Public Health Nutrition: 23(17), 3170–3180 doi:10.1017/S1368980019004002

The determinants of maternal homocysteine in pregnancy: findings from the Ottawa and Kingston Birth Cohort

Shazia H Chaudhry[1,2,3], Monica Taljaard[1,3], Amanda J MacFarlane[4,5], Laura M Gaudet[1,2,3], Graeme N Smith[6,7], Marc Rodger[1,3], Ruth Rennicks White[1,2], Mark C Walker[1,2,3] and Shi Wu Wen[1,2,3,*]

[1]The Ottawa Hospital Research Institute, Ottawa, Ontario, Canada: [2]OMNI Research Group, Department of Obstetrics, Gynecology, and Newborn Care, University of Ottawa, Faculty of Medicine, Ottawa, Ontario, Canada: [3]Faculty of Medicine, School of Epidemiology and Public Health, University of Ottawa, Ottawa, Ontario, Canada: [4]Nutrition Research Division, Health Canada, Ottawa, Ontario, Canada: [5]Department of Biochemistry, Microbiology and Immunology, University of Ottawa, Ottawa, Ontario, Canada: [6]Division of Maternal-Fetal Medicine, Department of Obstetrics & Gynaecology, Queen's University, Kingston, Ontario, Canada: [7]Kingston General Hospital Research Institute, Kingston, Ontario, Canada

Submitted 22 February 2019: Final revision received 13 August 2019: Accepted 16 September 2019: First published online 19 March 2020

Abstract

Objective: Observational studies have linked elevated homocysteine to vascular conditions. Folate intake has been associated with lower homocysteine concentration, although randomised controlled trials of folic acid supplementation to decrease the incidence of vascular conditions have been inconclusive. We investigated determinants of maternal homocysteine during pregnancy, particularly in a folic acid-fortified population.

Design: Data were from the Ottawa and Kingston Birth Cohort of 8085 participants. We used multivariable regression analyses to identify factors associated with maternal homocysteine, adjusted for gestational age at bloodwork. Continuous factors were modelled using restricted cubic splines. A subgroup analysis examined the modifying effect of MTHFR 677C>T genotype on folate, in determining homocysteine concentration.

Setting: Participants were recruited in Ottawa and Kingston, Canada, from 2002 to 2009.

Participants: Women were recruited when presenting for prenatal care in the early second trimester.

Results: In 7587 participants, factors significantly associated with higher homocysteine concentration were nulliparous, smoking and chronic hypertension, while factors significantly associated with lower homocysteine concentration were non-Caucasian race, history of a placenta-mediated complication and folic acid supplementation. Maternal age and BMI demonstrated U-shaped associations. Folic acid supplementation of >1 mg/d during pregnancy did not substantially increase folate concentration. In the subgroup analysis, MTHFR 677C>T modified the effect of folate status on homocysteine concentration.

Conclusions: We identified determinants of maternal homocysteine relevant to the lowering of homocysteine in the post-folic acid fortification era, characterised by folate-replete populations. A focus on periconceptional folic acid supplementation and improving health status may form an effective approach to lower homocysteine.

Keywords
Homocysteine
Hyperhomocysteinaemia
Pregnancy
Birth cohort
MTHFR (methylenetetrahydrofolate reductase)

Elevated plasma homocysteine is an independent risk factor for CVD[1,2] and has also been associated with vascular-related complications in pregnancy[3–5]. These include pre-eclampsia, small for gestational age and foetal demise[4–7].

In studies of the general population and pregnant women, folate intake and serum folate were identified as major nutritional determinants of homocysteine apart from genetic, health and lifestyle factors[4,5,8,9]. Clinical trials

*Corresponding author: Email swwen@ohri.ca

have been inconclusive in determining the effectiveness of folic acid supplementation to decrease the risk of vascular conditions, as a consequence of decreased homocysteine concentration[10–12]. This may be due to the relatively short duration of these trials[2,10,13,14] and due to the post-folic acid fortification era being characterised by folate-replete populations[15].

Homocysteine is an intermediate metabolite formed during the metabolism of the essential amino acid methionine in two main metabolic pathways: trans-sulfuration, in which vitamin B_6 acts as an enzyme co-factor, and remethylation, which depends on adequate serum folate and vitamin B_{12} as an enzyme co-factor[16,17]. Remethylation also requires a methyl group donated by 5-methyl tetrahydrofolate, a form of folate (vitamin B_9) that is formed from a reaction involving the enzyme methylenetetrahydrofolate reductase (MTHFR)[18]. A common SNP substitutes a T for a C nucleotide at position 677 of the gene encoding the MTHFR enzyme. The MTHFR 677C>T polymorphism produces an enzyme that is thermolabile—with enzyme activity reduced by half, which can result in moderately elevated homocysteine[14,19].

In pregnancy, homocysteine concentrations decrease as early as the first trimester and are lowest during the second trimester[20]. Concentrations rise into the third trimester and do not reach pre-pregnancy values until postpartum[21]. It is, therefore, essential that any studies investigating homocysteine concentrations during pregnancy account for gestational age at the time of bloodwork. Methods used to account for gestational age-related changes in homocysteine concentration include multivariable regression adjustment for gestational age and dichotomising the outcome relative to percentiles for gestational age at measurement[4,5].

We aimed to identify factors associated with maternal homocysteine in the early to mid-second trimester of pregnancy, particularly in a folic acid-fortified population. As a secondary objective, we examined the interaction of serum folate and the MTHFR 677C>T polymorphism in determining homocysteine concentration.

Methods

Study design

This study is based on the Ottawa and Kingston (OaK) Birth Cohort, which recruited 8085 participants from 2002 to 2009 at the Ottawa Hospital and Kingston General Hospital in the province of Ontario, Canada. OaK participants were recruited the early second trimester when presenting for prenatal appointments and were followed until delivery. Women were excluded from the analytic dataset of the current study if they were <12 or >20 weeks of gestation, carrying twins or multiples, and if they withdrew, were lost to follow-up or if the pregnancy was terminated.

The OaK Birth Cohort has been described previously[22]. Briefly, the baseline survey consisted of an interviewer-administered questionnaire on maternal characteristics, bloodwork and chart abstraction. Questionnaire responses were verified from medical records and brief telephone interviews to collect missing information.

Lab investigations

Plasma homocysteine measurement (primary outcome)

The primary outcome of this study was maternal plasma homocysteine concentration in μmol/l. The blood samples obtained for homocysteine measurement were collected in K_2EDTA Vacutainer tubes (Becton Dickinson, Lincoln Park, NJ). Samples for homocysteine measurement were immediately placed on ice and within 30 min centrifuged in 4°C at 3000***g*** for 10 min. Blood plasma was aliquoted and stored at −20°C. Plasma homocysteine (μmol/l) was measured within 1 month in batches on the Abbott AxSYM II Immunoassay System (Abbott Laboratories, Abbott Park, IL) using fluorescence polarisation immunoassay.

Serum folate measurement

Maternal blood samples obtained for folate measurement were collected in serum separator tubes (Becton Dickinson). The sample was left to clot and then centrifuged at 3000***g*** for 10 min. Serum was aliquoted and stored at −20°C. Serum folate (nmol/l) was measured within 1 month in batches on the Access 2 and UniCel® DxI 800 Immunoassay Systems using manufacturer's reagents (Beckman Coulter, Brea, CA).

DNA extraction and MTHFR genotyping

The blood samples obtained for MTHFR 677C>T genotyping (in a subset of participants due to logistics) were collected in K_2EDTA Vacutainer tubes (Becton Dickinson). DNA was extracted by manual extraction and later switched to automated extraction. In manual extraction, blood samples were centrifuged at 2500***g*** for 10 min and DNA extracted from the buffy coat using the FlexiGene DNA Kit (QIAGEN, Hilden, Germany). In automated extraction, blood samples were centrifuged at 1100***g*** and DNA extracted using the BioRobot M48 and MagAttract DNA Blood Midi Kit (QIAGEN). The MTHFR gene segment was amplified using PCR and genotyped using the ABI 3130xl Genetic Analyser and the ABI PRISM SNaPshot Multiplex Kit (Applied Biosystems, Waltham, MA).

Determinants of interest

Factors of interest and interactions were prespecified from a literature review of studies on homocysteine determinants[4,5,8,9,23] and from studies investigating the role of folates and homocysteine in placenta-mediated (i.e. vascular-related) pregnancy complications[4,5,8,24]. We considered the following factors: gestational age at bloodwork (abstracted from medical records), maternal

https://doi.org/10.1017/S1368980019004002 Published online by Cambridge University Press

age (years), race (based on self-reported ethnicity in terms of race/origin/ancestry), education (highest completed level), household income, parity, smoking during pregnancy, BMI (kg/m^2, from measured height and weight), diabetes (type 1 or 2), use of hormonal birth control prior to conception (i.e., oral, injection, IUD), chronic hypertension, history of a placenta-mediated pregnancy complication (i.e., small for gestational age, pre-eclampsia, placental abruption or pregnancy loss), folic acid supplementation and dose (from current prenatal vitamin brand, multivitamin brand and folic acid supplement), serum folate and the MTHFR 677C>T genotype.

Statistical analyses

Multivariable regression

We conducted multivariable linear regression analyses to examine the association of the identified factors of interest with homocysteine concentration while adjusting for gestational age at bloodwork as a continuous variable. Prior to analysis, a variable clustering algorithm was used to rule out multicollinearity among the prespecified variables of interest. Analyses were performed in RStudio version 0.99.892, R version 3.2.3.

Missing data were handled by multiple imputation using the package mice, which stands for multivariate imputation by chained equations[25]. In this approach, missing values are replaced by random draws of predicted values from a series of sequential multivariable models specified according to the type of incomplete variable: predictive mean matching for continuous variables, logistic regression for binary variables, multinomial logit model for categorical variables, and ordered logit model for ordinal variables. The number of imputations was set to 10, with 200 iterations. Ten to twenty imputations are considered adequate for this imputation method.[25]

The regression modelling strategies (rms) package within R was used for multivariable regression analyses[26]. Continuous variables were modelled with a restricted cubic spline function. Knots were set by default to the following quantiles: three knots at the 10th, 50th and 90th quantile, four knots at the 5th, 35th, 65th and 95th quantile, and five knots at the 5th, 27·5th, 50th, 72·5th and 95th quantile. To build the multivariable model, we first entered all prespecified variables into the model[27]. Continuous variables were modelled with five knots, and categorical and ordinal variables retained their original categories. Next, Akaike's Information Criterion (AIC) and the Bayesian Information Criterion (BIC) were used to examine both the goodness of fit of the multivariable model by altering the number of knots for continuous variables, as well as the effect of collapsing categories in categorical variables. AIC and BIC penalise the log likelihood for complexity of the model (i.e., number of parameters) with the aim to avoid overspecifying the model[27]. The final model was then refitted according to the lowest AIC and BIC values for each variable. Rubin's method was used to combine the results across the ten imputed datasets[25,27,28].

Results are presented using regression coefficients (representing mean differences) with 95 % CIs. For continuous factors modelled with restricted cubic splines, effect estimates (mean differences) are presented for the 75th *v.* 25th percentiles. Plots of modelled associations were generated to interpret the effects of continuous factors analysed as a restricted cubic spline. Model fit was assessed by plotting residuals against fitted values. Normality was assessed by visual inspection of normal probability plots.

Subgroup analysis

The multivariable analysis was repeated in the subgroup of participants with measured MTHFR 677C>T genotype. These participants had not self-selected into the study and were, therefore, expected to be representative of the entire cohort. The subgroup analysis examined the interaction of serum folate and the MTHFR 677C>T genotype in determining homocysteine concentration. We confirmed Hardy–Weinberg equilibrium of the MTHFR 677C>T genotype[29].

Secondary analyses

In addition to the primary analysis adjusting for gestational age at bloodwork as a continuous covariate, we conducted two secondary analyses to examine alternative methods of accounting for gestational age at bloodwork (see online supplementary material). The first method used continuous normalised score (Z-scores) as dependent variable. Z-scores were calculated for each participant by subtracting the mean and dividing by the SD of homocysteine concentration of all participants with the same gestational week at the time of bloodwork. The second method used a dichotomous outcome, with participants classified as having homocysteine concentration greater than the 90th percentile at each gestational week at the time of bloodwork. Similar cut-offs for elevated homocysteine have been used in studies investigating the effect of elevated homocysteine concentration on pregnancy outcomes[5,30–32]. The multivariable analysis was repeated for each version of the outcome. Model building followed the same procedures as described for the primary approach.

Results

Participant characteristics

We analysed data of 7587 participants from the OaK Birth Cohort (Fig. 1). Table 1 presents the characteristics of the studied population in terms of demographic characteristics, health indicators and factors associated with homocysteine metabolism. The majority of participants were non-smokers during pregnancy, reported taking folic acid-containing supplements at the time of recruitment, had a normal to

https://doi.org/10.1017/S1368980019004002 Published online by Cambridge University Press

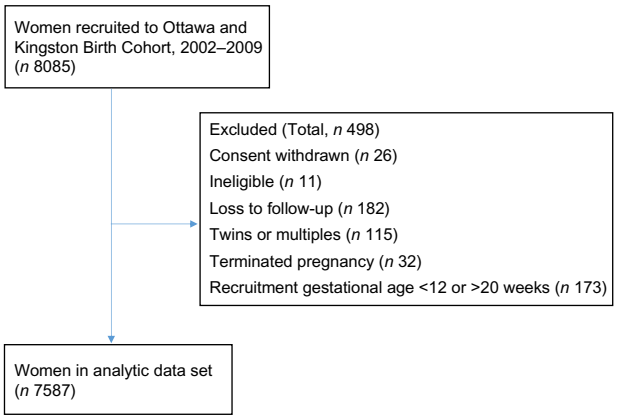

Fig. 1 (colour online) Participant flow diagram for the analytic dataset

overweight BMI and were normotensive and non-diabetic. Most participants were recruited around 12–13 weeks gestation and had a mean homocysteine concentration of 4·8 μmol/l (SD 1·3), with measured values ranging from 1 to 34 μmol/l. The MTHFR 677C>T genotype frequencies were not significantly different from Hardy–Weinberg equilibrium[29]. Around 12% of participants were homozygous for the TT mutant genotype.

Folic acid supplementation dose was categorised as 0–1 or >1 mg. We found a linear relation between serum folate and reported dosage up to approximately 1 mg, corresponding to a serum folate concentration of 45 nmol/l, above which serum folate was less responsive to increasing folic acid dose (Fig. 2). The serum folate distribution was wide and skewed; to normalise the distribution, a ceiling was set to the 90th percentile, ranging from 76 nmol/l at 12 weeks gestation to 48 nmol/l at 20 weeks. Although a serum folate deficiency cut-off in pregnant women has not been established, the WHO sets the cut-off for serum folate deficiency based on homocysteine concentration as a metabolic indicator at 10 nmol/l[33]. We, therefore, considered the 10 % of values beyond the 90th percentile cut-points as equally high and differentiating between these high values as unnecessary.

Determinants of homocysteine

Multivariable regression analysis

A variable clustering algorithm revealed that maternal education and household income were highly correlated; income was, therefore, dropped from the analysis because education was more strongly associated with homocysteine. The results for the primary multivariable regression analysis are presented in Table 2. All factors were significantly associated with plasma homocysteine concentration, except for maternal education, diabetes and hormonal birth control prior to conception (Table 2). Factors significantly associated with a higher homocysteine concentration were nulliparity, smoking during pregnancy (including exposure to second-hand smoke) and chronic hypertension. Factors significantly associated with lower homocysteine concentration were non-Caucasian race, history of a placenta-mediated (i.e., vascular-related) pregnancy complication and folic acid supplementation (Table 2). Inspection of residuals revealed a symmetrical distribution with two obvious outliers. These outliers were retained in the analysis because excluding them revealed no major differences in Wald tests of most meaningful hypotheses' *P* values and the error terms (results not shown).

Figure 3 shows plots of associations for continuous factors modelled using a restricted cubic spline function. For gestational age (five knots), the plot of association demonstrated a peak in homocysteine concentration close to 13 weeks gestation and a decrease thereafter. Plots of modelled association for maternal age and BMI (three knots) demonstrated a U-shaped pattern of increasing homocysteine concentration. For serum folate (five knots), the plot of modelled association demonstrated higher homocysteine concentration (i.e., a negative slope) at serum folate concentrations below approximately 30 nmol/l (Fig. 3).

Subgroup analysis

The subgroup analysis examining the interaction between MTHFR 677C>T genotype and serum folate is presented in Supplemental Table S1. In a subset of 4006 OaK participants with measured genotype, the interaction between MTHFR 677C>T genotype and serum folate was significant ($P < 0{\cdot}0001$). For the CC/CT genotypes, there was no association between serum folate and plasma homocysteine, whereas for the TT genotype, the association with homocysteine was a steep negative slope, which levelled off beyond a folate concentration of approximately 30 nmol/l (Fig. 4).

Folic acid supplementation was not associated with homocysteine in the subgroup analysis (see online supplementary material, Supplemental Table S1). As well, African race (compared to Caucasian) and exposure to second-hand smoke were no longer associated with homocysteine concentration. Plots of association of gestational age at bloodwork, maternal age and BMI modelled using restricted cubic spline functions all demonstrated similar patterns of association with homocysteine concentration, as in the primary multivariable analysis (see online supplementary material, Supplemental Fig. S1).

Secondary analyses

In the analysis with the dependent variable specified as a continuous Z-score (calculated from the mean homocysteine concentration and SD for each gestational week of recruitment), results were similar to those in the primary analysis (see online supplementary material, Supplemental Table S2). In the analysis with the dependent variable specified as a dichotomous variable (homocysteine concentration greater than the 90th percentile for each gestational week of

https://doi.org/10.1017/S1368980019004002 Published online by Cambridge University Press

Table 1 Participant characteristics and determinants of interest in the OaK Birth Cohort (*n* 7587), 2002–2009

Variable	Frequency	
	n	%
Demographic characteristics		
Age (years)		
Mean	30·3	
SD	5·06	
Race (missing/unknown, *n* 415, 5·5 %)		
African	152	2·12
Middle Eastern	224	3·12
Asian	422	5·88
Caucasian	6250	87·1
Other	124	1·72
College/university completed (missing, *n* 7, 0·09 %)		
Yes	5711	75·3
No	1869	24·6
Household income in CAD (missing, *n* 485, 6·4 %)		
<$50 000	1603	22·6
$50 000–<80 000	5499	77·4
Health indicators		
BMI (missing, *n* 136, 1·8 %)		
Mean	24·9	
SD	5·49	
Diabetes (missing, *n* 61, 0·80 %)		
Yes	115	1·53
No	7411	98·5
Chronic hypertension (missing, *n* 64, 0·84 %)		
Yes	91	1·21
No	7432	98·8
Pregnancy characteristics		
Nulliparous (missing, *n* 738, 9·7 %)		
Yes	3059	44·7
No	3790	55·3
Smoking during pregnancy (missing, *n* 26, 0·34 %)		
No	6714	88·8
Second-hand exposure	148	1·96
Medium/light smoker (<10 cigarettes per day)	449	5·93
Heavy smoker (≥10 cigarettes per day)	250	3·31
Hormonal birth control prior to conception (missing, *n* 45, 0·59 %)		
Yes	2749	36·6
No	4783	63·4
History of a placenta-mediated complication (pre-eclampsia, placental abruption, SGA, pregnancy loss) (missing, *n* 4, 0·053 %)		
Yes	794	10·5
No	6789	89·5
Any folic acid supplementation (folic acid alone or from prenatal vitamin or from multivitamins) (missing, *n* 1, 0·01 %)		
Yes	7184	94·7
No	402	5·30
Folic acid dose (mg) (missing, *n* 242, 3·19 %)		
0	402	5·47
>0 and ≤1	5876	80·0
>1	1067	14·5
Homocysteine metabolism		
Gestational age at bloodwork (weeks)		
Mean	13·7	
SD	2·09	
Homocysteine (μmol/l) (missing, *n* 87, 1·1 %)		
Mean	4·8	
SD	1·3	
Range	1–34	
Serum folate (nmol/l) (missing, *n* 987, 13·0 %)		
Median	37·4	
IQR	30·6–45·1	
Range	3·70–79·6	
MTHFR genotype*	*n* 4006	
CC (wild-type)	1768	44·1
CT (heterozygous)	1760	43·9
TT (homozygous)	478	11·9

*Measured in a subset of participants (*n* 4006).

https://doi.org/10.1017/S1368980019004002 Published online by Cambridge University Press

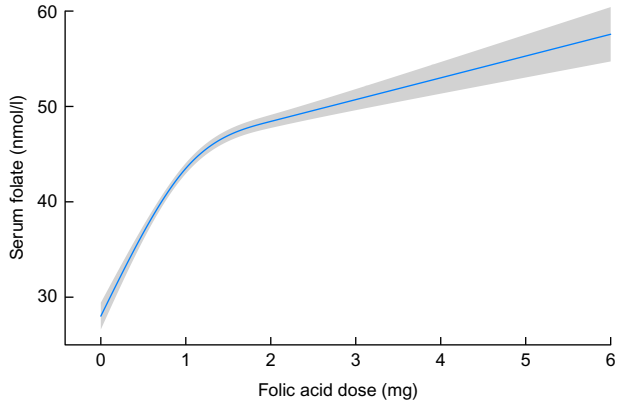

Fig. 2 (colour online) Modelled association between folic acid supplementation (restricted cubic spline with five knots) and serum folate, adjusting for gestational age at bloodwork and homocysteine. Shaded area represents 95 % CI

recruitment), there were some differences in results, presented as ORs and 95 % CIs in Supplemental Table S3. Maternal age and history of a placenta-mediated pregnancy complication were no longer associated with homocysteine concentration. Gestational age at bloodwork was significant despite gestational week at bloodwork being factored into the outcome.

Discussion

Using data on 7587 participants from the OaK Birth Cohort, a folic acid-fortified study population, we investigated the determinants of maternal plasma homocysteine concentration in the early second trimester of pregnancy. Factors associated with maternal homocysteine were demographic characteristics (age and race), health indicators (BMI and

Table 2 Multivariable linear regression analysis of the determinants of plasma homocysteine, with plasma homocysteine as a continuous dependent variable (*n* 7587)

Variable	Effect	95 % CI	*P*-value*
Demographic characteristics			
Age (rcs, three knots)			0·0013
34 *v.* 27 years	−0·027	−0·070, 0·016	
Race			<0·0001
Caucasian	Reference		
African	−0·370	−0·561, −0·179	
Middle Eastern	−0·438	−0·594, −0·282	
Asian	−0·468	−0·588, −0·349	
Other	−0·405	−0·612, −0·197	
Education			0·3987
College/university completed *v.* less than completed	−0·032	−0·105, 0·042	
Health indicators			
BMI (rcs, three knots)			0·0103
27·3 *v.* 21·1 kg/m^2	−0·070	−0·119, −0·022	
Diabetes			0·6197
Yes *v.* no	−0·056	−0·279, 0·166	
Chronic hypertension			0·0001
Yes *v.* no	0·502	0·250, 0·754	
Pregnancy characteristics			
Nulliparous			<0·0001
Yes *v.* no	0·140	0·073, 0·207	
Smoking			<0·0001
No	Reference		
Second-hand exposure	0·298	0·097, 0·499	
Med/light smoker (<10 cigarettes per day)	0·418	0·297, 0·539	
Heavy smoker (≥10 cigarettes per day)	0·864	0·705, 1·022	
Hormonal birth control prior to conception			0·1747
No	Reference		
Oral	−0·046	−0·108, 0·015	
Injection or IUD	0·091	−0·088, 0·270	
History of PMC (pre-eclampsia, placental abruption, SGA, stillbirth/loss)			0·0078
Yes *v.* no	−0·124	−0·215, −0·033	
Folic acid supplementation			<0·0001
None	Reference		
>0 and ≤1 mg	−0·310	−0·442, −0·178	
>1 mg	−0·329	−0·476, −0·181	
Homocysteine metabolism			
Gestational age at bloodwork (rcs, five knots)			<0·0001
13·4 *v.* 12·4 weeks	0·016	−0·063, 0·095	
Serum folate (rcs, five knots)			<0·0001
45·1 *v.* 30·6 nmol/l	0·137	0·065, 0·210	

rcs, restricted cubic spline; PMC, placenta-mediated complication.
*Wald test of most meaningful hypotheses, pooled across multiple imputation datasets.

https://doi.org/10.1017/S1368980019004002 Published online by Cambridge University Press

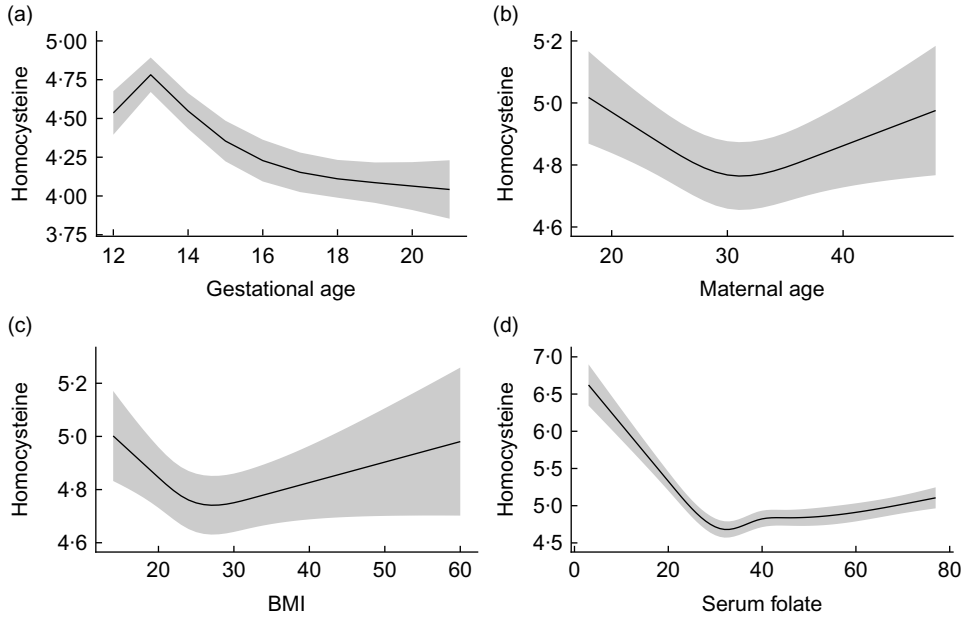

Fig. 3 Association between homocysteine and continuous variables modelled as a restricted cubic spline. (a) Gestational age at bloodwork with five knots at 12·1, 12·4, 12·8, 13·4 and 19; (b) maternal age with three knots at 24, 30 and 37; (c) BMI with three knots at 19·6, 23·5 and 32·2; and (d) serum folate with five knots at 20·7, 32·3, 39·4, 45 and 74·1. Shaded area represents 95 % CI

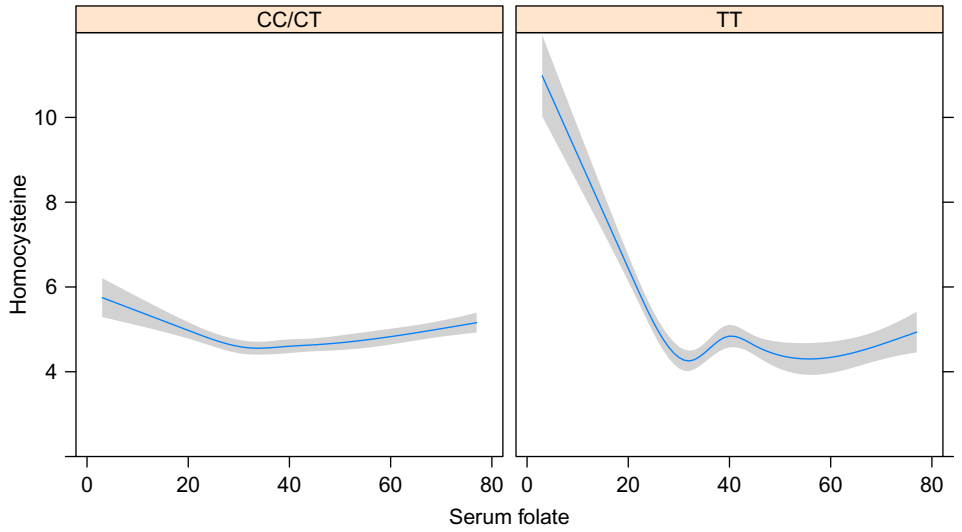

Fig. 4 (colour online) Modelled association between serum folate (restricted cubic spline) and homocysteine by MTHFR 677C>T genotype. Panels by MTHFR genotype, CC (wild-type) or CT (heterozygous) and TT (homozygous mutant). Folate variable has five knots at 20·7, 32·3, 39·4, 45 and 74·1. Shaded area represents 95 % CI

chronic hypertension), pregnancy characteristics (smoking, history of a placenta-mediated pregnancy complication and folic acid supplementation) and an important factor related to homocysteine metabolism (serum folate). Serum folate modified the effect of the MTHFR 677C>T genotype on plasma homocysteine concentration. The pattern in which these factors were associated with either higher or lower homocysteine concentration has practical implications for the lowering of homocysteine in women of reproductive age.

Our findings support the method of analysing homocysteine as a continuous variable. The traditional approach of dichotomising homocysteine concentration at a percentile cut-off relative to gestational age at measurement led to some differences in results compared to converting homocysteine to a Z-score or analysing homocysteine as a continuous variable while adjusting for gestational age in the multivariable model. Disadvantages associated with categorising continuous variables for analysis include bias and decreased precision; nevertheless, categorising is often

https://doi.org/10.1017/S1368980019004002 Published online by Cambridge University Press

driven by simplicity and clinical interpretability[34–36]. With no agreed-upon cut-off for elevated homocysteine during pregnancy, the use of data-derived cut-offs is additionally problematic because different distributions produce different cut-offs, limiting comparability between studies[34].

Interpretation

Our findings of demographic, health, pregnancy-related and metabolic (including genetic) determinants of plasma homocysteine in pregnant women agree with findings from previous studies[9,37,38]. The Hordaland Homocysteine study investigated the association of cardiovascular risk factors with non-fasting homocysteine. It is the largest study in the general population on homocysteine determinants; approximately 8000 men and 8000 women aged 40–67 years were recruited in Norway from 1992 to 1993. The strongest determinants of homocysteine were age, cigarette smoking dosage (stronger effects in women) and vitamin intake score[38]. The Generation R study also investigated factors associated with early-second-trimester homocysteine in a cohort of 5085 participants recruited in the Netherlands from 2002 to 2006[4]. Smoking, comorbidity, lower education and Caucasian ethnicity were all associated with higher homocysteine. In our adjusted analyses, we found that education level was not associated with homocysteine, which suggests that the effects of adequate folate intake and healthy behaviours can transcend socioeconomic status.

Maternal age and BMI were significant determinants of homocysteine in a U-shaped relation. We also found that nulliparity was associated with higher homocysteine, as did Bergen *et al.*[4], and this may be due to nullipara participants being younger than multipara. A U-shaped relation of homocysteine with age and BMI has been reported in pregnant women[4] but not in the general population[9,38]. A U-shaped association for BMI may be linked to physical activity; in the Hordaland study, BMI modified the effect of physical activity on homocysteine such that participants with lower BMI had an inverse relation between physical activity and homocysteine, whereas participants with a higher BMI had a positive relation between physical activity and homocysteine[38].

Homocysteine and folate distributions in the OaK cohort were similar to another Canadian birth cohort that recruited participants in the same time period[5], but the distributions of homocysteine and folate were lower and higher, respectively, than a European cohort[4]. Despite the lower distribution of homocysteine, our results were in agreement with other studies, including large meta-analyses, in showing a moderating effect of serum folate with the MTHFR 677C>T polymorphism in determining homocysteine concentration[13,14,39]. Our subgroup analyses found that folic acid supplementation was no longer associated with homocysteine concentration; this finding was likely due to the smaller available sample size for the MTHFR subgroup analysis.

Our study demonstrated that continued focus on increasing folate status during pregnancy, particularly via folic acid supplementation >1 mg/d, may no longer result in substantially higher folate and, therefore, lower homocysteine concentration. A Canadian open-label folic acid trial demonstrated that folate concentrations do not reach a steady state during pregnancy[40], but folic acid supplementation during pregnancy had not demonstrated a substantial benefit to reduce pregnancy outcomes such as preterm birth, stillbirth, low birthweight and neonatal death[41]. The multicentre folic acid clinical trial (FACT) found no benefit of high-dose folic acid (i.e., 4 mg daily) taken after the first trimester on the risk of pre-eclampsia in high-risk women[42]. Folate intake has, however, been identified among the major determinants of plasma homocysteine[9,43]; in the Hordaland study, a folate intake score was validated against plasma folate in a subsample of study participants. With or without additional changes, preconception folic acid intake has also demonstrated short-term benefits; in a Norwegian population-based cohort study, periconceptional folic acid intake modified the effect of continued smoking during pregnancy on homocysteine concentration in the first trimester[44].

A number of studies conducted in the post-folate fortification era in Canada and the USA demonstrated folate-replete populations[15,45–47]. Based on current evidence, WHO recommends population-level RBC folate concentrations >906 nmol/l in women of childbearing age to protect against neural tube defects[48]. A comparison of mean RBC folate concentrations in women aged 15–44 years in NHANES III (1988–1994) and NHANES 1999 demonstrated a significant increase 3 years after mandatory folic acid fortification of cereal grains was introduced[46]. However, the Canadian Health Measures Survey (CHMS) cycle 2007–9 found that although 1 % of the population-based sample was folate-deficient, 22 % of women aged 15–45 years had suboptimal RBC folate status <906 nmol/l[49]. In a more recent analysis of 1035 physician-ordered tests of RBC folate concentrations in Toronto, Canada, 7 % of women aged 15–45 years had suboptimal folate status[15].

Our study indicated that despite improvements in folate levels through fortification, women with the MTHFR 677C>T polymorphism are one subgroup that is susceptible to low folate and high homocysteine. A population-based folic acid trial in approximately 900 Northern Chinese women of reproductive age, who had no other source of folic acid and were not anaemic or vitamin B_{12}-deficient, found that folic acid dose did not significantly change the effect of the MTHFR variant. Throughout 6 months of supplementation and 3 months of discontinuation, the TT genotype was associated with response (i.e., folate and homocysteine concentrations) to the highest administered folic acid doses of 400 and 4000 μg/d[50].

Women taking hormonal contraceptives are another subgroup of women possibly susceptible to low folate status. A recent systematic review and meta-analysis found a

https://doi.org/10.1017/S1368980019004002 Published online by Cambridge University Press

significant RBC and serum folate-lowering effect of oral contraceptive use[51]. However, we found that hormonal contraceptive use prior to conception was not a determinant of homocysteine in the early second trimester of pregnancy. Although homocysteine was measured in the second trimester in OaK participants, introducing a gap in time from ceasing oral contraceptive use to homocysteine measurement, hormonal contraceptive use may be linked to a higher likelihood of planned pregnancy and, therefore, preconception folic acid supplementation. Preconception folic acid supplementation was shown to be associated with lower homocysteine in pregnant women[4,5]. Thus, improved folate intake in the preconception period could offset the folate-lowering effects of hormonal contraceptives.

In the Hordaland study, a 6-year follow-up of participants found reductions in homocysteine concentration associated with increased folate and vitamin B_{12} concentration, quitting smoking and weight loss[52]. This lowering of homocysteine may additionally explain our finding that history of a placenta-mediated complication was consistently associated with decreased homocysteine concentration, which appears contrary to findings of elevated homocysteine in women with a history of vascular-related pregnancy complications[3,53]. It is plausible that women who previously experienced pregnancy complications were more likely to make favourable lifestyle or nutritional changes or have been prescribed multivitamins or folic acid, which may have contributed to a lower homocysteine concentration.

Folic acid trials in women of childbearing age have demonstrated that plasma homocysteine and serum folate tend to return to baseline levels after discontinuing supplementation. In the folic acid trial in Northern Chinese women, homocysteine concentration decreased 17 % with a folic acid dose of 400 μg/d, and approximately 22 % with a dose of 4000 μg/d. However, 3 months after cessation, the effects of intervention were diminished[50]. Similarly, in a trial of twenty-seven Dutch women, 500 μg of folic acid was administered for 8 weeks; although homocysteine and folate reached a steady state, RBC folate did not. Moreover, 8 weeks after discontinuation, homocysteine and folate returned to baseline levels[54].

Strengths and limitations

To our knowledge, this is the largest comprehensive study of homocysteine determinants in pregnant women. We used prospective data on second-trimester maternal plasma homocysteine. Our analysis used multiple imputation to deal with missing data and included a wide range of potential determinants. We analysed continuous variables using flexible parametric forms, which allowed for possible non-linear associations with homocysteine.

Although we accounted for a range of potential determinants, we did not have data on physical activity and caffeine consumption, which are among other determinants of homocysteine identified in population-based studies and in studies of pregnant women[43,55]. Additionally, non-fasting blood samples were collected from OaK participants. Although fasting affects folate concentration, fasting for varying lengths of time has demonstrated no measurable effect on homocysteine concentration[37].

Conclusion

Research is on-going into the role of homocysteine in the development of CVD and vascular-related pregnancy complications. Our findings of the determinants of maternal plasma homocysteine are especially relevant to the lowering of homocysteine in women of childbearing age in the post-folic acid fortification era, which is characterised by folate-replete populations. Clinical trials may point to a benefit of preconception folic acid and/or multivitamin intake on the risk of pregnancy complications. Periconceptional uptake of folic acid is suboptimal worldwide, including in Canada[56–63]. Therefore, promoting timely uptake in the wider population of reproductive-age women is necessary in addition to targeting at-risk groups. A targeted approach based on factors associated with higher homocysteine may prove beneficial. This includes demographic characteristics (younger or older age and considering race or ethnic background), health indicators (lower or higher BMI and chronic conditions such as hypertension) and pregnancy-related factors such as smoking status. Thus, in the post-folic acid fortification era, a focus on periconceptional folic acid supplementation in addition to other modifiable factors – for example, healthy weight and lifestyle choices and other nutritional factors such as vitamin B_{12} intake – may be an effective approach to lower maternal homocysteine concentration over the long term[9,64].

Acknowledgements

Acknowledgements: Shazia Chaudhry is recipient of a PhD scholarship from the Canadian Institutes of Health Research–Quebec Training Network in Perinatal Research (CIHR-QTNPR). *Financial support:* This study was supported by grants from the CIHR, grants MOP 53188 and 82802, and FDN 148438. CIHR had no role in the design, analysis or writing of this article. *Conflict of interest:* None. *Authorship:* S.W., M.W., R.R.W., G.S. and M.R. are lead investigators of the OaK Birth Cohort. S.C., S.W. and M.T. designed the study. S.C. analysed patient data and wrote the draft manuscript. S.C., S.W., M.T. and A.M. were involved in data interpretation. L.G., M.W., R.R.W., G.S. and M.R. critically revised the manuscript and contributed to the final version. All authors read and approved the final manuscript. *Ethics of human subject participation:* The OaK Birth Cohort

https://doi.org/10.1017/S1368980019004002 Published online by Cambridge University Press

study was conducted according to the guidelines laid down in the Declaration of Helsinki, and all procedures involving research study participants were approved by the Ottawa Health Science Network Research Ethics Board (OHSN-REB), formerly the Ottawa Hospital Research Ethics Board (protocol numbers 2002343 and 2007034). Participants' written informed consent was sought for participation in the cohort study as well as for banking maternal blood, cord blood and contact for long-term follow-up. Ethics approval for secondary analyses of the OaK Birth Cohort was obtained from the OHSN-REB on 31 May 2016 (protocol 20160163-01H).

Supplementary material

For supplementary material accompanying this paper visit https://doi.org/10.1017/S1368980019004002.

References

1. The Homocysteine Studies Collaboration (2002) Homocysteine and risk of ischemic heart disease and stroke: a meta-analysis. *JAMA J Am Med Assoc* **288**, 2015–2023.
2. Wald DS, Morris JK & Wald NJ (2011) Reconciling the evidence on serum homocysteine and ischaemic heart disease: a meta-analysis. *PLoS One* **6**, e16473.
3. Vollset SE, Refsum H, Irgens LM *et al.* (2000) Plasma total homocysteine, pregnancy complications, and adverse pregnancy outcomes: the Hordaland Homocysteine study. *Am J Clin Nutr* **71**, 962–968.
4. Bergen NE, Jaddoe VW, Timmermans S *et al.* (2012) Homocysteine and folate concentrations in early pregnancy and the risk of adverse pregnancy outcomes: the Generation R Study. *BJOG* **119**, 739–751.
5. Dodds L, Fell DB, Dooley KC *et al.* (2008) Effect of homocysteine concentration in early pregnancy on gestational hypertensive disorders and other pregnancy outcomes. *Clin Chem* **54**, 326–334.
6. Mignini LE, Latthe PM, Villar J *et al.* (2005) Mapping the theories of preeclampsia: the role of homocysteine. *Obstet Gynecol* **105**, 411–425.
7. Hogeveen M, Blom HJ & den Heijer M (2012) Maternal homocysteine and small-for-gestational-age offspring: systematic review and meta-analysis. *Am J Clin Nutr* **95**, 130–136.
8. Refsum H, Nurk E, Smith AD *et al.* (2006) The Hordaland Homocysteine Study: a community-based study of homocysteine, its determinants, and associations with disease. *J Nutr* **136**, Suppl. 6, 1731S–1740S.
9. Jacques PF, Bostom AG, Wilson PWF *et al.* (2001) Determinants of plasma total homocysteine concentration in the Framingham Offspring cohort. *Am J Clin Nutr* **73**, 613–621.
10. Clarke R, Halsey J, Lewington S *et al.* (2010) Effects of lowering homocysteine levels with B vitamins on cardiovascular disease, cancer, and cause-specific mortality: meta-analysis of 8 randomized trials involving 37 485 individuals. *Arch Intern Med* **170**, 1622–1631.
11. Martí-Carvajal AJ, Solà I & Lathyris D (2017) Homocysteine-lowering interventions for preventing cardiovascular events. *Cochrane Database Syst Rev* **8**, CD006612.
12. Sayyah-melli M, Ghorbanihaghjo A, Alizadeh M *et al.* (2016) The effect of high dose folic acid throughout Pregnancy on Homocysteine (Hcy) concentration and pre-eclampsia: a randomized clinical trial. *PLoS One* **11**, e0154400.
13. Holmes MV, Newcombe P, Hubacek JA *et al.* (2011) Effect modification by population dietary folate on the association between MTHFR genotype, homocysteine, and stroke risk: a meta-analysis of genetic studies and randomised trials. *Lancet* **378**, 584–594.
14. Clarke R, Bennett DA, Parish S *et al.* (2012) Homocysteine and coronary heart disease: meta-analysis of MTHFR case-control studies, avoiding publication bias. *PLoS Med* **9**, e1001177.
15. Shere M, Kapur BM & Koren G (2015) Folate status of women in Toronto: implications of folate fortification and supplementation. *Can J Public Health* **106**, e509–e513.
16. Dasarathy J, Gruca LL, Bennett C *et al.* (2010) Methionine metabolism in human pregnancy. *Am J Clin Nutr* **91**, 357–365.
17. Blom HJ & Smulders Y (2011) Overview of homocysteine and folate metabolism. With special references to cardiovascular disease and neural tube defects. *J Inherit Metab Dis* **34**, 75–81.
18. Salway JG (1999) *Metabolism at a Glance*. Malden, MA: Blackwell Science Publishing.
19. Sibani S, Leclerc D, Weisberg IS *et al.* (2003) Characterization of mutations in severe methylenetetrahydrofolate reductase deficiency reveals an FAD-responsive mutation. *Hum Mutat* **21**, 509–520.
20. Murphy MM, Scott JM, McPartlin JM *et al.* (2002) The pregnancy-related decrease in fasting plasma homocysteine is not explained by folic acid supplementation, hemodilution, or a decrease in albumin in a longitudinal study 1–3. *Am J Clin Nutr* **76**, 614–619.
21. Walker MC, Smith GN, Perkins SL *et al.* (1999) Changes in homocysteine levels during normal pregnancy. *Am J Obstet Gynecol* **180**, 660–664.
22. Walker MC, Finkelstein SA, Rennicks White R *et al.* (2011) The Ottawa and Kingston (OaK) Birth Cohort: development and achievements. *J Obstet Gynaecol Canada* **33**, 1124–1133.
23. Selhub J, Jacques PF, Rosenberg IH *et al.* (1999) Serum total homocysteine concentrations in the third National Health and Nutrition Examination survey (1991–1994): population reference ranges and contribution of vitamin status to high serum concentrations. *Ann Intern Med* **131**, 331–339.
24. Kahn SR, Platt R, McNamara H *et al.* (2009) Inherited thrombophilia and preeclampsia within a multicenter cohort: the Montreal Preeclampsia study. *Am J Obstet Gynecol* **200**, 151.e1–151.e9.
25. van Buuren S & Groothuis-oudshoorn K (2011) Mice: multivariate imputation by chained equations in R. *J Stat Softw* **45**, 1–67.
26. Harrell FE Jr (2016) Rms: Regression Modeling Strategies. *R Packag version 4.5-0*. https://cran.r-project.org/package=rms (accessed January 2019).
27. Harrell FE (2015) *Regression Modeling Strategies with Applications to Linear Models, Logistic and Ordinal Regression, and Survival Analysis*, 2nd ed. Springer Series in Statistics. New York: Springer.
28. Harrell FE Jr & With contributions from Charles Dupont and many others (2016) Hmisc: Harrell Miscellaneous. *R Packag version 3.17-4*. https://cran.r-project.org/package=Hmisc (accessed January 2019).
29. Graffelman J (2015) Exploring diallelic genetic markers: the HardyWeinberg package. *J Stat Softw* **63**, 1–22.
30. Sorensen TK, Malinow MR, Williams MA *et al.* (1999) Elevated second-trimester serum homocyst(e)ine levels and subsequent risk of preeclampsia. *Gynecol Obstet Invest* **48**, 98–103.
31. Polat M, Biberoglu EH, Guler I *et al.* (2016) Coexistence of preeclampsia and inherited thrombophilia in Turkish pregnant women. *Turkish J Med Sci* **46**, 1094–1100.

32. Onalan R, Onalan G, Gunenc Z *et al.* (2006) Combining 2nd-trimester maternal serum homocysteine levels and uterine artery Doppler for prediction of preeclampsia and isolated intrauterine growth restriction. *Gynecol Obstet Invest* **61**, 142–148.
33. World Health Organization (2012) Serum and red blood cell folate concentrations for assessing folate status in populations. *Vitamin and Mineral Nutrition Information System*. Document: WHO/NMH/NHD/EPG/15.01. http://apps.who.int/iris/bitstream/10665/75584/1/WHO_NMH_NHD_EPG_12.1_eng (accessed January 2019).
34. Bernette C & Vickers A (2012) Against quantiles: categorization of continuous variables in epidemiologic research, and its discontents. *BMC Med Res Methodol* **12**, 21.
35. Fedorov V, Mannino F & Zhang R (2009) Consequences of dichotomization. *Pharm Stat* **8**, 50–61.
36. Royston P, Altman DG & Sauerbrei W (2006) Dichotomizing continuous predictors in multiple regression: a bad idea. *Stat Med* **25**, 127–141.
37. Jacques PF, Rosenberg IH, Rogers G *et al.* (1999) Serum total homocysteine concentrations in adolescent and adult Americans: results from the third National Health and Nutrition Examination survey. *Am J Clin Nutr* **69**, 482–489.
38. Nygard O, Vollset SE, Refsum H *et al.* (1995) Total plasma homocysteine and cardiovascular risk profile. The Hordland homocysteine study. *JAMA* **274**, 1526–1533.
39. Jin H, Cheng H, Chen W *et al.* (2018) An evidence-based approach to globally assess the covariate-dependent effect of the MTHFR single nucleotide polymorphism rs1801133 on blood homocysteine: a systematic review and meta-Analysis. *Am J Clin Nutr* **107**, 817–825.
40. Shere M, Nguyen P, Tam C *et al.* (2015) Pregnancy-induced changes in the long-term pharmacokinetics of 1.1 mg vs. 5 mg folic acid: a randomized clinical trial. *J Clin Pharmacol* **55**, 159–167.
41. Lassi ZS, Salam RA, Haider BA *et al.* (2013) Folic acid supplementation during pregnancy for maternal health and pregnancy outcomes. *Cochrane Database Syst Rev* **3**, CD006896.
42. Wen SW, White RR, Rybak N *et al.* (2018) Effect of high dose folic acid supplementation in pregnancy on pre-eclampsia (FACT): double blind, phase III, randomised controlled, international, multicentre trial. *BMJ* **362**, k3478.
43. Nygård O, Refsum H, Ueland PM *et al.* (1998) Major lifestyle determinants of plasma total homocysteine distribution: the Hordaland Homocysteine Study. *Am J Clin Nutr* **67**, 263–270.
44. Bakker R, Timmermans S, Steegers EAP *et al.* (2011) Folic acid supplements modify the adverse effects of maternal smoking on fetal growth and neonatal complications 1–3. *J Nutr* **141**, 2172–2179.
45. Joelson DW, Fiebig EW & Wu AHB (2007) Diminished need for folate measurements among indigent populations in the post folic acid supplementation era. *Arch Pathol Lab Med* **131**, 477–480.
46. CDC: National Center for Health Statistics; National Center for Chronic Disease Prevention and Health Promotion; and National Center for Environmental Health (2000) *Folate status in women of childbearing age–United States, 1999. Morb Mortal Wkly Rep* **49**, 962–965.
47. Caudill MA, Le T, Moonie SA *et al.* (2001) Folate status in women of childbearing age residing in Southern California after folic acid fortification. *J Am Coll Nutr* **20**, 129–134.
48. WHO (2015) *Guideline: Optimal Serum and Red Blood Cell Folate Concentrations in Women of Reproductive Age for Prevention of Neural Tube Defects*. Geneva: World Health Organization.
49. Colapinto CK, O'Connor DL & Tremblay MS (2011) Folate status of the population in the Canadian Health Measures survey. *Can Med Assoc J* **183**, E100–E106.
50. Crider KS, Zhu J, Hao L *et al.* (2011) MTHFR 677C>T genotype is associated with folate and homocysteine concentrations in a large, population-based, double-blind trial of folic acid supplementation. *Am J Clin Nutr* **93**, 1365–1372.
51. Shere M, Bapat P, Nickel C *et al.* (2015) Association between use of oral contraceptives and folate status: a systematic review and meta-analysis. *J Obstet Gynaecol Canada* **37**, 430–438.
52. Nurk E, Tell GS, Vollset SE *et al.* (2004) Changes in lifestyle and plasma total homocysteine: the Hordaland Homocysteine Study. *Am J Clin Nutr* **79**, 812–819.
53. Visser S, Hermes W, Ket JCF *et al.* (2014) Systematic review and metaanalysis on nonclassic cardiovascular biomarkers after hypertensive pregnancy disorders. *Am J Obstet Gynecol* **211**, 373.e1–373.e9.
54. Bakker DJ, de Jong-van den Berg LTW & Fokkema MR (2009) Controlled study on folate status following folic acid supplementation and discontinuation in women of child-bearing age. *Ann Clin Biochem* **46**, 231–234.
55. Shiraishi M, Haruna M, Matsuzaki M *et al.* (2014) Relationship between plasma total homocysteine level and dietary caffeine and vitamin B6 intakes in pregnant women. *Nurs Health Sci* **16**, 164–170.
56. Ray J, Singh G & Burrows S (2004) Evidence for suboptimal use of periconceptual folic acid supplements globally. *BJOG* **111**, 399–408.
57. Tam LE, McDonald SD, Wen SW *et al.* (2005) A survey of periconceptional folic acid use in a group of Canadian women. *J Obstet Gynaecol Canada* **27**, 232–236.
58. McNulty B, Pentieva K, Marshall B *et al.* (2011) Women's compliance with current folic acid recommendations and achievement of optimal vitamin status for preventing neural tube defects. *Hum Reprod* **26**, 1530–1536.
59. Richard-Tremblay AA, Sheehy O, Audibert F *et al.* (2012) Concordance between periconceptional folic acid supplementation and Canadian clinical guidelines. *J Popul Ther Clin Pharmacol* **19**, 150–159.
60. Nelson CRM, Leon JA & Evans J (2014) The relationship between awareness and supplementation: which Canadian women know about folic acid and how does that translate into use? *Can J Public Heal* **105**, e40–e46.
61. Nilsen RM, Leoncini E, Gastaldi P *et al.* (2016) Prevalence and determinants of preconception folic acid use: an Italian multicenter survey. *Ital J Pediatr* **42**, 1–10.
62. Yamamoto S & Wada Y (2018) Awareness, use and information sources of folic acid supplementation to prevent neural tube defects in pregnant Japanese women. *Public Health Nutr* **21**, 732–739.
63. Cawley S, Mullaney L, Kennedy R *et al.* (2017) Duration of periconceptional folic acid supplementation in women booking for antenatal care. *Public Health Nutr* **20**, 371–379.
64. MacFarlane AJ, Greene-finestone LS & Shi Y (2011) Vitamin B-12 and homocysteine status in a folate-replete population: results from the Canadian Health Measures Survey. *Am J Clin Nutr* **94**, 1079–1087.

https://doi.org/10.1017/S1368980019004002 Published online by Cambridge University Press