International Journal of Epidemiology, 2014, 1487–1497
doi: 10.1093/ije/dyu132
Advance Access Publication Date: 22 July 2014
Original article

Original article

Maternal homocysteine in pregnancy and offspring birthweight: epidemiological associations and Mendelian randomization analysis

Chittaranjan S Yajnik,[1]* Giriraj R Chandak,[2,3]* Charudatta Joglekar,[1] Prachi Katre,[4] Dattatray S Bhat,[1] Suraj N Singh,[2] Charles S Janipalli,[2] Helga Refsum,[5,6] Ghattu Krishnaveni,[7] Sargoor Veena,[7] Clive Osmond[8] and Caroline HD Fall[8]

[1]Diabetes Unit, King Edward Memorial Hospital and Research Centre, Pune, India, [2]CSIR-Centre for Cellular and Molecular Biology (CSIR-CCMB), Hyderabad, India, [3]Adjunct Group Leader, Adjunct Group, Genome Institute of Singapore, Singapore, [4]Persistent Systems Ltd, Pune, India, [5]Department of Nutrition, University of Oslo, Oslo, Norway, [6]Department of Pharmacology, University of Oxford, Oxford, UK, [7]Epidemiology Research Unit, CSI Holdsworth Memorial Hospital, Mysore, India and [8]MRC Lifecourse Epidemiology Unit, University of Southampton, Southampton, UK

*Corresponding authors. Chittaranjan S. Yajnik, Diabetes Unit, King Edward Memorial Hospital and Research Centre, Pune, India 411 011. E-mail: diabetes@kemdiabetes.org; Giriraj R. Chandak, CSIR-Centre for Cellular & Molecular Biology, Hyderabad, India, 500007. E-mail: chandakgrc@ccmb.res.in

Accepted 4 June 2014

Abstract

Background: Disturbed one-carbon (1-C) metabolism in the mother is associated with poor fetal growth but causality of this relationship has not been established.

Methods: We studied the association between maternal total homocysteine and offspring birthweight in the Pune Maternal Nutrition Study (PMNS, Pune, India) and Parthenon Cohort Study (Mysore, India). We tested for evidence of causality within a Mendelian randomization framework, using a methylenetetrahydrofolatereductase (*MTHFR*) gene variant rs1801133 (earlier known as 677C→T) by instrumental variable and triangulation analysis, separately and using meta-analysis.

Results: Median (IQR) homocysteine concentration and mean (SD) birthweight were 8.6 μmol/l (6.7,10.8) and 2642 g (379) in the PMNS and 6.0 μmol/l (5.1,7.1) and 2871 g (443) in the Parthenon study. Offspring birthweight was inversely related to maternal homocysteine concentration—PMNS: –22 g/SD [95% confidence interval (CI): (–50, 5), adjusted for gestational age and offspring gender]; Parthenon: –57 g (–92, –21); meta-analysis: –40 g (–62, –17)]. Maternal risk genotype at rs1801133 predicted higher homocysteine concentration [PMNS: 0.30 SD/allele (0.14, 0.46); Parthenon: 0.21 SD (0.02, 0.40); meta-analysis: 0.26 SD (0.14, 0.39)]; and lower birthweight [PMNS: –46 g (–102, 11, adjusted for gestational age, offspring gender and rs1801133 genotype); Parthenon: –78 g

(–170, 15); meta-analysis: –61 g (–111, –10)]. Instrumental variable and triangulation analysis supported a causal association between maternal homocysteine concentration and offspring birthweight.

Conclusions: Our findings suggest a causal role for maternal homocysteine (1-C metabolism) in fetal growth. Reducing maternal homocysteine concentrations may improve fetal growth.

Key words: Maternal homocysteine, offspring birthweight, causality, *MTHFR* variant, folate, vitamin B_{12}, Mendelian randomization analysis

Key Messages

- Maternal 1-C metabolism is thought to be important for fetal growth.
- Indian mothers have high homocysteine concentrations due to dietary deficiencies (vitamin B12 and protein).
- Higher maternal homocysteine concentrations predict earlier delivery and lower birthweight.
- Mendelian randomization analysis linked variant rs1801133 of the MTHFR gene with neonatal birthweight.
- This supports causality of the association between maternal 1-C metabolism and fetal growth.
- Reducing maternal homocysteine concentrations may improve fetal growth.

Introduction

Small size at birth is associated with poor perinatal outcomes and an increased risk of type 2 diabetes and cardiovascular disease (CVD) in later life.[1–3] Indian babies are among the smallest in the world and this is attributed to the small size and poor nutrition of their mothers.[4] Studies relating maternal nutrition to newborn size have mostly investigated macronutrients; recent observational and interventional studies have shown a role for maternal micronutrient nutrition.[5–6]

The Pune Maternal Nutrition Study (PMNS) investigated the effect of maternal nutrition on fetal growth and future risk of diabetes and CVD. We found that higher frequency of intake of micronutrient-rich foods and higher circulating folate and vitamin C predicted larger newborn size.[7] In a subset of PMNS participants, we found that mothers of small-for-gestational-age (SGA) babies had higher total homocysteine concentrations compared with those in appropriate-for-gestational-age pregnancies.[6] Homocysteine is a key metabolite in one-carbon (1-C) metabolism, and homocysteine levels influence several cellular processes including DNA methylation and synthesis of nucleic acids and proteins.[8] Homocysteine concentration is influenced by a number of genetic polymorphisms that affect the function of different enzymes regulating 1-C metabolism, and also by dietary intake of 'methyl donors' (folate, vitamin B_{12}, choline and betaine) and other vitamins (B_6 and B_2, co-factors for enzymes regulating the 1-C metabolism). The association of elevated maternal homocysteine with fetal growth restriction suggests a role of maternal 1-C metabolism in fetal growth and development, and a potential for improvement by dietary manipulations.[9–11] However, observational associations may be confounded by various lifestyle factors and may suffer from reverse causality. Causality is best proved by conducting randomized controlled trials which are time- and labour-intensive, expensive and sometimes unethical if they deviate from local established standards of care. Mendelian randomization is an alternative method that uses genetic variants associated with environmental and lifestyle exposures to overcome these limitations. The genotype is randomized at conception and is unaffected by lifestyle confounders, and there cannot be reverse causality. Therefore the 'instrumental component' calculated from genotype can only be causally related. Demonstration of such a relationship, therefore, improves confidence in the causality of associations.[12]

Methylenetetrahydrofolatereductase (encoded by *MTHFR*) is an important enzyme in 1-C metabolism that helps the conversion of homocysteine to methionine by providing the methyl donor tetrahydrofolate.[13] Although several homocysteine-raising variants of *MTHFR* have been identified, the rs1801133 variant (earlier known as 677C→T polymorphism) significantly reduces the enzyme efficiency (by 75%), and individuals carrying the risk genotype 'TT' have ~20% higher circulating homocysteine levels than those with the wild CC genotype.[14] This polymorphism has been used as an instrument for investigating

causality of associations of homocysteine with various cardiometabolic phenotypes,[15,16] and in clinical practice to predict high risk of adverse outcomes in pregnancy (neural tube defects and other congenital anomalies).[17]

We aimed to investigate the association between maternal homocysteine concentration and offspring birthweight in a large number of mother-offspring pairs by combining data from two Indian birth cohorts. We also used the rs1801133 variant for a Mendelian randomization analysis (instrumental variable and triangulation methods) to test the hypothesis that homocysteine concentrations in mothers have a causal role in determining fetal growth and size at birth.

Methods

Participants

The PMNS was started in 1993 in six villages near Pune to study the relationship between maternal nutrition and fetal growth.[7] Women were studied twice during pregnancy (18 ± 2 and 28 ± 2 weeks of gestation) for nutritional, biochemical and demographic information. Socioeconomic status (SES) was assessed using a standardized questionnaire for rural India.[18] None of the women were receiving vitamin supplements at enrolment. According to national guidelines, the women were supplemented with iron (60 mg) and folic acid (500 μg) tablets, one daily for 100 days from 18 weeks of gestation onwards.

The Parthenon Cohort Study was set up in 1997 to investigate the relationship between maternal glucose tolerance and fetal growth in the antenatal clinic at the Holdsworth Memorial Hospital (HMH), Mysore.[19] Women were studied at 30 ± 2 weeks of gestation for anthropometric, biochemical and demographic information. SES was assessed using the Kuppuswamy scoring system for urban India.[20] The number of deliveries reflects only those who delivered at the HMH. Maternal supplement use was recorded when the women were recruited in mid pregnancy, but not at 30 weeks of gestation. At recruitment, 188 women (36.5%) reported taking vitamin supplements containing both vitamin B_{12} and folic acid, and 52 (10%) reported taking folic acid alone though the dosage was not recorded.

The institutional ethics committees at both centres approved the study and informed consent was obtained from all women and their families.

Data collection

In the PMNS, neonatal birthweight was measured to the nearest 50 g using a Salter spring balance (Salter Abbey, Suffolk, UK) within 72 h of birth, and in the Parthenon Cohort Study within 24 h, using a digital weighing scale (Seca, Germany) to the nearest 5 g. In the PMNS, an early morning fasting blood sample was collected at home, transported on ice to the local research centre and centrifuged, and plasma aliquots were transferred to the KEM Hospital Research Centre (KEMHRC) and stored at –80°C until further use. In the Parthenon Cohort study, fasting blood was collected in the clinic, plasma separated within 2 h and aliquots stored at –80°C.

Biochemical and genetic analyses

Red cell folate concentrations in PMNS were measured by radioimmunoassay (Becton Dickinson, Oxford, UK),[7] plasma vitamin B_{12} by microbial assay[21,22] and homocysteine by the fully automated GC-MS method.[23] For the Parthenon study, plasma folate and plasma vitamin B_{12} were measured by microbial assays[21,22,24] and homocysteine by fluorescence polarization immune assay at KEMHRC.[25] Plasma glucose and triglyceride concentrations were measured by standard enzymatic kits using auto analyzer (Abbott VP Super System, Irving, TX). The inter- and intra-assay coefficient of variation was <5% for all measurements.

Genotyping for both studies was carried out at the CSIR-Centre for Cellular and Molecular Biology, Hyderabad. Genomic DNA was isolated from blood samples using the salting-out method and DNA plated in uniform concentrations. We used a Sequenom-based MassARRAY technology to genotype the rs1801133 variant. The genotyping success rate was >95%. We confirmed the genotypes for ~10% of the samples by sequencing the purified polymerase chain reaction (PCR) products on an ABI 3730 Genetic Analyzer. Inconsistency of only 0.003% (1/384) suggested high genotyping accuracy.

Definitions

Low vitamin B_{12} refers to a plasma concentration <150 pmol/l[23] and hyperhomocysteinaemia to a concentration >10 μmol/l.[26] Low folate status was defined as an erythrocyte folate concentration <283 nmol/l and plasma folate concentration <7 nmol/l.[27] Prematurity refers to delivery at <37 weeks of gestation, low birthweight (LBW) as a birthweight <2500 g and SGA as per Oken *et al.*[28]

Statistical methods

Data are presented as mean (SD) for continuous variables, median (25th-75th centiles) for skewed variables and percentages for categorical variables. Vitamin B_{12},

homocysteine and folate concentrations had skewed distributions and were log-transformed for the analyses. They were adjusted for the gestational age at which the sample was taken. Because of differences in methods between the two cohorts, we used study-specific SD scores for folate concentrations and SES scores in the combined analysis. Higher scores refer to better SES. Associations between exposures and birthweight were analysed using multiple linear regression adjusted for gestation and gender. Effect sizes are represented as change in physiological units [95% confidence intervals (CI)] per SD change in the exposure. In the genotype homocysteine association, the effect size is represented as SD change in homocysteine per allele. This aligned the units with those used in the triangulation analysis. Genotype distribution at rs1801133 was tested for compliance with the Hardy-Weinberg equilibrium (HWE). Associations between maternal genotype and continuous variables were examined using an additive model. Since genotype frequency, homocysteine concentrations and birthweight were different between the two centres, we examined associations in each cohort separately and then combined by meta-analysis using a fixed effect inverse variance model after testing for between-study heterogeneity (I^2).

We tested the causality of the association between maternal homocysteine concentration and offspring birthweight by instrumental variable and triangulation analysis.[29] For the instrumental variable analysis, we used a two-stage least-square process. In the first stage, we calculated the SD change in homocysteine concentration per maternal rs1801133 allele in the PMNS and Parthenon cohorts. In the second stage, this instrument was used to estimate the association between maternal homocysteine and offspring birthweight. The difference between estimates from the observed and instrumental variable analyses was tested using the Durbin-Wu-Hausman test. We also performed a meta-analysis of these results.

In the triangulation method (see Supplementary data available at *IJE* online), we calculated the expected effect of maternal homocysteine on offspring birthweight by dividing the observed effect between maternal rs1801133 variant and offspring birthweight, and the observed effect between maternal rs1801133 variant and homocysteine concentration, and compared it with the observed effect.

Results

We restricted the analysis to 526 mother-baby pairs in the PMNS and 515 mother-baby pairs in the Parthenon cohort study, whose maternal homocysteine concentrations, maternal rs1801133 genotype and offspring birthweight data were available. These mothers and babies were no different from others in the original cohorts with respect to maternal age, body mass index (BMI), SES and offspring birthweight (data not shown). Mothers in the PMNS were younger and thinner than Parthenon mothers, and more likely to be vegetarian (Table 1). PMNS babies were lighter at birth than Parthenon babies, and had a higher prevalence of SGA births.

Maternal homocysteine, folate and vitamin B_{12} concentrations

Median maternal homocysteine concentration was 8.6 μmol/l in the PMNS and 6.0 μmol/l in the Parthenon cohort (32.3% and 3.7% had hyperhomocystenaemia, respectively) (Table 1). Low folate was rare in both cohorts but 70% of mothers in the PMNS and 43% in the Parthenon cohort had low vitamin B_{12} concentrations. Both vitamin B_{12} and folate concentrations were inversely related to homocysteine concentrations (PMNS: $r = -0.27$ and -0.39, respectively; Parthenon: $r = -0.24$ and $r = -0.28$, respectively; $P < 0.001$ for all). Maternal BMI was inversely associated with vitamin B_{12} concentrations in the Parthenon cohort (standardized $\beta = -0.4$, $P = 0.02$) but not in the PMNS (standardized $\beta = -0.09$, $P = 0.3$). Maternal folate concentration was directly associated with SES in the Parthenon cohort ($r = 0.3$, $P < 0.001$). Maternal B_{12} was directly associated with SES in the PMNS ($r = 0.1$, $P = 0.04$). Non-vegetarians had lower median homocysteine concentrations than vegetarians in the Parthenon cohort (5.9 vs 6.7 μmol/l, $P < 0.01$).

Maternal homocysteine, folate and vitamin B_{12} concentrations and offspring birthweight

Folate concentrations were positively associated with birthweight (PMNS: $\beta = 22.8$ g/SD, 95% CI: 4.7, 40.9, $P = 0.01$; Parthenon: $\beta = 8.2$ g/SD, 95% CI: −7, 23.8, $P = 0.3$). Vitamin B_{12} concentrations were not associated with birthweight in either cohort (PMNS: $\beta = 5.1$ g/SD, 95% CI: −14.5, 24.6, $P = 0.6$; Parthenon: $\beta = 1.6$ g/SD, 95% CI: −13.9, 17.1, $P = 0.8$). Mothers with higher plasma homocysteine concentrations delivered earlier and had lighter babies (Table 2). One SD increase in plasma homocysteine concentration predicted a 0.1-week earlier delivery ($P = 0.15$) in the PMNS, and a 0.2-week earlier delivery ($P = 0.03$) in the Parthenon cohort. The effect in both cohorts combined, using fixed effects meta-analysis, was 0.1 week/SD ($P = 0.01$), P for heterogeneity = 0.5 and $I^2 = 0\%$. After adjusting for gestational age and gender, one SD increase in plasma homocysteine concentration predicted a 22-g lower birthweight ($P = 0.1$) in the PMNS,

Table 1. Maternal and offspring characteristics in Pune Maternal Nutrition Study (PMNS) and Parthenon Study

Characteristic	PMNS	Parthenon	*P*-value[d]
Number	526	515	
Maternal			
Age (years)[a]	21.5 (3.5)	23.9 (4.1)	<0.001
Primipara[b]	160 (30.4)	256 (49.7)	<0.001
SES score[a]	27.1 (6.6)	34.0 (6.4)	NA
Vegetarian diet[b]	164 (31.6)	44 (9.3)	<0.001
Tobacco use[b]			
Smoking	0 (0)	0 (0)	NA
Chewing	68 (13.0)	0 (0)	0.01
BMI (kg/m^2)[a]	20.4 (1.9)	23.7 (3.6)	<0.001
Gestation at measurement of biomarkers (weeks)	29.4 (1.2)	29.1 (1.6)	0.51
Plasma homocysteine (μmol/l)[c]	8.6 (6.7, 10.8)	6.0 (5.1, 7.1)	<0.001
>10 μmol/l[b]	170 (32.3)	19 (3.7)	<0.001
Erythrocyte folate (nmol/l)[c]	958 (734, 1261)	–	NA
<283 nmol/l[b]	1 (0.2)	–	NA
Plasma folate (nmol/l)[c]	–	34.4 (16.8, 51.2)	NA
<7 nmol/l[b]	–	24 (4.7)	NA
Plasma vitamin B_{12} (pmol/l)[c]	122 (94, 164)	162 (123, 223)	<0.001
<150 pmol/l[b]	368 (70.4)	221 (43.0)	<0.001
Offspring			
Gender (boys)[b]	280 (53.2)	247 (48.0)	0.09
Gestation (weeks)[a]	39.0 (1.7)	39.1 (1.7)	0.93
Preterm (<37 weeks)[b]	47 (8.9)	46 (8.9)	0.99
Birthweight (g)[a]	2642 (379)	2871 (443)	<0.001
Low birthweight (<2500 g)[b]	160 (30.4)	91 (17.7)	<0.001
SGA[b]	356 (67.7)	217 (42.1)	<0.001
Maternal genotype			
rs1081133[b]			
CC	378 (71.9)	415 (80.6)	0.003
CT	132 (25.1)	92 (17.9)	
TT	16 (3.0)	8 (1.5)	
Offspring genotype			
rs1081133[b]			
CC	381 (74.9)	393 (78.9)	0.029
CT	113 (22.2)	101 (20.3)	
TT	15 (2.9)	4 (0.8)	

SES, socioeconomic status; BMI, body mass index; SGA, small-for-gestational-age.

NA refers to measurements which are not comparable; SES can not be compared as the scales of measurements vary according to rural/urban area.

Values are mean (SD)[a] or number (%)[b] or median (25th, 75th centile)[c].

[d]The *P*-value is for the difference between the PMNS and Parthenon cohorts derived using t-tests for continuous and chi square tests for categorical variables.

57 g ($P = 0.002$) in the Parthenon cohort and 40 g ($P < 0.001$) in the meta-analysis, P for heterogeneity = 0.15.

Maternal rs1801133 genotype, homocysteine concentrations and offspring birthweight

The genotype frequencies in the mothers and children did not show evidence of deviating from HWE in both the cohorts ($P > 0.05$). The maternal frequency of the risk allele ('T') at rs1801133 was 15% in the PMNS and 11% in the Parthenon cohort; it was similar in mothers and children (Table 1). The TT genotype was uncommon in both cohorts (≤3%), but was more common in the mothers and babies in the PMNS.

The maternal T allele at rs1801133 was associated with higher maternal plasma homocysteine concentrations and TT mothers had the highest plasma homocysteine concentrations (Table 3). Per allele, there was a 0.30-SD ($P < 0.001$) increase in homocysteine in the PMNS, a 0.21-SD ($P = 0.04$) in the Parthenon cohort and 0.26-SD ($P < 0.001$; P for

Table 2. Association between maternal plasma total homocysteine (tHcy) and offspring birthweight and gestational age at birth

Quintiles of tHcy (μmol/l) (median, range)	Birthweight (g) (mean, SD)	Gestation (weeks) (mean, SD)
PMNS (*N*=526)		
1. 5.44 (1.78–6.34)	2713 (348)	39.4 (1.6)
2. 7.14 (6.38–7.78)	2605 (402)	38.9 (1.7)
3. 8.59 (7.79–9.37)	2638 (392)	39.1 (1.8)
4. 10.38 (9.38–11.38)	2655 (389)	39.1 (1.6)
5. 13.33 (11.41–27.59)	2601 (357)	39.1 (1.7)
P^1	0.02	0.15
β1 (95% CI)	–39 (–71, –7)	–0.10 (–0.25, 0.04)
P^2	0.11	0.16
β2 (95% CI)	–22 (–50, 5)	–0.10 (–0.25, 0.04)
Parthenon (*N*=515)		
1. 4.49 (2.26–4.85)	2921 (431)	39.3 (1.6)
2. 5.31 (4.87–5.68)	2928 (365)	39.4 (1.3)
3. 6.03 (5.70–6.41)	2909 (405)	39.0 (1.6)
4. 6.82 (6.42–7.47)	2821 (445)	38.8 (1.7)
5. 8.51 (7.48–40.73)	2772 (536)	38.8 (2.2)
P^1	<0.001	0.03
β1 (95% CI)	–74 (–112, –36)	–0.17 (–0.32, –0.02)
P^2	0.002	0.02
β2 (95% CI)	–57 (–92, –21)	–0.17 (–0.32, –0.02)
Meta-analysis		
P^1	<0.001	0.01
β1 (95% CI)	–56 (–81, –32)	–0.14 (–0.24, –0.03)
P for heterogeneity	0.16	0.5
I^2	48.4%	0.0%
P^2	<0.001	0.009
β2 (95% CI)	–40 (–62, –17)	–0.14 (–0.24, –0.04)
P for heterogeneity	0.15	0.6
I^2	51.0%	0.0%

tHcy concentrations are shown as quintiles for ease of interpretation. However, in regression analysis tHcy is used as a continuous variable in SD units.

P^1: unadjusted; P^2: adjusted for gender and gestational age as appropriate.

β1, β2 represent corresponding effect sizes.

heterogeneity = 0.5) in the meta-analysis. The T allele was not associated with other maternal characteristics which influence birthweight (circulating folate, vitamin B_{12}, fasting glucose, triglycerides, BMI and SES) (data not shown).

The maternal T allele at rs1801133 was associated with lower offspring birthweight (Table 3). In the meta-analysis, birthweight decreased by 46 g per allele ($P = 0.051$, adjusted for gestation and gender)and by 61 g ($P = 0.019$) after further adjustment for fetal genotype. Higher maternal folate protected the fetus against the birthweight-lowering effect of the TT genotype. Thus, the mean birthweight of babies born to TT mothers who had folate concentrations in the highest tertile was 2827 g compared with 2266 g among those in the lowest tertile. ($P = 0.047$, in the combined data) (Table 4).

Instrumental variable analysis

We calculated the effect of the rs1801133 genotype on homocysteine concentration, which is the 'gene effect' and therefore the 'instrumental variable' (Table 5). We expressed this as the SD change in homocysteine concentration per allele of the rs1801133 variant. In the second step, we used this variable to predict birthweight. In Pune, birthweight decreased by 84 g ($P = 0.3$, adjusted for gestation and gender) per SD change in tHcy concentration; in the Parthenon cohort by 309 g ($P < 0.001$) and in the meta-analysis by 224 g ($P < 0.001$). The difference between Pune and Parthenon of 231 g [standard error (SE) 130 g] was not statistically significant ($P = 0.08$). These results were unaffected by additional adjustment for the fetal genotype. There was a difference between the estimates from the

Table 3. Association of maternal *MTHFR* rs1801133 genotype with homocysteine, offspring birthweight and gestational age at birth

MTHFR rs1801133 genotype	N	tHcy (μmol/l) (median, 25th, 75th centile)	Birthweight (g) (mean, SD)	Gestation (weeks) (mean, SD)
PMNS	526			
CC	378	8.4 (6.6, 10.4)	2651 (383)	39.1 (1.8)
CT	132	9.3 (7.3, 11.9)	2620 (349)	39.0 (1.6)
TT	16	9.3 (6.9, 13.8)	2615 (530)	39.8 (1.6)
	P^1	<0.001	0.41	0.77
	β1 (95% CI)	0.30 (0.14, 0.46)	–26 (–88, 36)	0.04 (–0.23, 0.31)
	P^2		0.28	0.77
	β2 (95% CI)		–29 (–81, 23)	0.04 (–0.23, 0.31)
	P^3		0.11	0.72
	β3 (95% CI)		–46 (–102, 11)	–0.05 (–0.34, 0.24)
Parthenon	515			
CC	415	6.1 (5.1, 7.1)	2865 (438)	39.0 (1.8)
CT	92	5.8 (4.8, 6.9)	2911 (449)	39.5 (1.5)
TT	8	7.7 (6.5, 10.6)	2679 (610)	39.2 (1.4)
	P^1	0.04	0.56	0.02
	β1 (95% CI)	0.21 (0.02, 0.40)	–26 (–113, 61)	0.39 (0.05, 0.72)
	P^2		0.10	0.02
	β2 (95% CI)		–67 (–148, 13)	0.39 (0.06, 0.72)
	P^3		0.10	0.05
	β3 (95% CI)		–78 (–170, 15)	0.40 (0.01, 0.79)
Meta-analysis				
	P^1	<0.001	0.32	0.09
	β1 (95% CI)	0.26 (0.14, 0.39)	–26 (–78, 26)	0.18 (–0.03, 0.39)
	P-heterogeneity, I^2	0.49, 0.0%	0.99, 0.0%	0.10, 60%
	P^2		0.051	0.09
	β2 (95% CI)		–46 (–92, 0)	0.18 (–0.03, 0.39)
	P-heterogeneity, I^2		0.40, 0.0%	0.11, 61%
	P^3		0.02	0.32
	β (95% CI)		–61 (–111, –10)	0.12 (–0.12, 0.35)
	P-heterogenity, I^2		0.44, 0.0%	0.07, 71%

tHcy concentrations are adjusted for gestation at measurement.

P^1: derived by regression using genotype as a continuous variable; P^2: additionally adjusted for gender and gestational age as appropriate; P^3: additionally adjusted for offspring *MTHFR* rs1801133 genotype.

β1, β2, β3 represent corresponding effect sizes per allele change. For homocysteine this is in SD units, for others in physiological units

observed and instrumental variable analysis (test of 'endogenecity'). Thus, the instrumental variable effect was larger than the observed effect. These results suggest that the effect of homocysteine on birthweight is causal. Triangulation analysis also provided a similar conclusion (see Supplementary data, available at *IJE* online).

Discussion

In this study from two Indian birth cohorts, higher maternal homocysteine concentration predicted lower offspring birthweight. This effect was contributed both by a shorter gestation and by growth restriction. The causality of the association is supported by the instrumental variable and triangulation analyses in the Mendelian randomization framework using maternal genotype at the rs1801133 variant of *MTHFR*. This showed that the instrumental variable estimate of the maternal homocysteine effect on offspring birthweight was larger than the observed effect of 28-week homocysteine measurements. The instrumental variable is free of confounding and measurement error, is fixed at birth and therefore represents 'lifetime' exposure of the conceptus to elevated maternal homocysteine concentration from the periconceptional period to the time of birth. It also eliminates the possibility of reverse causality. Thus it suggests a true underlying causal association between the exposure (homocysteine concentration) and the outcome (birthweight). This association was independent of offspring genotype, suggesting that maternal homocysteine metabolism rather than fetal homocysteine metabolism is

responsible. The two Indian cohorts in this study are geographically separated and have demographic and nutritional differences. Moreover, the distribution of the rs1801133 genotype and homocysteine concentrations (exposure) and birthweight (outcome) were different. Such stratification might produce spurious associations. However, the meta-analysis confirms the associations observed in the individual cohorts and there was no evidence of heterogeneity in the results, supporting a true association.

There are only a few human studies which have investigated the role of maternal 1-C metabolism and related nutritional and genetic factors in fetal growth. These are predominantly in well-nourished European populations. The Hordaland study in Norway observed that high maternal plasma homocysteine concentrations (measured many years after the pregnancy) were associated with adverse pregnancy outcomes including low birthweight, and maternal rs1801133 polymorphism increased the risk of intrauterine growth restriction.[30] A study in Newcastle, UK[31] and the Generation R cohort in The Netherlands[32] both demonstrated that lower maternal folate concentrations predicted lower offspring birthweight. The variant rs1801133 of *MTHFR* by itself was unrelated to birthweight, but in combination with low folate predicted lower birthweight. In our study, women with TT genotype gave birth to lighter babies, but higher folate levels provided some protection against this effect. This suggests that both maternal nutrition and her genotype influencing 1-C metabolism have a causal influence on fetal growth and neonatal birthweight.

Mothers in our study are relatively thin, have low intakes of energy, protein and micronutrients and their babies are amongst the smallest in the world. Moreover, mothers in the Indian subcontinent have a different balance between folate and vitamin B_{12} compared with Europeans: adequate in folate due to vegetarian food and supplements but low in B_{12} due to a low intake of foods of animal origin.[7,33] Unlike in Europeans, in whom low folate is a major contributor to hyperhomocysteinaemia, vitamin B_{12} contributed much more to the risk (49%) compared with folate (8%) in our population, similar to the findings in Nepalese pregnant women.[34] Low maternal vitamin B_{12} predicted intrauterine growth restriction in a study in Bangalore, India[35] and in babies of migrant Indians in the UK,[36] whereas high maternal homocysteine and low folate predicted intrauterine growth restriction in Pakistan.[37] These studies support a role for maternal 1-C metabolism in fetal growth and suggest that disturbances in this

Table 4. Mean offspring birthweight (g) according to maternal *MTHFR* rs1801133 genotype by tertiles of folate concentrations in combined data

Maternal *MTHFR* rs1801133 genotype	Tertile of SD score for folate[a]		
	1	2	3
CC	2747	2739	2814
CT	2639	2729	2903
TT	2266	2572	2827

[a]We used SD scores of folate concentrations because of different units of measurements in the two cohorts.

Table 5. Observed and instrumental variable analysis of the maternal homocysteine offspring birthweight association

Analysis	Observed change in birthweight (g) per SD change in total homocysteine			Instrumental variable estimate of change in birthweight (g) per SD change in total homocysteine			P^1
	Effect size (g)	95% CI	*P*	Effect size (g)	95% CI	*P*	
Unadjusted							
PMNS	–39	–71, –7	0.02	–89	–295, 118	0.40	0.63
Parthenon	–74	–112, –36	<0.001	–268	–462, –75	0.007	0.045
Meta-analysis	–56	–81, –32	<0.001	–198	–338, –57	0.006	0.044
Heterogenity *P* (I^2%)	0.16 (48.4)			0.20 (33.5)			
Adjusted for gestational age and gender							
PMNS	–22	–50, 5	0.11	–84	–259, 90	0.34	0.48
Parthenon	–57	–92, –21	0.002	–309	–488, –131	0.001	0.005
Meta-analysis	–40	–62, –17	<0.001	–224	–349, –100	<0.001	0.003
Heterogenity *P* (I^2%)	0.15 (51.0)			0.078 (68.0)			
Additionally adjusted for fetal genotype							
PMNS	–27	–55, 1	0.06	–135	–324, 53	0.16	0.25
Parthenon	–58	–94, –22	0.002	–308	–490, –125	0.001	0.006
Meta-analysis	–43	–65, –20	<0.001	–248	–378, –118	<0.001	0.002
Heterogenity *P* (I^2%)	0.29 (37.9)			0.076 (39.4)			

P^1: *P*-value for the Durbin-Wu-Hausman test for the difference between the estimates from observed and instrumental variable analysis.

pathway may lead to fetal programming, so called 'nutrient-mediated teratogenesis'.[38] On this background, it is surprising that a recent study in Norwegian pregnant women failed to show an association between dietary folate intake, supplemental folic acid, maternal plasma folate, homocysteine concentrations in the second trimester and offspring birth size.[39]

The possible mechanisms of these associations include the role of 1-C (-CH_3) groups in nucleic acid synthesis and methylation of DNA which contributes to regulation of gene expression.[33] In addition, 1-C metabolism provides the essential amino acid methionine for protein synthesis. The importance of dietary methyl donors in fetal programming is well illustrated in a number of animal studies.[40,41] Hyperhomocysteinaemia could also have a direct damaging effect on endothelium by lowering nitric oxide and stimulating pro-inflammatory and oxidative stress pathways, which could impair placental perfusion and function.[42] Recently a number of studies have demonstrated an association between factors influencing fetal growth (nutrition and smoking), birth size and a number of epigenetic (DNA methylation) markers in the cord blood.[43–50]

Our study has several strengths and some limitations. One of the major strengths is the simultaneous measurement of maternal nutritional and genetic markers in relation to fetal growth. Inclusion of two Indian birth cohorts increases the generalizablity of the results within India. The populations studied have a high incidence of micronutrient deficiencies and of low birthweight, increasing the statistical power and relevance of the investigation. Both studies have a comprehensive range of measurements including many well-known confounders and effect modifiers, allowing appropriate statistical adjustments in the analysis. The similarity of the results in the two cohorts, despite many differences in the design and methods, and results of meta-analysis suggests biological consistency. Findings in the instrumental variable and triangulation analysis strengthen the causality of association between maternal homocysteine and offspring birthweight.

One of the limitations was the difference in methods for some of the measurements in the two cohorts. We adjusted for these by using cohort-specific SD scores in the analysis. Another limitation is a relatively small number of mothers with the risk genotype TT because of the low frequency of the risk allele at rs1801133 variant which, however, is consistent with other Indian studies.[51] Given the differences in risk allele frequency and other maternal and neonatal characteristics in the two cohorts, it is difficult to discount an effect of population stratification, but significant results in the meta-analysis argue against stratification. Use of a single genetic instrument, as opposed to multiple instruments that would allow checks for pleiotropy, may also constitute a limitation. However, the rs1801133 variant in *MTHFR* is a well-established instrument for homocysteine levels and has been used in many different studies for association with many diverse traits.[14,15] Potential pleotropic effects cannot be ruled out. In the presence of vertical pleiotropy or pathway-specific effects, this genetic variant will exert its effect on birthweight through a direct pathway. Whereas this is a potentially informative relationship, it is difficult to rule out alternative intermediate effects through DNA methylation and alteration in function of other genes. This could have horizontal pleiotropic effects, leading potentially to widespread biological effects and more global patterns of secondary effect.[52] We acknowledge this biological complexity, but would like to emphasize that we have taken the best measures and their instruments to make appropriate use of the data and investigate biological relationships.

Our finding of an important role of maternal 1-C metabolism in fetal growth is likely to promote interest in public health interventions to improve vitamin B_{12} and folate status in young Indian women. The role of folate in prevention of neural tube defects is well established and it is current standard practice to improve folate intake in the pre- and periconceptional period in developed countries. The role of maternal vitamin B_{12} is only just emerging; it influences fetal growth, neural tube development and neurocognitive development.[53,54] In a recent vitamin B_{12} intervention trial in India, started in the first trimester of pregnancy, there was a borderline reduction in incidence of intrauterine growth retardation.[55] An imbalance with low B_{12} and high folate in the mother predicts insulin resistance in the child, and high folate predicts higher adiposity in the child.[33] Thus, balanced B_{12} and folate nutrition in the mothers, taking care to avoid folate excess, may improve fetal growth and reduce risk of later diabetes and CVD, in line with the Developmental Origins of Health and Disease (DOHaD) theory of 'fetal programming'. Our estimates suggest that if all the mothers had circulating homocysteine concentrations in the lowest quintile (median 4.7 μmol/l) seen in our study, the incidence of low birthweight would fall from 26% to 19%. This would be a substantial public health achievement and should be tested.

Supplementary Data

Supplementary data are available at *IJE* online.

Funding

This work was supported by funds from the Medical Research Council, UK, the Department for International Development, UK, the Wellcome Trust, UK, Parthenon Trust, Switzerland and Council

of Scientific and Industrial Research (CSIR), Ministry of Science and Technology, Government of India, India [XII Five-Year Plan titled 'CARDIOMED (BSC0122)']. Some aspects of this work were supported by GEoCoDE, an International Research Staff Exchange Scheme supported by EU which helped travel and interaction to promote analysis and writting of this manuscript.

Acknowledgements

We are grateful to all the women who took part in this study. We thank the late BanooCoyaji and the late V NRao, initiators of the rural primary health care programme in the study area around Pune. We acknowledge major contributions by S Rao, S Hirve and P Gupta, H Lubree and S Rege, and the invaluable community work contributed by T Deokar, S Chaugule, A Bhalerao and V Solat. We appreciate the help of O Netland, H Bergesen, E Blomdal and B Olsen with the biochemicalanalyses in Bergen (B_{12} and homocysteine), and the Special Haematology Laboratory, Southampton General Hospital, UK, for folate assays. We also thank BDR Paul, former Director, S C Karat, the current Director, and the obstetric and research staff of the Holdsworth Memorial Hospital. We acknowledge the help of Nic Timpson and Rachel Freathy in discussing and editing the manuscript. We also acknowledge the support of SNEHA-INDIA.

Author Contributions: Chittaranjan S Yajnik, Giriraj Ratan Chandak, Caroline HD Fall and Helga Refsum planned the study and wrote the manuscript. Charles S Janipalli and Suraj Nongmaithen Singh performed genotyping, collated the data and performed genetic and association analysis. Dattatray S Bhat performed laboratory measurements. Clive Osmond and Charudatta Joglekar performed the statistical analysis and data management. Ghattu Krishnaveni and Sargoor Veena coordinated the Parthenon study, provided relevant data and helped in writing the manuscript. Prachi Katre helped in writing the manuscript, data mining and organizing the references and also coordinated the manuscript writing.

Conflict of interest: None declared.

References

1. Barker DJ, Winter PD, Osmond C, Margetts B, Simmonds SJ. Weight in infancy and death from ischaemic heart disease. *Lancet* 1989;**2**:577–80.
2. Hales CN, Barker DJ, Clark PM *et al*. Fetal and infant growth and impaired glucose tolerance at age 64. *BMJ* 1991;**303**:1019–22.
3. Newsome CA, Shiell AW, Fall CH, Phillips DI, Shier R, Law CM. Is birthweight related to later glucose and insulin metabolism? A systematic review. *Diabet Med* 2003;**20**:339–48.
4. Yajnik CS, Fall CH, Coyaji KJ *et al*. Neonatal anthropometry: the thin-fat Indian baby. The Pune Maternal Nutrition Study. *Int J Obes Relat Metab Disord* 2003;**27**:173–80.
5. Fall CH, Fisher DJ, Osmond C *et al*. Multiple micronutrient supplementation during pregnancy in low-income countries: a meta-analysis of effects on birth size and length of gestation. Maternal Micronutrient Supplementation Study Group. *Food Nutr Bull* 2009;**30**(**4 Suppl**):S533–46.
6. Yajnik CS, Deshpande SS, Panchanadikar *et al*. Maternal total homocysteine concentration and neonatal size in India. *Asia Pac J Clin Nutr* 2005;**14**:179–81.
7. Rao S, Yajnik CS, Kanade A *et al*. Intake of micronutrient-rich foods in rural Indian mothers is associated with the size of their babies at birth: Pune Maternal Nutrition Study. *J Nutr* 2001;**131**:1217–24.
8. Selhub J. Public health significance of elevated homocysteine. *Food Nutr Bull* 2008;**29**:S116–25.
9. Rush EC, Katre P, Yajnik CS. Vitamin B12: one carbon metabolism, fetal growth and programming for chronic disease. *Eur J Nutr* 2014:**68**:2–7.
10. Yajnik CS, Deshmukh US. Maternal nutrition, intrauterine programming and consequential risks in the offspring. *Rev Endocr Metab Disord* 2008;**9**:203–11.
11. Deshmukh U, Katre P, Yajnik CS. Influence of maternal vitamin B12 and folate on growth and insulin resistance in the offspring. *Nestle Nutr Inst Workshop Ser* 2013;**74**:145–56.
12. Davey Smith G, Ebrahim S. 'Mendelian randomization': can genetic epidemiology contribute to understanding environmental determinants of disease? *Int J Epidemiol* 2003; **32**:1–22.
13. http://omim.org/entry/607093 (29 March 2014, date last accessed).
14. Frosst P, Blom HJ, Milos R *et al*. A candidate genetic risk factor for vascular disease: a common mutation in methylenetetrahydrofolate reductase. *Nat Genet* 1995;**10**:111–13.
15. Casas JP, Bautista LE, Smeeth L, Sharma P, Hingorani AD. Homocysteine and stroke: evidence on a causal link from mendelian randomisation. *Lancet* 2005;**365**:224–32.
16. Lewis SJ, Ebrahim S, Davey Smith G. Meta-analysis of MTHFR 677C->T polymorphism and coronary heart disease: does totality of evidence support causal role for homocysteine and preventive potential of folate? *BMJ* 2005; **331**:1053.
17. Ueland PM, Hustad S, Schneede J, Refsum H, Vollset SE. Biological and clinical implications of the MTHFR C677T polymorphism. *Trends Pharmacol Sci* 2001;**22**:195–201.
18. Pareek U, Trivedi G. Reliability and validity of socio-economic scales. *Ind J Appl Psychol* 1964;**1**:34–40.
19. Hill JC, Krishnaveni GV, Annamma I, Leary SD, Fall CHD. Glucose tolerance in pregnancy in South India: Relationships to neonatal anthropometry. *Acta Obstet Gynecol Scand* 2005;**84**:159–65.
20. Kuppuswamy B. *Manual of Socio-Economic Status Scale*. Delhi: Manasayan Publications, 1962.
21. Kelleher BP, Broin SD. Microbiological assay for vitamin B_{12} performed in 96-well microtitre plates. *J Clin Pathol* 1991;**44**:592–95.
22. Kelleher BP, Walshe KG, Scott JM, O'Broin SD. Microbiological assay for vitamin $B_{12}$with use of a colistin-sulfate-resistant organism. *Clin Chem* 1987;**33**:52–54.
23. Refsum H, Yajnik CS, Gadkari M *et al*. Hyperhomocysteinemia and elevated methylmalonic acid indicate a high prevalence of cobalamin deficiency in Asian Indians. *Am J Clin Nutr* 2001;**74**:233–41.
24. Horne DW, Patterson D. Lactobacillus casei microbiological assay of folic acid derivatives in 96-well microtiter plates. *Clin Chem* 1988;**34**:2357–59.
25. Shipchandler MT, Moore EG. Rapid, fully automated measurement of plasma homocyst(e)ine with the Abbott IMx analyzer. *Clin Chem* 1995;**41**:991–4.

26. Refsum H, Smith AD, Ueland PM *et al*. Facts and recommendations about total homocysteine determinations: an expert opinion. *Clin Chem* 2004;**50**:3–32.
27. Clarke R, Grimley EJ, Schneede J *et al*. Vitamin B12 and folate deficiency in later life. *Age Ageing* 2004;**33**:34–41.
28. Oken E, Kleinman KP, Rich-Edwards J, Gillman MW. A nearly continuous measure of birthweight for gestational age using a United States national reference. *BMC Pediatr* 2003;**3**:6.
29. Freathy RM, Timpson NJ, Lawlor DA *et al*. Common variation in the FTO gene alters diabetes-related metabolic traits to the extent expected given its effect on BMI. *Diabetes* 2008;**57**:1419–26.
30. Vollset SE, Refsum H, Irgens LM *et al*. Plasma total homocysteine, pregnancy complications, and adverse pregnancy outcomes: the Hordaland Homocysteine study. *Am J Clin Nutr* 2000;**71**:962–68.
31. Relton CL, Pearce MS, Burn J, Parker L. An investigation of folate-related genetic factors in the determination of birthweight. *Paediatr Perinatal Epidemiol* 2005;**19**:360–67.
32. Bergen NE, Jaddoe VW, Timmermans S *et al*. Homocysteine and folate concentrations in early pregnancy and the risk of adverse pregnancy outcomes: the Generation R Study. *BJOG* 2012;**119**:739–51.
33. Yajnik CS, Deshpande SS, Jackson AA *et al*. Vitamin B12 and folate concentrations during pregnancy and insulin resistance in the offspring: the Pune Maternal Nutrition Study. *Diabetologia* 2008;**51**:29–38.
34. Bondevik GT, Schneede J, Refsum H, Lie RT, Ulstein M, Kvale G. Homocysteine and methylmalonic acid levels in pregnant Nepali women. Should cobalamin supplementation be considered? *Eur J Clin Nutr* 2001;**55**:856–64.
35. Muthayya S, Kurpad AV, Duggan CP *et al*. Low maternal vitamin B_{12} status is associated with intrauterine growth retardation in urban South Indians. *Eur J Clin Nutr* 2006;**60**:791–801.
36. Robert PD, James H, Petrie A, Morgan JO, Hoffbrand AV. Vitamin B_{12} status in pregnancy among immigrants to Britain. *BMJ* 1973;**3**:67–72.
37. Lindblad B, Zaman S, Malik A *et al*. Folate, vitamin B_{12}, and homocysteine levels in South Asian women with growth-retarded fetuses. *Acta Obstet Gynecol Scand* 2005;**84**:1055–61.
38. Yajnik CS. Nutrient-mediated teratogenesis and fuel-mediated teratogenesis: two pathways of intrauterine programming of diabetes. *Int J Gynaecol Obstet* 2009;**104**:S27–31.
39. Nilsen RM, Vollset SE, Monsen AL *et al*. Infant birth size is not associated with maternal intake and status of folate during the second trimester in Norwegian pregnant women. *J Nutr* 2010;**140**:57–79.
40. Waterland RA, Jirtle RL. Early nutrition, epigenetic changes at transposons and imprinted genes, and enhanced susceptibility to adult chronic diseases. *Nutrition* 2004;**20**:63–68.
41. Sinclair KD, Allegrucci C, Singh R *et al*. DNA methylation, insulin resistance, and blood pressure in offspring determined by maternal periconceptional B vitamin and methionine status. *Proc Natl Acad Sci U S A* 2007;**104**:19351–56.
42. Stanger O, Weger M. Interactions of homocysteine, nitric oxide, folate and radicals in the progressively damaged endothelium. *Clin Chem Lab Med* 2003;**41**:1444–54.
43. Steegers-Theunissen RP, Twigt J, Pestinger V *et al*. The periconceptional period, reproduction and long-term health of offspring: the importance of one-carbon metabolism. *Hum Reprod Update* 2013;**19**:640–55.
44. Steegers-Theunissen RP, Obermann-Borst SA, Kremer D *et al*. Periconceptional maternal folic acid use of 400 microg per day is related to increased methylation of the IGF2 gene in the very young child. *PLoS One* 2009;**4**:e7845.
45. Joubert BR, Håberg SE, Nilsen RM *et al*. 450K epigenome-wide scan identifies differential DNA methylation in newborns related to maternal smoking during pregnancy. *Environ Health Perspect* 2012;**120**:1425–31.
46. Heijmans BT, Tobi EW, Stein AD *et al*. Persistent epigenetic differences associated with prenatal exposure to famine in humans. *Proc Natl Acad Sci U S A* 2008;**105:**17046–49.
47. Waterland RA, Kellermayer R, Laritsky E *et al*. Season ofconception in rural Gambia affects DNA methylation at putative human metastableepialleles. *PLoS Genet* 2010; **23**:e1001252.
48. Relton CL, Groom A, St Pourcain B *et al*. DNA methylation patterns in cord blood DNA and body size in childhood. *PLoS One* 2012;**7**:e31821.
49. Cooper WN, Khulan B, Owens S *et al*. DNA methylation profiling at imprinted loci after periconceptional micronutrient supplementation in humans: results of a pilot randomized controlled trial. *FASEB J* 2012;**26**:1782–90.
50. Engel SM, Joubert BR, Wu MC *et al*. Neonatal genome-wide methylation patterns in relation to birthweight in the Norwegian mother and child cohort. *Am JEpidemiol* 2014;**179**:834–42
51. Kumar J, Garg G, Kumar A *et al*. Single nucleotide polymorphisms in homocysteine metabolism pathway genes: association of CHDH A119C and MTHFR C677T with hyperhomocysteinemia. *Circ Cardiol Genet* 2009;**2**:599–606.
52. Hodgkin J. Seven types of pleiotropy. *Int J Dev Biol* 1998;**42**:501–05.
53. Godbole K, Gayathri P, Ghule S *et al*. Maternal one-carbon metabolism, MTHFR and TCN2 genotypes and neural tube defects in India. *Birth Defects Res A Clin Mol Teratol* 2011;**91**:848–56.
54. Bhate V, Deshpande S, Bhat D *et al*. Vitamin B12 status of pregnant Indian women and cognitive function in their 9-year-old children. *Food Nutr Bull* 2008;**29**:249–54.
55. Duggan C, Srinivasan K, Thomas T *et al*. Vitamin B-12 supplementation during pregnancy and early lactation increases maternal breast milk, and infant measures of vitamin B-12 status. *J Nutr* 2014;**144:**758–64.