British Journal of Nutrition (2005), **93**, 593–599
DOI: 10.1079/BJN20041395

The influence of erythrocyte folate and serum vitamin B_{12} status on birth weight

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(Received 9 June 2004 – Revised 9 December 2004 – Accepted 9 December 2004)

The extent to which maternal folate and vitamin B_{12} modulate infant birth weight is unclear. The present study investigated mothers in early gestation (mean 11·5 (SD 5·8) weeks) and neonates, at delivery. Erythrocyte (RBC) folate (mothers: *n* 683; neonates: *n* 614) and vitamin B_{12} (mothers: *n* 534; neonates: *n* 614) were measured. Data on smoking habits were available for 44 % of pregnancies (*n* 443). The relationship between vitamin levels and birth weight standardized for gender and gestational age was investigated, using linear regression and adjusting for possible confounding variables (maternal age, parity). Results are presented as standardized regression coefficients (*b*). Increasing maternal age was associated with elevated RBC folate (*b* 0·11 (95 % CI 0·08, 0·15), $P<0·001$; *n* 674) and smoking was associated with a decrease in maternal RBC folate (*b* −1·38 (95 % CI −1·92, −0·86), $P=0·001$; *n* 319). Neonatal RBC folate was predicted by maternal RBC folate (*b* 0·08 (95 % CI 0·04, 0·11), $P=0·001$; *n* 315) and maternal vitamin B_{12} (*b* 0·08 (95 % CI 0·01, 0·16), $P=0·02$; *n* 252). Smoking influenced maternal vitamin B_{12} status (*b* −0·88 (95 % CI −1·49, −0·27), $P=0·005$; *n* 231). Using univariate regression, smoking significantly influenced infant birth weight (*b* −2·15 (95 % CI −3·24, −1·04), $P<0·001$; *n* 437). However, the effect of smoking on birth weight was statistically non-significant when considered in a multivariate regression model, leaving maternal RBC folate as the only significant predictor of birth weight (*b* 0·25 (95 % CI 0·08, 0·42), $P=0·005$; *n* 145). These findings suggest that maternal folate status is an important determinant of infant birth weight. The combined effects of smoking and reduced RBC status on birth weight require further investigation.

Folate: Birth weight: Smoking

Nutritional status during pregnancy has a direct influence upon birth weight. Adequate supply of micronutrients is known to be very important in pregnancy and there is much evidence to support a role for folate in early embryonic development (Rosenquist & Finnell, 2001). A clear causal association has been shown between reduced maternal folate and neural tube defects (MRC Vitamin Study Research Group, 1991; Czeizel & Dudas, 1992; Eskes & Steegers-Theunissen, 1994) and other congenital malformations (Botto *et al.* 1996; van Rooij *et al.* 2003) as well as early pregnancy loss (Nelen *et al.* 2000).

Maternal diet is highly important for the health of the fetus, both in the peri-conceptional period and later in pregnancy (Bailey, 1995). Research into micronutrient deficiency and birth weight in animal studies has shown profound effects of dietary micronutrient restriction on early development (Keen *et al.* 2003). The relationship between maternal folate status and birth weight has been investigated by a number of groups, most commonly in relation to intrauterine growth restriction (reviewed by Scholl & Johnson, 2000). Earlier studies relied on assessment of maternal folate intake or serum folate levels; Scholl *et al.* (1996) reported a twofold greater risk of infant low birth weight in women with a low mean daily folate intake at 28 weeks gestation (<240 μg/d). Although this may appear not to be a particularly low level of folate intake, it accounted for both dietary and supplementary forms of folate and was 60 % of the recommended daily intake for pregnant women (400 μg/d). Neggers *et al.* (1997) reported a small but significant positive association between maternal folate intake at 18 and 30 weeks gestation and infant birth weight and a significant association between low serum folate levels at 30 weeks gestation and fetal growth restriction was reported by Goldenberg *et al.* (1992). Erythrocyte (RBC) folate is a far more robust measure of physiological folate status than serum measures, reflecting longer-term folate intake and bioavailability and not influenced by daily fluctuations in intake as serum folate can be. A study of birth weight and maternal RBC folate status at delivery reported a positive correlation with birth weight (Ek, 1982). More recently Rao *et al.* (2001) observed a positive association between infant birth weight and dietary intake of folate-rich foods in rural Indian women. Furthermore, infant birth weight and maternal RBC folate status were positively correlated at 28 weeks gestation (Rao *et al.* 2001). Such studies suggest a role for maternal folate in the determination of infant birth weight. Elevated total homocysteine (tHcy) concentration measured in serum or plasma is a sensitive marker of impaired folate status (Green & Miller, 1999). Several studies have explored the relationship

Abbreviations: RBC, Erythrocyte; tHcy, total homocysteine.

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between tHcy and birth weight, with mixed findings (Burke *et al.* 1992; Vollset *et al.* 2000; Ronnenberg *et al.* 2002; Infante-Rivarde *et al.* 2003). It remains unclear whether tHcy is merely a biomarker of folate status or is deleterious in itself.

Vitamin B_{12} plays an integral role in folate-dependent homocysteine metabolism (Swanson *et al.* 2001) as a rate-limiting co-factor in the conversion of homocysteine to methionine (Fig. 1) and a limited supply of vitamin B_{12} during pregnancy may have very important consequences for fetal growth. Furthermore, low serum levels of maternal vitamin B_{12} have been associated with risk of neural tube defect-affected pregnancy (Kirke *et al.* 1993; Suarez *et al.* 2003). Vitamin B_{12} is known to be a significant predictor of tHcy levels in neonates, with relatively greater importance in the first few months of life, before folate assumes primacy as the predictor of tHcy (Ueland & Bjorke Monsen, 2003). The role of vitamin B_{12} in pregnancy outcome has been explored by Molloy *et al.* (2002), who suggest that vitamin B_{12} status may be an important factor in maintaining optimal tHcy during pregnancy. The potential role of vitamin B_{12} in the determination of birth weight has yet to be explored.

The present study investigates the relationship between RBC folate status and vitamin B_{12} status in a large population of pregnant mothers and neonates in relation to maternal smoking, age, parity and infant birth weight.

Materials and methods

Participants

The study population comprised mothers and newborn babies from 998 pregnancies delivered at West Cumberland Hospital, Whitehaven, Cumbria, UK between 2000 and 2002. Maternal blood samples were collected during routine antenatal care at the first antenatal visit (mean gestational age 11·5 (SD 5·8) weeks; Fig. 2). Cord blood samples were obtained from newborn babies immediately after ligation of the umbilical cord following delivery. Of the 998 pregnancies, 315 mother–child pairs were collected, in the remaining 683 pregnancies only mother or child were collected. Data from mother–child pairs were used only to assess the correlation between maternal and neonatal vitamin levels. Of the total 998 pregnancies involved in the study, RBC folate measurements were obtained for 683 mothers; of these, vitamin B_{12} data were available for 504 individuals. Corresponding figures for neonates are 614 with RBC folate values figures, of which 534 had vitamin B_{12} data available. For both maternal and neonatal data, a proportion of pregnancies also recorded smoking habits (Fig. 2). All participants were recruited and gave their informed consent as part of the North Cumbria Community Genetics Project, a community-based DNA banking project (Chase *et al.* 1998). Ethical approval was obtained for the collection and storage of biological samples, delivery details, and demographic and lifestyle information for use in genetic epidemiological research. Multiple births, gestational age <37 weeks and all births with congenital anomalies identified upon delivery were excluded.

Biochemical analysis

Blood samples for determining RBC folate and vitamin B_{12} concentrations were collected in vacutainers containing EDTA and taken to the Department of Haematology, West Cumberland Hospital, Whitehaven within 24 h of collection and full blood count analysis was undertaken. Lysing agent (1ml, 0·1% ascorbic acid) was added to 50 μl whole blood for RBC folate analysis. Remaining blood was allowed to clot and was then centrifuged for 30 min at 3000 ***g*** to separate serum. Samples were then stored at -20 °C for up to 7 d to facilitate batch analysis, which was then carried out with inter- and intra-batch controls. Intra-sample variation was accepted at <6 %. RBC folate was measured using an ion capture assay and serum vitamin B_{12} using an enzyme immunoassay (Abbott IMx®; Abbott GmBH, Germany).

Epidemiological data

Epidemiological information was collected by means of a self-completed mother's questionnaire (never/ever/current smoking) and from delivery records (birth weight, gestational age, maternal

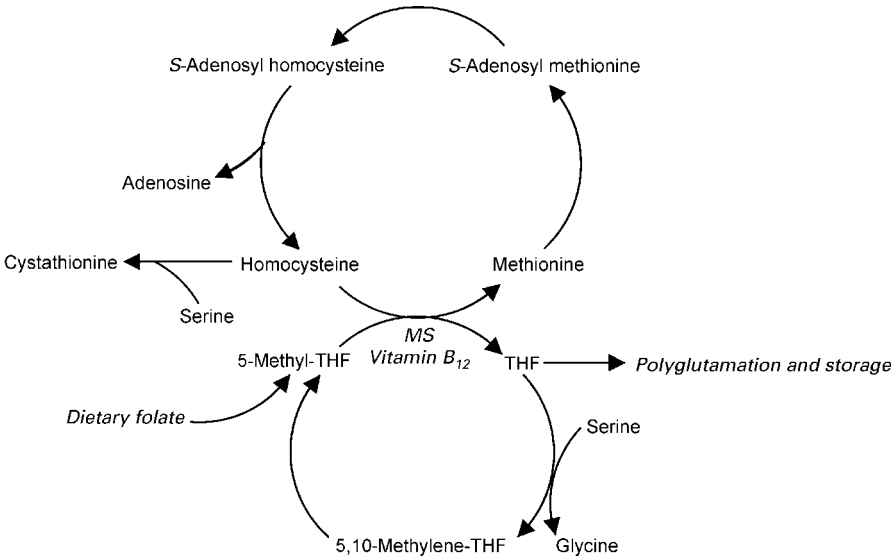

Fig. 1. Folate-dependent homocysteine metabolic pathway. 5-Methyl THF, 5-methyltetrahydrofolate; THF, tetrahydrofolate.

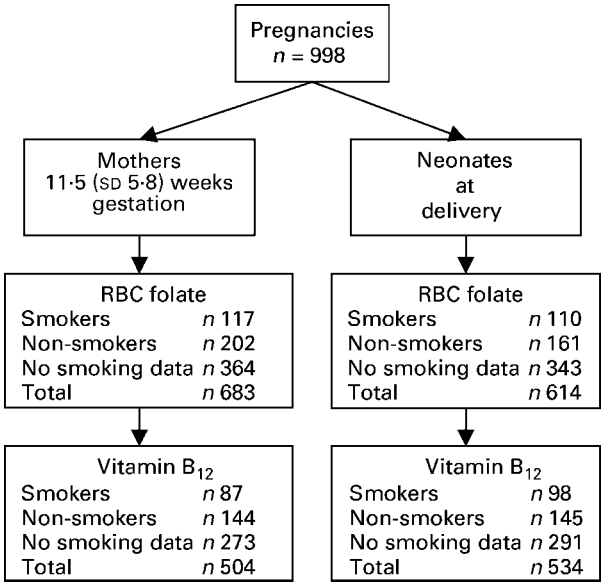

Fig. 2. Samples analysed.

age, gender, parity), as part of the North Cumbria Community Genetics Project (Chase *et al.* 1998). Birth weights were adjusted for gender and gestational age and presented as *z*-scores (Freeman *et al.* 1995).

Statistical analysis

RBC folate levels were not normally distributed and were therefore log-transformed prior to analysis. The influence of each parameter on RBC folate status, vitamin B_{12} status and standardized birth weight (*z*-score) was assessed using multiple linear regression. Standardized regression coefficients, *b*, denoting the increase in the dependent variable for a standard deviation increase in the explanatory variable, are presented with accompanying 95 % confidence intervals. R^2 values denoting the variation in the outcome variable explained by the variable included in the regression model are also presented. Smoking data were available for 44 % of the study group. *t* Tests were therefore undertaken to ensure that there was no significant difference in dependent variables between smoking responders and smoking non-responders. All statistical analysis was undertaken using the STATA™ (Texas, USA; version 8) statistical software package.

Results

Maternal mean RBC folate and vitamin B_{12} levels were within the normal ranges (160–640 ng/ml and 160–925 pg/ml, respectively; Hoffbrand & Pettit, 1997) (Table 1). Neonatal levels were higher than those observed in maternal samples drawn during early pregnancy. There was a significant correlation between RBC folate concentration in maternal antenatal blood and cord blood (Fig. 3; *r* 0·258; *n* 315), and similarly for vitamin B_{12} levels (Fig. 4; *r* 0·516, *n* 249), although wide variation was observed across the study population.

Factors influencing vitamin status

The results of linear regression analysis carried out on log-transformed vitamin data to identify factors that influenced maternal and neonatal vitamin status are detailed in Tables 2 and 3. Univariate linear regression analysis showed that increasing maternal vitamin B_{12} and maternal age were positively associated with maternal RBC folate status. Increasing delivery parity and smoking were associated with a significant decrease in maternal RBC folate status (Table 2). When analysed using a multivariate linear regression model, all variables associated with maternal and neonatal RBC folate retained a high degree of statistical significance. Multivariate linear regression analysis showed both maternal RBC folate and maternal vitamin B_{12} to be important determinants of neonatal RBC folate status (Table 2).

Univariate linear regression analysis showed that maternal age and smoking habits were predictive of maternal vitamin B_{12} status (Table 3). Maternal vitamin B_{12}, maternal age and delivery parity (but not smoking) were all associated with neonatal vitamin B_{12}

Table 1. Summary of variables

Variable	Number of observations	Mean	SD
Maternal RBC folate (ng/ml)	683	418	178
Log_{10} maternal RBC folate (ng/ml)	683	5·95	0·41
Neonatal RBC folate (ng/ml)	614	514	154
Log_{10} neonatal RBC folate (ng/ml)	614	6·19	0·29
Maternal vitamin B_{12} (pg/ml)	504	324	132
Log_{10} maternal vitamin B_{12} (pg/ml)	504	5·71	0·39
Neonatal vitamin B_{12} (pg/ml)	534	362	187
Log_{10} neonatal vitamin B_{12} (pg/ml)	534	5·77	0·49
Birth weight (kg)	981	3·43	0·47
Birth weight (*z*-score)	980	−0·010	0·98
Maternal age (years)	974	27·8	5·83
Gestational age at sampling (weeks)	678	11·5	5·82
Delivery parity	997	1 = 43 % 2 = 36 % >3 = 21 %	
Gender (female:male)	982	1:1·05	
Maternal smoking habits	443	Ever smoker = 173 Current smoker = 105 Never smoker = 270	

RBC, erythrocyte.

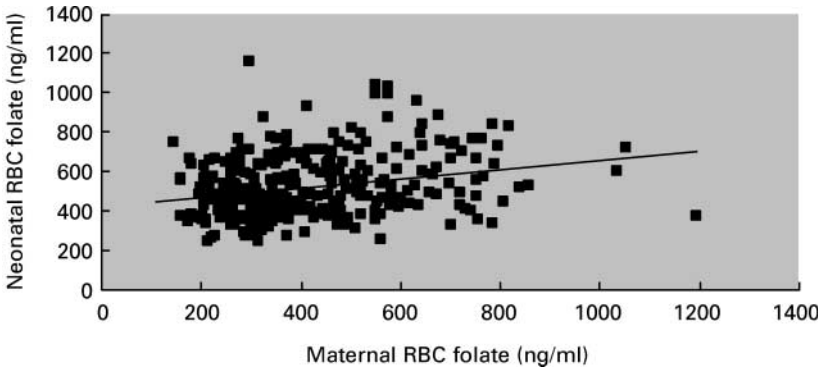

Fig. 3. Graph showing paired maternal and neonatal erythrocyte (RBC) folate levels (*n* 315, *r* 0·258).

status (Table 3). Multivariate linear regression analysis reduced the effect of maternal age and delivery parity to non-significance, leaving maternal vitamin B_{12} as the sole significant determinant of neonatal vitamin B_{12}.

Factors influencing birth weight

No significant difference in infant birth weight was observed between mothers who reported smoking habits and those who did not. Univariate linear regression analysis showed maternal RBC folate status to be a significant predictor of birth weight (Table 4). This effect remained highly significant in a multivariate model (*b* 0·25 (95 % CI 0·08, 0·42), *P*=0·005; *n* 145). Smoking was associated with a significant decrease in birth weight when considered in a univariate model (Table 4); however, multivariate analysis resulted in a dramatic reduction in the influence of smoking on birth weight, which was no longer significant (*b* 0·88 (95 % CI −1·17, 2·93), *P*=0·40; *n* 145). This suggests that smoking and maternal RBC folate status are not independent of each other. Neonatal vitamin B_{12} was shown to exert a small but significant influence on infant birth weight when analysed in a univariate model (*b* −0·09 (−0·17, −0·01), *P*=0·02; *n* 522) (Table 4). However, when considered in a multivariate model this effect was not significant.

Discussion

We found maternal RBC folate status to be an important predictor of infant birth weight, with increasing maternal RBC folate being associated with increasing infant birth weight (corrected for gender and gestational age). The standardized regression coefficient given indicates a 0·14 unit increase in birth weight *z*-score for each standard deviation increase in maternal RBC folate. We confirmed that smoking during pregnancy results in a reduction in infant birth weight (Kramer, 1987; de Weerd *et al.* 2003; Spencer, 2003). However, when smoking and maternal RBC folate status were considered together, only maternal RBC folate was a significant determinant of infant birth weight, implying that the effect of smoking may be mediated, at least in part, through its association with maternal RBC folate. This is supported by a clear association between smoking and reduced maternal RBC folate in the present study population. No association between neonatal RBC folate status and infant birth weight was observed. Maternal vitamin B_{12} status was also shown to be significantly associated with RBC folate status both in mother and neonate, but not with birth weight.

The folate pathway has the potential to influence fetal growth by a number of mechanisms. Folate has many roles of fundamental importance to cell function, in particular in the synthesis and repair of DNA and in gene expression, through the process of DNA methylation (Lucock *et al.* 2003). Reduced folate status is associated with elevated tHcy, and the elevated serum levels of this amino acid have been associated with a wide range of clinical conditions, including placental vasculopathy (van der Molen *et al.* 2000; Medina *et al.* 2001), which have well-established effects upon fetal growth (Baschat & Hecher, 2004). Processes in early embryo–fetal development may be influenced directly by elev-

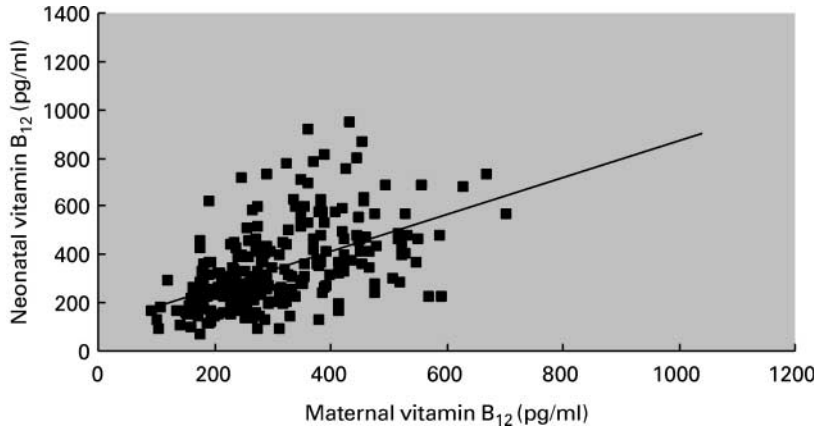

Fig. 4. Graph showing paired maternal and neonatal vitamin B_{12} levels (*n* 249, *r* 0·516).

Table 2. Factors influencing erythrocyte (RBC) folate status (univariate regression analysis, standardized regression coefficients are shown)

	Log (maternal RBC folate)					Log (neonatal RBC folate)				
Factor	*n*	Coeff.	95 % CI	R^2	*P*	*n*	Coeff.	95 % CI	R^2	*P*
Maternal RBC folate (ng/ml)†	–					315	0·08	0·04, 0·11	0·067	<0·001*
Maternal vitamin B_{12} (pg/ml)†	502	0·11	0·08, 0·14	0·073	<0·001*	252	0·08	0·01, 0·16	0·092	0·02*
Neonatal vitamin B_{12} (pg/ml)†	–					533	0·03	−0·03, 0·10	0·062	0·33
Maternal age (years)	674	0·11	0·08, 0·15	0·074	<0·001*	599	−0·05	−0·11, 0·02	0·008	0·19
Delivery parity	682	−0·03	−0·06, 0·001	0·006	0·05*	613	0·02	−0·05, 0·09	0·001	0·60
Current smoker	319	−1·38	−1·92, −0·86	0·077	<0·001*	271	0·31	−0·62, 1·25	0·009	0·50

* $P<0·05$.
† Log-transformed values.

Table 3. Factors influencing vitamin B_{12} status (univariate regression analysis, standardized regression coefficients are shown)

	Log (maternal vitamin B_{12})					Log (neonatal vitamin B_{12})				
Factor	*n*	Coeff.	95 % CI	R^2	*P*	*n*	Coeff.	95 % CI	R^2	*P*
Maternal vitamin B_{12} (pg/ml)†	–					249	0·28	0·23, 0·33	0·307	<0·001*
Maternal age (years)	499	0·04	0·01, 0·08	0·013	0·01*	523	0·04	0·001, 0·08	0·007	0·05*
Delivery parity	503	0·02	0·02, 0·06	0·002	0·27	533	−0·05	−0·09, −0·002	0·008	0·04*
Current smoker	231	−0·88	−1·49, −0·27	0·034	0·005*	243	0·23	−0·50, 0·96	0·002	0·54

* $P<0·05$.
† Log-transformed values.

ated tHcy or through other downstream effects of reduced folate status. More subtle effects may include the modulation of gene expression through altered DNA methylation.

The present finding of a clear association between maternal RBC folate and birth weight is in agreement with some, but not all, previous reports, which include a positive correlation between maternal serum folate and infant birth weight reported by Tamura *et al.* (1997). de Weerd *et al.* (2003) reported no association between RBC folate and birth weight in a cohort of 240 women; however, their cohort included 15 women with a previous neural tube defect pregnancy and 119 epileptic women. Both conditions are known to be associated with perturbation of folate metabolism (Finnell *et al.* 2003). Three intervention studies have shown a significant increase in birth weight associated with the use of prenatal multivitamin supplements, which included folate, whilst three other studies have failed to show an effect (Spencer, 2003). The importance of folate and genetic determinants of folate status on infant birth weight is an area of increasing research interest (Kramer *et al.* 2001).

In the present study, smoking is associated with a significant reduction in maternal, but not neonatal RBC folate level. A reduction in serum folate in pregnant women who smoke has been reported previously (McDonald *et al.* 2002; van Wersch *et al.* 2002) and the influence of smoking on RBC folate in normal adult populations is well documented (Cogswell *et al.* 2003; Mannino *et al.* 2003). Maternal but not neonatal RBC folate and vitamin B_{12} status are sensitive to smoking habits. However, an indirect effect on fetal growth could result from inadequate maternal nutritional reserves as a consequence of smoking. The current study suggests that the influence of smoking and RBC folate status on birth weight are not independent, and that the effect of smoking may be mediated in part through folate, although these results require confirmation by other investigators. RBC folate status may be influenced by smoking through a number of possible mech-

Table 4. Factors influencing birth weight (univariate regression analysis, standardized regression coefficients are shown)

	Birth weight (*z*-score)				
Factor	*n*	Coeff.	95 % CI	R^2	*P*
Maternal RBC folate (ng/ml)†	677	0·14	0·07, 0·23	0·021	<0·001*
Neonatal RBC folate (ng/ml)†	597	0·06	−0·01, 0·14	0·005	0·10
Maternal vitamin B_{12} (pg/ml)†	500	0·03	−0·05, 0·12	0·001	0·41
Neonatal vitamin B_{12} (pg/ml)†	522	−0·09	−0·17, −0·01	0·011	0·02*
Maternal age (years)	966	0·04	−0·02, 0·11	0·002	0·16
Delivery parity	980	0·06	−0·005, 0·12	0·003	0·07
Current smoker	437	−2·15	−3·24, −1·04	0·033	<0·001*

* $P<0·05$.
† Log-transformed values.

anisms; intermediates in the folate-dependent homocysteine metabolic pathway (Fig. 1) are extremely labile and sensitive to the redox balance within the cell. Cigarette smoking is a significant source of oxidative stress (Alberg, 2002) and may alter the ability of the cell to metabolize and ultimately to store folate. It is also possible that women who smoke are also less likely to eat a diet rich in folate or use folic acid supplements (Ortega *et al.* 2004). However, no significant difference in folate intake (including the contribution of folic acid supplements) between smokers and non-smokers was reported in a recent study of Canadian women (McDonald *et al.* 2002).

We report a positive association between maternal vitamin B_{12} level and both neonatal vitamin B_{12} levels and RBC folate levels. Maternal status appears to be a far more important predictor of neonatal status in the case of vitamin B_{12} than in the case of folate, accounting for 31 and 7 % of the variation in neonatal status, respectively. This disparity may be due to a number of factors influencing the transport, absorption and storage of RBC folate, whereas fewer parameters, and therefore less variation, are involved in determining serum vitamin B_{12}. The inference, which has been corroborated elsewhere (Bjorke Monsen *et al.* 2001; Guerra-Shinohara *et al.* 2002), is that pregnant women with low vitamin B_{12} are unable to provide the necessary amount of B_{12} to their fetus. This has clear clinical implications and serves to emphasize the importance of vitamin B_{12} in maternal nutrition during pregnancy.

The relationship between maternal vitamin B_{12} and neonatal RBC folate is less obvious, but becomes apparent when considered in the context of the folate-dependent homocysteine metabolic pathway (Fig. 1). Bioavailable folate at a cellular level is present in the form of 5-methyltetrahydrofolate. This is taken into the cell as a prerequisite for the conversion of homocysteine to methionine by the action of the enzyme methionine synthase. This enzyme is highly important in early development and this has been clearly demonstrated by the targeted disruption of the methionine synthase gene in mice (Swanson *et al.* 2001). Vitamin B_{12} is a strong determinant of methionine synthase activity, thereby influencing the rate of conversion of Hcy to methionine (Brunaud *et al.* 2003). Vitamin B_{12} is also an important co-factor in the conversion of of 5-methyltetrahydrofolate to tetrahydrofolate, and limited vitamin B_{12} can induce a 'methyl trap' (Ueland & Bjorke Monsen, 2003). Measurement of RBC folate reflects the amount of tetrahydrofolate stored in the cell. The observation that both maternal and neonatal vitamin B_{12} levels are important determinants of neonatal RBC folate is consistent with the 'methyl trap' hypothesis. Vitamin B_{12} status is not directly associated with birth weight in our current study, however, for reasons outlined above, it is an important component in maternal nutrition during pregnancy.

In summary, the current study reports a statistically significant association between maternal RBC folate, during early gestation, and infant birth weight. Folate fulfils many important roles and there exist a number of biologically plausible hypotheses to explain the observed association. RBC folate was significantly lower in smoking mothers compared to non-smokers and the subsequent influence that this may have on birth weight requires further investigation. Maternal vitamin B_{12} also represents an important determinant of folate status in the neonate and the present study provides evidence for a role of this micronutrient in maternal nutrition during pregnancy. A detailed exploration of the role of folate and vitamin B_{12} in embryo–fetal development and the modulation of their effects by genetic variation in folate-related genes and by nutritional intervention is recommended.

Acknowledgements

The authors would like to acknowledge the assistance of Mr Terry Burgess in undertaking RBC folate and vitamin B_{12} analyses. Professor John Mathers is thanked for his helpful comments on the manuscript. Dr Caroline Relton is funded by Westlakes Research Institute and Dr Mark Pearce is funded by the Minnie Henderson Trust.

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