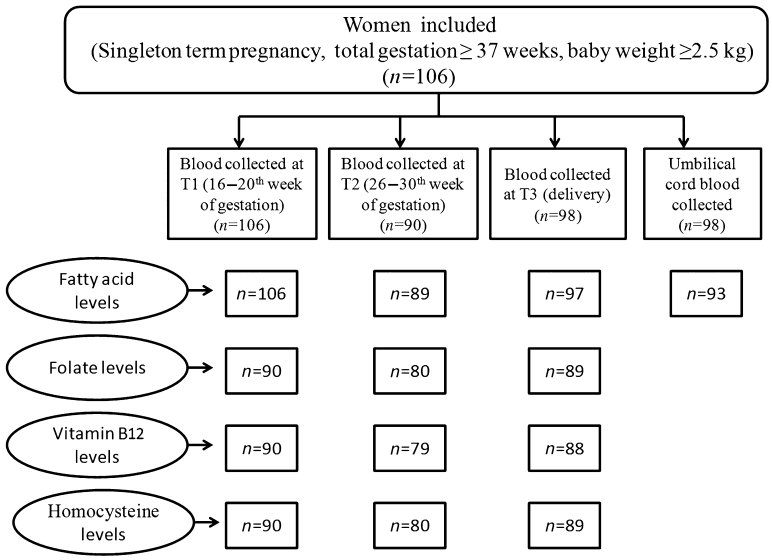

Fig. 1. Flow chart showing number of maternal and cord plasma samples analysed for different parameters at various time points (T1 = 16–20th weeks, T2 = 26–30th week, T3 = at delivery).

samples (10 mL) were collected into ethylenediaminetetraacetic acid (EDTA) tubes at each time point and at delivery. Cord blood was obtained immediately post-partum from the umbilical cord. All blood samples were immediately layered on histopaque (Sigma-Aldrich, St. Louis, MO, USA) and centrifuged at 2000 rpm for 30 min to separate the plasma and erythrocytes and were stored at –80°C until further analysis. As the folate in plasma is known to be unstable with long storage times, analysis for folate, vitamin B12 and homocysteine was carried out immediately. The storage cut-off was 3 months. Care was taken not to perform analysis on samples that were stored for a longer duration. Furthermore, haemolysed samples were not used for analysis.

Biochemical estimations

All biochemical analyses were performed at laboratories separate from subject recruitment sites. Investigators were blinded to subject identity, which was indicated by a code number maintained by the clinical staff until analysis was completed.

Fatty acid analysis

The procedure for fatty acid analysis used in our study was as reported in our several previous studies (Mehendale *et al*. 2008; Kilari *et al*. 2009, 2010; Dangat *et al*. 2010; Kale *et al*. 2010; Dhobale *et al*. 2011; Kulkarni *et al*. 2011b). Briefly, transesterification of the total plasma fatty acids was performed using hydrochloric acid–methanol. Methyl esters were separated using a PerkinElmer gas chromatograph (SP 2330, 30-m capillary Supelco column; PerkinElmer, Shelton, CT, USA). Peaks were identified by comparison with standard fatty acid methyl esters (Sigma-Aldrich). Fatty acids were expressed as g per 100 g fatty acid. The saturated fatty acids (SFAs) include myristic acid (Myr), palmitic acid (Pal) and stearic acid (Ste), while the monounsaturated fatty acids (MUFAs) include myristoleic acid (Myro), palmitoleic acid (Palo), oleic acid (Ole) and nervonic acid (NA). The *n*-3 fatty acids included alpha-linolenic acid (ALA), EPA and DHA, while *n*-6 fatty acids included linoleic acid (LA), gamma linolenic acid (GLA), di-homo-gammalinolenic acid (DGLA), docosapentaenoic acid (DPA) and AA.

Folate, vitamin B12 and homocysteine estimations

Folate, vitamin B12 and homocysteine levels were estimated by the chemiluminescent microparticle immunoassay (CMIA) technology (Abbott Diagnostics, Abbott Park, IL, USA) (Lee & Griffiths 1985) and described by us earlier (Dhobale *et al*. 2012). Briefly, 100 μL of plasma was used for analysis of vitamin B12, folate and homocysteine. The vitamin B12, folate and homocysteine assay was a two-step assay with an automated sample pre-treatment for determining the presence of vitamin B12, folate and homocysteine in human plasma. The reference range for plasma vitamin B12 assays was 187–883 pg mL^{-1}, for plasma folate assays was 2.34–17.56 ng mL^{-1}, while for homocysteine assay was 5.08–15.39 μmol L^{-1}.

Low plasma folate and vitamin B12 concentrations were defined as <10 ng mL^{-1} and <150 pg mL^{-1}, respectively, and elevated plasma total homocysteine concentrations as a concentration >10 μmol L^{-1} (Kulkarni *et al*. 2011b).

Statistical analysis

Values are reported as mean ± SD. The data were analysed using the SPSS/PC+ package (Version 20, SPSS Inc., Chicago, IL, USA). Skewed variables were transformed to normality using the following transformations: log to the base 10. Mean values of the various parameters were compared using least significance difference estimated from one-way analysis of variance (ANOVA) ($P < 0.05$). Correlation between variables was studied using Pearson's correlation analysis after adjusting for gestation, age and body mass index (BMI). Bonferroni adjustment was applied as correction for multiple testing, and after adjusting for multiple comparisons, the acceptance level for statistical significance was 0.016. Statistical analysis was carried out on two sets of data. First set of data consists of all the women who have participated in the study. The second set of data consists of women from whom all the parameters (folic acid,

vitamin B12, DHA and homocysteine levels) were analysed for all time points as well as in cord ($n = 74$). Statistical analysis performed on the first set of data has been reported in this manuscript. Similar results and trends were observed for the second set of data (data not shown). The variable sample number (n) in different measures was due to insufficient sample volume available. Although the data were incomplete, they are unlikely to have biased the results.

Results

Table 1 shows the demographic characteristics of normotensive mothers and their neonates.

Association between frequency of maternal intake of micronutrient and fatty acid-rich foods and their plasma values

There was a positive association between the frequency of intake of vitamin B12-rich foods and plasma vitamin B12 levels at T1 ($P < 0.05$) and delivery ($P < 0.01$). There was a positive association between frequency of intake of DHA-rich foods and plasma DHA levels at T1 ($P < 0.01$) and T2 ($P < 0.01$), but not at delivery. There was, however, no association between frequency of consumption of folate-rich foods and folate levels.

Table 1. Demographic characteristics of normotensive mothers and their neonates

	Mean ± SD
	Normotensive women ($n = 109$)
Maternal characteristics	
Age (years)	24.95 ± 3.94
Height (cm)	154.86 ± 5.76
Weight (kg)	50.62 ± 8.15
Body mass index (kg m^{-2})	21.17 ± 3.46
Gestation (weeks)	19.22 ± 2.15
Systolic blood pressure (mm Hg)	114.62 ± 6.56
Diastolic blood pressure (mm Hg)	75.46 ± 5.4
Income (Rs.)	8667.92 ± 5462.21
Neonatal characteristics	
Baby weight (kg)	2.89 ± 0.27
Baby length (cm)	48.23 ± 2.64
Baby head circumference (cm)	33.52 ± 1.21
Baby chest circumference (cm)	32.09 ± 1.63

Levels of maternal plasma folate, vitamin B12, homocysteine and fatty acids over time during pregnancy

Plasma folate levels decreased as gestation progressed and was significantly reduced at T3 ($P < 0.05$) as compared with T1 (Fig. 2). Similar trend was seen for plasma vitamin B12 level and the levels were significantly lower at T2 ($P < 0.05$) and T3 ($P < 0.01$) as compared with T1 (Fig. 2). Homocysteine levels, on the other hand, were higher at T3 ($P < 0.01$) as compared with T1 and T2 (Fig. 2). The plasma folate levels were <10 ng mL^{-1} in 46.66%, 56.25% and 61.79% of pregnant women at T1, T2 and T3, respectively, and the plasma vitamin B12 levels were <150 pg mL^{-1} in 22.22%, 31.64% and 42.04% of pregnant women at T1, T2 and T3, respectively, whereas the plasma homocysteine levels were >10 nmol L^{-1} in 7.77%, 7.5% and 22.47% of pregnant women at T1, T2 and T3, respectively.

Table 2 shows the fatty acid levels at three time points during pregnancy. The relative amounts of SFA in maternal plasma were higher at T3 ($P < 0.01$) as compared with T1 and T2. The relative amounts of MUFA in maternal plasma were higher at T2 ($P < 0.05$) and T3 ($P < 0.01$) as compared with T1. The relative amounts of LA in maternal plasma were lower at T3 ($P < 0.01$) as compared with T1 and T2, whereas there was no significant difference observed in the relative amounts of AA. However, the n-6 fatty acids were lower at T3 ($P < 0.01$ for both) as compared with T1 and T2. There was no change in the relative amounts of maternal plasma ALA or n-3 fatty acid throughout pregnancy, but the relative amounts of DHA in maternal plasma were lower at T2 and T3 ($P < 0.05$) as compared with T1.

Associations between folate, vitamin B12, homocysteine and fatty acids in maternal plasma

There was a negative association between folate and homocysteine in maternal plasma at T1 ($r = -0.245$,

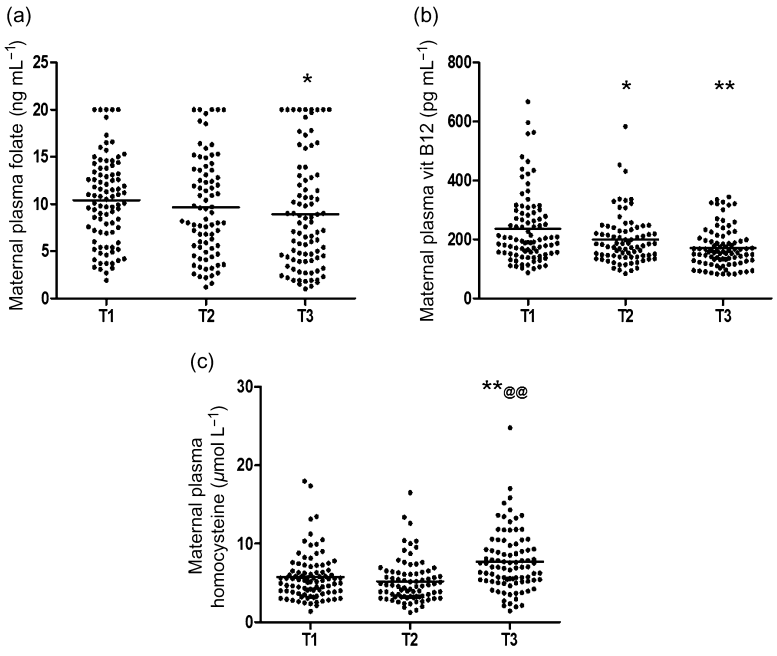

Fig. 2. Levels of plasma (a) folate, (b) vitamin B12 and (c) homocysteine at various time points during pregnancy. T1 = 16–20th weeks, T2 = 26–30th week, T3 = At Delivery. $^{**}P < 0.01$, $^{*}P < 0.05$ as compared with T1, @@$P < 0.01$ as compared with T2.

$P < 0.05$, $n = 81$). Furthermore, there was a negative association between vitamin B12 and homocysteine in maternal plasma at both T1 and T2 ($r = –0.412$, $P < 0.001$, $n = 81$; $r = –0.391$, $P < 0.01$, $n = 72$, respectively). There was a positive association between folate and DHA ($r = 0.225$, $P < 0.05$, $n = 77$) and between folate and AA ($r = 0.300$, $P < 0.01$, $n = 77$) in maternal plasma at T3. There was, however a negative association between DHA and homocysteine at T2 and T3 ($r = –233$ 0.242, $P < 0.05$, $n = 71$; $r = –0.381$, $P < 0.01$, $n = 77$, respectively) and a negative association between *n*-3 fatty acids and homocysteine in maternal plasma at T3 ($r = –0.240$, $P < 0.05$, $n = 77$). Similarly, there was a negative association between AA and homocysteine in maternal plasma at T1 and T2 ($r = –0.234$, $P < 0.05$, 236, $n = 71$; $r = –0.238$, $P < 0.05$, $n = 71$, respectively).

Cord plasma fatty acid composition and its association with maternal plasma fatty acids

The relative amounts of both SFA and MUFA in cord plasma were higher ($P < 0.01$) as compared with maternal values at all time points (Table 2), whereas the total *n*-6 fatty acids in cord plasma were lower ($P < 0.01$) at all time points. The relative amounts of LA in cord plasma were lower ($P < 0.01$), whereas the relative amounts of AA in cord plasma were higher ($P < 0.01$) as compared with maternal values at all time points. Total *n*-3 fatty acids in cord plasma were higher ($P < 0.01$) and so were the relative amounts of DHA in cord plasma ($P < 0.01$) as compared with maternal values at all time points, but the relative amounts of ALA in cord plasma were lower as compared with maternal values at T2 ($P < 0.05$).

There was a negative association between SFA in cord plasma and maternal plasma at T3 ($r = –0.300$, $P < 0.01$, $n = 89$), whereas there was a positive association between MUFA in cord plasma and maternal plasma at T3 ($r = 0.213$, $P < 0.05$, $n = 89$). There was a negative association between cord plasma and maternal plasma at T3 for LA, AA and *n*-6 fatty acids ($r = –0.777$, $P < 0.001$, $n = 89$; $r = –0.642$, $P < 0.001$, $n = 89$; r = –0.491, $P < 0.001$, $n = 89$, respectively), whereas there was a strong positive association between cord plasma and maternal plasma at T3 for DHA and *n*-3 fatty acids ($r = 0.351$, $P < 0.001$, $n = 87$ and $r = 0.417$, $P < 0.001$, $n = 87$, respectively) (Table 2).

Table 2. Mean (SD) maternal plasma at three time points during normal pregnancy (T1 = 16–20th week, T2 = 26–30th week, T3 = at delivery) and cord plasma fatty acid composition (% total fatty acids)

Fatty acids (g/100 g fatty acids)	Maternal plasma fatty acids (mean ± SD)			Cord fatty acids (mean ± SD)	Pearson's correlation coefficients between T3 and cord fatty acids ($n = 89$)	
	T1 ($n = 106$)	T2 ($n = 89$)	T3 ($n = 97$)	Cord ($n = 93$)	r	P
Myr	0.62 ± 0.30	0.80 ± 0.31**	0.72 ± 0.30	0.76 ± 0.40*	0.172	0.112
Myro	0.03 ± 0.03	0.03 ± 0.02	0.04 ± 0.03	0.08 ± 0.10**@@##	0.364	0.001
Pal	24.77 ± 2.35	25.86 ± 2.60*	27.04 ± 2.69**@@	28.34 ± 2.61**@@##	0.191	0.077
Palo	0.59 ± 0.37	0.77 ± 0.52	1.20 ± 0.72**@@	2.25 ± 0.84**@@##	–0.117	0.281
Ste	5.81 ± 1.60	5.25 ± 0.71	6.34 ± 4.16@	10.18 ± 2.45**@@##	–0.398	0.001
Ole	16.38 ± 2.16	17.58 ± 2.40*	17.80 ± 2.79**	18.72 ± 3.49**#	0.244	0.023
LA	38.72 ± 3.85	37.92 ± 4.09	33.50 ± 9.26**@@	14.66 ± 7.87**@@##	–0.777	0.001
GLA	0.07 ± 0.98	0.06 ± 0.08	0.11 ± 0.10@	0.25 ± 0.15**@@##	–0.135	0.214
ALA	0.46 ± 0.20	0.49 ± 0.24	0.44 ± 0.24	0.37 ± 0.30#	0.130	0.226
DGLA	1.12 ± 032	1.11 ± 0.33	1.18 ± 0.49	1.98 ± 0.60**@@##	–0.229	0.033
AA	7.08 ± 2.12	6.38 ± 1.35	7.04 ± 3.46	13.80 ± 3.78**@@##	–0.642	0.001
EPA	0.32 ± 0.35	0.27 ± 0.26	0.24 ± 0.27	0.41 ± 0.50##	–0.054	0.621
NA	0.34 ± 0.34	0.29 ± 0.22	0.35 ± 0.23	0.68 ± 0.42**@@##	–0.128	0.232
DPA (*n*-6)	0.20 ± 0.09	0.20 ± 0.08	0.19 ± 0.11	0.21 ± 0.12	–0.071	0.511
DHA	1.07 ± 0.36	0.92 ± 0.36*	0.95 ± 0.44*	1.56 ± 0.69**@@##	0.351	0.001
SFA	31.22 ± 3.12	31.92 ± 2.69	34.10 ± 5.90**@@	39.29 ± 3.75**@@##	–0.3	0.004
MUFA	17.35 ± 2.36	18.68 ± 2.54*	19.40 ± 3.03**	21.75 ± 3.40**@@##	0.213	0.046
Total *n*-3	1.85 ± 0.59	1.69 ± 0.58	1.64 ± 0.67	2.35 ± 0.85**@@##	0.417	0.001
Total *n*-6	47.21 ± 4.33	45.70 ± 4.14	42.04 ± 6.65**@@	30.93 ± 5.30**@@##	–0.491	0.001

**$P < 0.01$, *$P < 0.05$ as compared with T1, @@$P < 0.01$, @$P < 0.05$ as compared with T2, ##$P < 0.01$, #$P < 0.15$ as compared with T3. Myr, myristic acid; Myro, myristoleic acid; Pal, palmitic acid; Palo, palmitoleic acid; Ste, stearic acid; Ole, oleic acid; LA, linoleic acid; GLA, gamma linolenic acid; ALA, alpha linolenic acid; DGLA, di-homo-gamma linolenic acid; AA, arachidonic acid; EPA, eicosapentaenoic acid; NA, nervonic acid; DPA, *n*-6 docosapentaenoic acid; DHA, docosahexaenoic acid. Saturated fatty acids (SFAs): (myristic acid + palmitic acid + stearic acid). Monounsaturated fatty acids (MUFAs): (myristoleic acid + palmitoleic acid + oleic acid + nervonic acid). Total *n*-3: (alpha linolenic acid + eicosapentaenoic acid + docosahexaenoic acid). Total *n*-6: (linoleic acid + gamma linolenic acid+ di-homo-gamma-linoleic acid + arachidonic acid + docosapentaenoic acid). For Pearson's correlation coefficients, $P < 0.05$.

Associations between maternal folate, vitamin B12, homocysteine and LCPUFA and birthweight

Correlation between maternal micronutrient and LCPUFA status was studied after adjusting for gestational age, maternal age and BMI. There was a positive association between baby weight and maternal plasma folate at T1 and T3 ($r = 0.240$, $P < 0.05$, $n = 75$; $r = 0.272$, $P < 0.05$, $n = 76$, respectively) and also for plasma vitamin B12 at T3 ($r = 0.223$, $P < 0.05$, $n = 76$). There was, however, a negative association between maternal homocysteine at T1 and baby weight ($r = –0.252$, $P < 0.05$, $n = 75$). There was a positive association between maternal plasma *n*-3 fatty acids at T1 and baby chest circumference ($r = 0.236$, $P < 0.05$, $n = 92$). Furthermore, there was a positive association between baby weight and maternal plasma AA at T1 ($r = 0.214$, $P < 0.05$, $n = 92$) and total *n*-6 fatty acids at T1 ($r = 0.199$, $P = 0.055$, $n = 92$).

Discussion

The present study reveals several important and interesting findings in a very large number of unique racial homogenous and with similar lifestyle healthy mothers delivering term: (1) significant reductions in the maternal plasma levels of folate and vitamin B12 as gestation advances; (2) positive association between maternal folate and maternal DHA at T3; (3) negative association between maternal homocysteine and maternal DHA and total *n*-3 fatty acids

at T2 and T3; (4) positive association between maternal DHA and cord DHA at T3; (5) negative association between maternal homocysteine and baby weight at T1; and (6) positive association between folate, vitamin B12 and baby weight.

In our study, maternal plasma folate levels were reduced as gestation advances and were lowest at T3. Reports on levels of folate during pregnancy are inconsistent with many studies reporting a decrease in serum folate levels as gestation advances (Cikot *et al*. 2001; López-Quesada *et al*. 2003; Milman *et al*. 2006; Wallace *et al*. 2008; Ubeda *et al*. 2011), while others report that folate levels of pregnant women are low initially and then rise later (Parazzini *et al*. 2011; Ozkan *et al*. 2012). Takimoto *et al*. (2007) reported that serum folate concentrations decrease between the first and second trimesters but increase between the second and third trimesters. It has been suggested that vitamin B12 deficiency leads to a folate trap and can interfere with folate metabolism, leading to low serum folate (Klee 2000).

Maternal plasma vitamin B12 levels in our study were reduced as gestation advances and were lowest at T3 and were similar to the findings seen for folate. Many others have also reported that vitamin B12 concentrations progressively decline during pregnancy (Cikot *et al*. 2001; Velzing-Aarts *et al*. 2005; Takimoto *et al*. 2007; Wallace *et al*. 2008; Ubeda *et al*. 2011) and has been attributed to the increased fetal requirement (Gadowsky *et al*. 1995; Guerra-Shinohara *et al*. 2002). Others suggest that an increase in plasma volume and a change in hormonal status can result in low levels of vitamin B12 (Bruinse & van der Berg 1995).

Folate and vitamins B12 and B6 are required for DNA synthesis and cell growth and are involved in homocysteine metabolism (Furness *et al*. 2013). It is well documented that deficiency of either folate or vitamin B12 results in hyperhomocysteinaemia (Takimoto *et al*. 2011). In our study, maternal homocysteine levels significantly increased at T3. One study indicates that homocysteine levels are higher in the first trimester as compared with the third trimester in pregnant women (Ozkan *et al*. 2012). Others indicate that homocysteine concentrations decline slightly in the first trimester and remained approximately constant during the second and third trimesters (Cikot *et al*. 2001); still others report that it increases in a stepwise manner during pregnancy (Holmes *et al*. 2005; Wallace *et al*. 2008). Recent studies indicate that serum homocysteine concentrations are significantly lower in the second trimester and increased significantly in the third trimester of pregnancy (Ubeda *et al*. 2011).

There are some studies that have examined the levels of LCPUFA at various time points during gestation (Al *et al*. 1995; Matorras *et al*. 2001; Otto *et al*. 2001; De Vriese *et al*. 2003; Herrera *et al*. 2004; Stewart *et al*. 2007; Alvino *et al*. 2008; Dwarkanath *et al*. 2009). Most of the reported studies have been carried out on a small sample size (Otto *et al*. 2001; Alvino *et al*. 2008) or only during the period of early pregnancy (Otto *et al*. 2001; van Eijsden *et al*. 2008) or towards the end of the last trimester of pregnancy (Wijendran *et al*. 1999; Parra *et al*. 2002). In our study, relative amounts of maternal plasma LA significantly were reduced at T3 as compared with T1 and T2, whereas the relative amounts of cord LA were reduced significantly as compared with maternal values. Although there was no significant difference in the relative amounts of maternal AA levels at T1, T2 and T3, relative amounts of cord AA significantly increased compared with maternal values. Similar increase in cord blood plasma AA with a decline in the third trimester has been reported earlier (Herrera *et al*. 2004). Others report a decline in erythrocyte membrane AA (Dwarkanath *et al*. 2009).

In our study, relative amounts of maternal plasma DHA were reduced at T2 and T3 as compared with T1, whereas the relative amounts of cord DHA increased significantly as compared with maternal values. In contrast, others observed an increase in DHA levels across pregnancy (Stewart *et al*. 2007); still others report a decrease in DHA during pregnancy (Al *et al*. 1995; Matorras *et al*. 2001). A preferential accumulation of AA and DHA in umbilical plasma at birth has been reported earlier (De Vriese *et al*. 2003).

The current study thus demonstrates lower levels of LA and ALA in maternal plasma and cord plasma and higher levels of AA and DHA in the cord. These results confirm the greater selectivity of placenta for LCPUFA than for essential fatty acids (EFAs)

(Santos *et al*. 2012). It may be possible that there may be an increased conversion of maternal LA to AA and ALA to DHA in the placenta (reduction in cord) as the placenta is, for the greater part, a fetal organ that has a fatty acid composition more similar to that of fetal plasma than of maternal plasma (Chambaz *et al*. 1985; Al *et al*. 1990).

Moreover, it is also known that the developing fetal brain requires DHA and AA for incorporation into membranes (Herrera 2002). Therefore, higher levels of AA and DHA in cord vs. maternal plasma can be explained by a selective transfer of these LCPUFAs by the placenta resulting in the 'biomagnification' of these fatty acids (Haggarty 2002) and has been extensively reviewed (Duttaroy 2009). Furthermore, others indicated that the fetal synthesis of these fatty acids from their precursors may also be possible (Herrera *et al*. 2004). Our findings of higher levels of both DHA and AA in the cord and lower LA and ALA in cord support the above studies.

In our study, there was also an increase in the relative amounts of SFA in cord plasma as compared with maternal values. It is known that there is a limited placental transfer for SFAs as compared with PUFA (Campbell *et al*. 1996; Haggarty *et al*. 1997) and our findings support the concept of an active lipogenesis in the fetus (Dunlop & Court 1978).

Our study reports, for the first time, a positive association between maternal folate and DHA during pregnancy. Furthermore, there was a negative association between maternal DHA and homocysteine, suggesting the associations of these micronutrients in the one-carbon cycle as has been reported by us earlier in animal studies (Rao *et al*. 2006; Dangat *et al*. 2011; Kulkarni *et al*. 2011a; Roy *et al*. 2012; Sable *et al*. 2012; Wadhwani *et al*. 2012). Our findings indicate that homocysteine levels in pregnancy are not only determined by folate and vitamin B12 but also by DHA. Durand *et al*. (1996) suggested that by reducing homocysteine concentrations, folate may reduce the generation of reactive oxygen species and thus spare DHA, which is a target for lipid peroxidation. Furthermore, intervention studies and recent meta-analysis document that the high consumption of *n*-3 PUFA decreases plasma homocysteine (Huang *et al*. 2011). The role of fatty acids in pregnancy and fetal growth has been well established; however, our data for the first time, in addition, link these through one-carbon cycle to influence pregnancy outcome.

It is known that gestation (Amini *et al*. 1994), maternal age (Lee *et al*. 1988) and maternal BMI (Löf *et al*. 2008) are associated with birthweight, and hence all associations examined in the current study with birth outcome were adjusted for the above. We observed a strong positive association between maternal and cord *n*-3 fatty acids during gestation. Positive associations of fish intake and birthweight have been reported earlier (Olsen *et al*. 1993; Rogers *et al*. 2004; Guldner *et al*. 2007), suggesting that *n*-3 fatty acids contribute to enhanced fetal growth (Takimoto *et al*. 2011). Muthayya *et al*. (2009) also found increases of between 100 and 200 g in birthweight between the lowest and highest fish/DHA intake groups. We found a negative association between maternal plasma AA levels at T3 and cord AA levels. One possible explanation may be that the placenta preferentially retains AA with respect to other fatty acids (Haggarty *et al*. 1997). It is also known that the fetus is less dependent on the maternal supply of AA compared with DHA (Haggarty 2010). Furthermore, a negative association between maternal plasma LA levels at T3 and cord LA levels was observed. A recent study suggests that there is a lower placental transfer of LA as a specific mechanism to prevent inhibition of Δ6 desaturase activity and enable higher rates of Δ6 desaturation and acylation of AA and DHA in fetal tissues (Novak *et al*. 2012).

There was a negative association between maternal homocysteine only at T1 and baby weight and a positive association of maternal folate and vitamin B12 at T3 and baby weight. This may be attributed to the low levels of both folate and vitamin B12 in the Indian population habitually consuming a vegetarian diet. A low maternal RBC folate and high homocysteine values in mid pregnancy are reported to be associated with subsequent reduced fetal growth (Furness *et al*. 2013). Takimoto *et al*. (2007) suggested that higher plasma homocysteine in the third trimester is a predictor of LBW. Our findings suggest that supplementation of both folic acid and vitamin B12 may be useful to improve baby

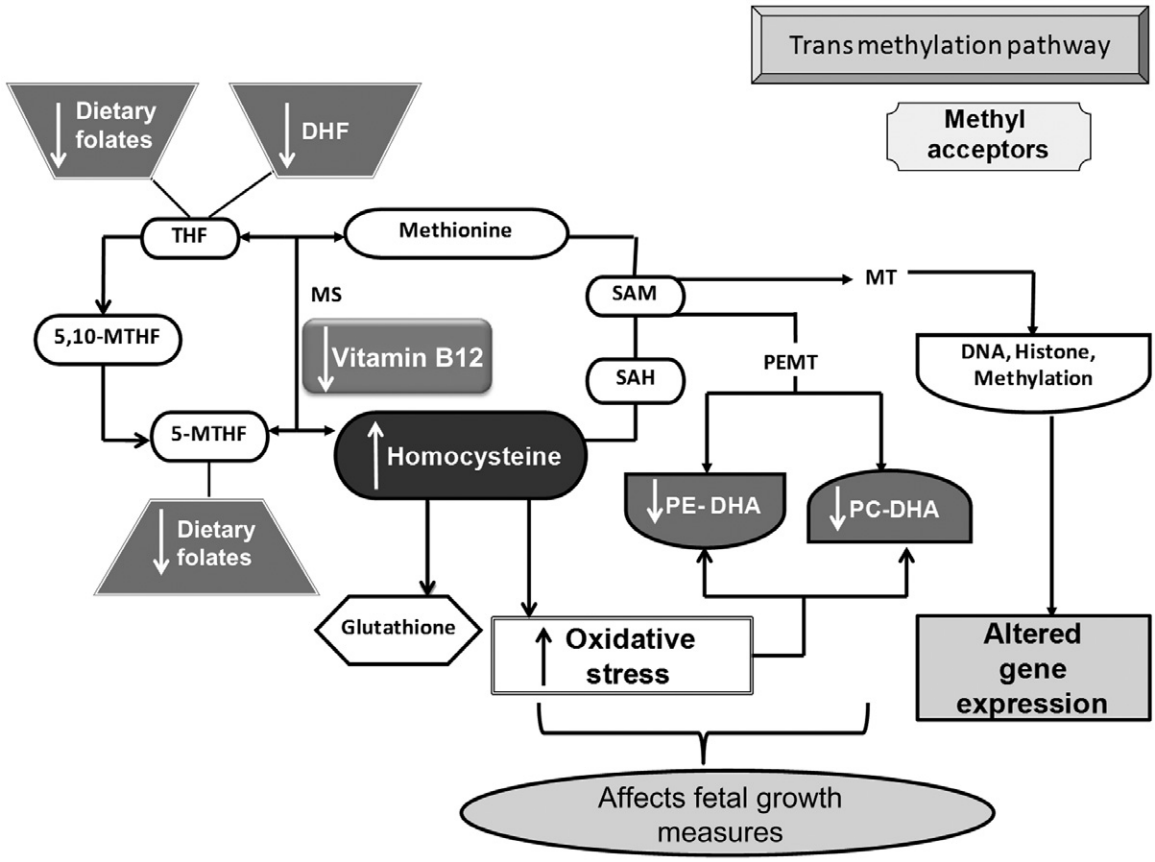

Fig. 3. Diagrammatic presentation of mechanisms explaining the interrelationship between folate, vitamin B12 and docosahexaenoic acid (DHA) in one-carbon cycle. The key metabolic components: THF, tetrahydrofolate, 5,10-MTHF; 5,10-methylene tetrahydrofolate; 5-MTHF, 5-methylene tetrahydrofolate; B12, vitamin B12; methionine; SAM, *S*-adenosyl methionine; SAH, *S*-adenosyl homocysteine; homocysteine; glutathione; PE-DHA, phosphatidylethanolamine with docosahexaenoic acid attached to position 2; PC-DHA, phosphatidylcholine with docosahexaenoic acid attached to position 2; DNA, deoxyribonucleic acid, histone. Key enzymes: MS, methionine synthase; PEMT, phosphatidyl ethanolamine methyl transferase; MT, methyl transferase. ↑ – increased levels, ↓ – reduced levels.

weight, especially when diet is insufficient in these micronutrients.

In the current study, while recruiting patients from the hospital, details about education and parity were recorded and were found to be similar. Furthermore, all study participants neither consumed alcohol nor smoked and came from a low socioeconomic background, which represents a large percentage of the Indian population and has been described by us recently (Sundrani *et al*. 2013). The well-matching cohort (ethnic, age, dietary habits, lifestyles and socioeconomic) described here eliminates or attenuates the contribution of a large number of variables. This is important as it allows us to propose and test our hypothesis, which are indicative of changes over time in a normotensive pregnancy and are not confounded by other factors. Our separate studies are ongoing to address issues, e.g., socio-economic and ethnic diversity, that will help understand the contribution of such variables to make our hypothesis relevant to global population.

To discuss the results in order to easily understand the complex inter-relationships among the studied micronutrients and their possible role in pregnancy and birth outcome, we have first provided a diagrammatic view (Fig. 3) of our hypothesis stated earlier.

This hypothesis, we believe with already some isolated published studies including our own studies in human and animals, will be able to explain mechanistically the possible causal relationship of these vital micronutrients. This also will help us understand the significance of some of the key associations. Briefly, the figure shows the key biochemical mechanisms and their consequences. It is known that reduced folate levels affect fetal growth (Furness *et al*. 2013). In addition, it will affect the restoration of methionine and, together with reduced vitamin B_{12}, will increase levels of homocysteine (Takimoto *et al*. 2011), in turn, leading to increased oxidative stress (Forges *et al*. 2007). During oxidative stress, free radicals initiate lipid peroxidation by attacking PUFAs in cell membranes (Madazli *et al*. 1999). We have previously discussed that reduced DHA may impair the phospholipid methylation and lead to an increase in DNA methylation (Kale *et al*. 2010), leading to altered programming of critical genes like desaturases (Wadhwani *et al*. 2012, 2013), resulting in poor pregnancy outcome.

It is known that a homogenous population provides better control for confounding factors that vary in the general population (Nilsen *et al*. 2010). The major strengths of our study include the size of the population and the longitudinal design with measurements of several indices of folate, vitamin B12, LCPUFA and homocysteine status at three different time points during pregnancy and cord blood samples. Furthermore, all women were extremely well matched for race and lifestyle patterns with no smoking, drug or alcohol use to reduce confounds to intake and metabolism of these key components. However, as with all observational studies, our study can only suggest associations between various key analytes (e.g. folate, vitamin B12, DHA and homocysteine) and supports their role in the one-carbon metabolism. Also, one cannot overlook the fact that although many of the correlations are statistically significant, the magnitudes of the correlations are small. Nevertheless, the associations of all the metabolites of the one-carbon cycle with each other as well as with baby weight, if confirmed on a large sample size, may have implications for increased risk for non-communicable diseases in adult life.

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Conflicts of interest

The authors declare that they have no conflicts of interest.

Contributions

SRJ, SSM and NSW contributed substantially to conception and design of, or acquisition of data or analysis and interpretation of data. NSW, HRP, SRJ drafted the article or revised it critically for important intellectual content. All authors approved the final version for publication.

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