The association of homocysteine with placenta-mediated pregnancy complications

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ABSTRACT

Background

Preeclampsia, small for gestational age (SGA), placental abruption, and fetal death are pregnancy complications linked to the utero-placental vasculature with serious consequences for maternal and infant well-being. Elevated homocysteine, a marker of cardiovascular disease risk, is postulated to play a role in placenta-mediated complications, but epidemiologic studies have reported inconsistent findings. The two primary objectives of this thesis were to 1: comprehensively investigate the association of homocysteine with placenta-mediated complications and examine modifying effects of pre-specified factors on this association, and 2: comprehensively investigate determinants of maternal homocysteine during pregnancy.

Methods

A systematic review and meta-analysis of prospective studies was conducted to address thesis objective 1. The Ottawa and Kingston (OaK) Birth Cohort, a prospective cohort study that recruited pregnant women between 2002 and 2009, was used to address thesis objectives 1 and 2. Homocysteine concentration was measured between 12 and 20 weeks gestation. Analyses based on the OaK Birth Cohort consisted of multivariable regressions using restricted cubic splines to model associations with continuously distributed variables.

Results

Objective 1: In an analysis of 7587 participants, a significant association between homocysteine concentration and a composite outcome of any placenta-mediated complication was observed (odds ratio (OR) for a 5 µmol/L increase: 1.63, 95% Confidence Interval (CI) 1.23-2.16) and SGA (OR 1.76, 95% CI 1.25-2.46), with potential modifying effects of the methylene tetrahydrofolate reductase (MTHFR) 677C>T variant (SGA) and high-risk pregnancy (preeclampsia).

In the systematic review identifying 30 prospective cohort or nested case-control studies, a random effects meta-analysis of pooled mean differences in homocysteine between cases and controls in 28 studies revealed significantly higher means for SGA: 0.35 µmol/L (95% CI 0.19 to 0.51, I^2=33%); and preeclampsia: 0.87 µmol/L (95% CI 0.52 to 1.21, $I^{2=}$92%). Significant sources of heterogeneity were study region (SGA and preeclampsia), adjusting for covariates (preeclampsia), folate status (preeclampsia), and severity (preeclampsia).

Objective 2: In 7587 OaK participants, factors related to favourable health status were associated with lower maternal homocysteine concentrations. Folic acid supplementation during pregnancy of >1 mg/day did not substantially increase serum folate concentration.

Conclusion

This thesis suggests an independent effect of slightly higher homocysteine concentration in the early to mid-second trimester on the risk of any placenta-mediated complication, SGA, and preeclampsia. Modifying effects explain some of the variability in previous studies. Favourable preconception health status was associated with lower maternal homocysteine.

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PREFACE

Statement of originality

I, Shazia Chaudhry, confirm that this thesis is an original work by myself. I performed the research, conducted the analyses, wrote the draft manuscripts, and interpreted the data. The co-Authors of the manuscripts were involved in interpreting the data and critically revising the manuscripts. The thesis comprises three manuscripts:

- Chapter 4: *The role of maternal homocysteine concentration in placenta-mediated complications: findings from the Ottawa and Kingston birth cohort*. This manuscript was published in BMC (BioMed Central) Pregnancy and Childbirth: Chaudhry SH, Taljaard M, Macfarlane AJ, et al. The role of maternal homocysteine concentration in placenta-mediated complications: findings from the Ottawa and Kingston birth cohort. *BMC Pregnancy Childbirth*. 2019;19(75)
- Chapter 5: *The association of maternal homocysteine with placenta-mediated complications, A systematic review*. This manuscript will be submitted to the Journal Obstetrics & Gynecology.
- Chapter 6: *The determinants of maternal homocysteine in pregnancy*. This manuscript was submitted to the Journal Public Health Nutrition. The current status is revisions requested.

Interchangeable terms

Throughout the thesis the following terms may be used interchangeably:

- Small for gestational age (SGA), intrauterine growth restriction (IUGR), and fetal growth restriction
- Fetal death and pregnancy loss

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Ethics

Chapters 3, 4, and 6 are based on the Ottawa and Kingston (OaK) Birth Cohort. Ethics approval for the OaK Birth Cohort was obtained from 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 participating in the cohort study as well as banking maternal blood, banking 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).

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LIST OF ABBREVIATIONS

ACMG	American College of Medical Genetics and Genomics
ACOG	American College of Obstetricians and Gynecologists
AIC	Akaike's information criterion
AJCN	American Journal of Clinical Nutrition
BIC	Bayesian information criterion
BMI	Body mass index
CHD	Coronary heart disease
CI	Confidence Interval
CIHR	Canadian Institutes of Health Research
CVD	Cardiovascular disease
FCS	Fully conditional specification
GA	Gestational age
Hhcy	Hyperhomocysteinemia (i.e., elevated homocysteine)
HBC	Hormonal birth control
H_x	History of
IUGR	Intrauterine growth restriction
MAR	Missing at random
MCAR	Missing completely at random
MNAR	Missing not at random
MTHFR	Methylenetetrahydrofolate reductase
NA	Not applicable
NOS	Newcastle-Ottawa Scale
NR	Not reported
NT	No threshold

NTD	Neural tube defect
OaK	Ottawa and Kingston
OG	Obstetrics & Gynecology (Journal)
OR	Odds ratio
PCR	Polymerase chain reaction
PMC	Placenta-mediated complication
RBC	Red blood cell
rcs	Restricted cubic spline
RCT	Randomized controlled trial
RR	Risk ratio/ relative risk
SD	Standard deviation
SES	Socioeconomic status
SGA	Small for gestational age
SNP	Single nucleotide polymorphism
SOGC	Society of Obstetricians and Gynaecologists of Canada
WHO	World Health Organization

CHAPTER 1. BACKGROUND AND THESIS OBJECTIVES

Chapter 1 presents the framework of the research and thesis objectives. The Chapter begins with a brief background, followed by the rationale for the research and specific research objectives. The chapter-by-chapter organization of the thesis is outlined.

1.1 BACKGROUND

Preeclampsia, placental abruption, fetal growth restriction, and fetal death are pregnancy complications linked to the utero-placental vasculature.[1] These conditions, referred to as placenta-mediated complications, share a number of risk factors and pathophysiological mechanisms,[2] which signifies common origins.[3,4] Subsequent to findings in cardiovascular disease research, elevated maternal homocysteine concentration is postulated to play a role in alterations of the utero-placental vasculature.[5,6]

Homocysteine is an intermediate metabolite formed from the breakdown of the essential amino acid methionine. Elevated homocysteine concentration is associated with nutritional, lifestyle, and biological factors,[7–9] and with polymorphisms in genes encoding enzymes involved in homocysteine metabolism.[10] In particular, a relatively common polymorphism methylene tetrahydrofolate reductase (MTHFR) 677C>T produces an enzyme with reduced activity, which can lead to moderately elevated blood homocysteine concentration.[11,12] In healthy individuals, higher folate (i.e., Vitamin B_9) concentrations attenuate the effect of the MTHFR variant on homocysteine concentrations.[12,13] During pregnancy, maternal homocysteine concentrations change throughout gestation.[14]

Epidemiological studies have suggested an association between elevated maternal homocysteine and placenta-mediated complications, but results across studies were inconsistent.[15–20] The inconsistencies have limited the interpretation of the current evidence and require further investigation, which motivated this thesis.

1.2 RATIONALE AND OBJECTIVES

The first aim of this thesis was to comprehensively investigate the association of maternal homocysteine with placenta-mediated pregnancy complications, namely small for gestational age (SGA) infant, preeclampsia, placental abruption, and pregnancy loss. Due to the variability in findings among previous studies, we also sought to investigate potential modifiers of the associations. To investigate the associations of homocysteine with placenta-mediated complications, we conducted analyses based on the Ottawa and Kingston (OaK) Birth Cohort, which recruited approximately 8000 pregnant women in Ottawa and Kingston, Canada between 2002 and 2009 (Chapter 4), and we conducted a systematic review and meta-analysis (Chapter 5).

Because elevated homocysteine is implicated in the development of pregnancy complications linked to the utero-placental vasculature, we sought to identify factors amenable to the lowering of maternal homocysteine, to reduce the risk of placenta-mediated complications. The second aim of this thesis was therefore to investigate determinants of maternal homocysteine. We again conduced analyses using data from the OaK Birth Cohort, in which maternal homocysteine was measured in the early to mid-second trimester of pregnancy (Chapter 6).

For the sake of clinical interpretability, previous investigations on homocysteine had in many instances dichotomized or grouped the homocysteine exposure variable based on percentile cut-offs (e.g., elevated homocysteine defined as greater than the 90^{th} percentile) or based on cut-offs obtained from earlier studies. The downsides of these approaches are the loss of precision and potential bias in estimating the true association between homocysteine and placenta-mediated complications (which may explain the variability in findings from previous studies). With the likely existence of potential moderating factors, there is a need to be precise in modeling homocysteine concentration as a

continuous variable. Therefore we designed our analyses based on the OaK Birth Cohort using methods that allowed homocysteine and other continuous variables to be analyzed using flexible non-linear forms (Chapters 4 and 6). Moreover, in our investigation of homocysteine determinants, we compared the traditional approach of analyzing homocysteine as a dichotomous variable representing elevated homocysteine, to a more sophisticated approach of analyzing homocysteine as a continuous variable or a z-score (Chapter 6).

1.3 ORGANIZATION OF THE THESIS

The thesis is organized as follows:

- Chapter 2 consists of a review of the literature on homocysteine and the association of homocysteine with placenta-mediated complications.
- Chapter 3 describes the analytic data set based on the OaK Birth Cohort. The OaK Birth Cohort recruited a total of 8085 participants from 2002 to 2009. To our knowledge this is the largest cohort study to prospectively measure maternal plasma homocysteine during pregnancy. The study also measured factors related to plasma homocysteine concentration: serum folate, the MTHFR 677C>T genotype, and folic acid supplementation during pregnancy. Moreover, participants were recruited from a post folic acid-fortified population, which would allow us to understand the effects of maternal homocysteine in a folate replete population, given that folate is a strong determinant of homocysteine concentration and mitigates the effect of the MTHFR 677C>T polymorphism. The Chapter describes inclusion and exclusion criteria for the analytic dataset, participant characteristics, and procedures to handle missing data. This dataset formed the basis of two investigations (Chapters 4 and 6). Both investigations consisted of rigorous analyses that used multiple imputation to account for missing data; confounders, effect modifiers and other factors

were pre-specified and continuous factors were analyzed in their original form to explore non-linear associations.

- Chapter 4 is a manuscript entitled, *The role of maternal homocysteine concentration in placenta-mediated complications: findings from the Ottawa and Kingston birth cohort*. This manuscript was published in BMC (BioMed Central) Pregnancy and Childbirth. Using data from the OaK Birth Cohort, the purpose of this study was to investigate whether elevated maternal plasma homocysteine concentration in the early to mid-second trimester is associated with an increased risk of placenta-mediated complications, namely SGA, preeclampsia, placental abruption, pregnancy loss, and a composite outcome. We examined potential moderating factors that may explain discrepancies among previous studies: high-risk pregnancy and the MTHFR 677C>T polymorphism.
- Chapter 5 is a manuscript entitled, *The association of maternal homocysteine with placenta-mediated complications, A systematic review*. This manuscript will be submitted to the Journal Obstetrics & Gynecology. The goal of this study was to conduct a systematic review and meta-analysis of prospective studies on the association of homocysteine with the placenta-mediated complications SGA, preeclampsia, placental abruption, pregnancy loss, or a composite outcome. Between-study heterogeneity has limited the interpretability of previous findings, thus one of the objectives of this systematic review was to investigate potential sources of heterogeneity. These included study quality, adjustment for gestational age of homocysteine measurement and for other factors, either in the study design or analysis, and probable folate status of the study population based on year of participant recruitment and national fortification policies.[18,19] Serum folate level as an effect modifier could explain some of the observed inconsistencies between previous studies because folate can normalize differences in homocysteine levels.[21,22] We limited the review to

studies with temporality of an association, in which maternal homocysteine was measured from preconception to the mid-second trimester of an index pregnancy.

- Chapter 6 is a manuscript entitled, *The determinants of maternal homocysteine in pregnancy*. This manuscript was submitted to the Journal Public Health Nutrition; the current status is revisions requested. Using data from the OaK Birth Cohort, the purpose of this study was to investigate determinants of maternal homocysteine in a folic acid-fortified population. We focused on maternal demographic, genetic, and health characteristics. The findings from this study can inform practical efforts to lower maternal homocysteine concentrations.
- Chapter 7 summarizes the findings from different chapters of this thesis and discusses the interpretation and implications.

CHAPTER 2. LITERATURE REVIEW

Chapter 2 reviews the development of the homocysteine hypothesis in cardiovascular disease research and the extension of this hypothesis to placenta-mediated complications of pregnancy, namely preeclampsia, intrauterine growth restriction, placental abruption, and pregnancy loss.

2.1 HOMOCYSTEINE

2.1.1 The homocysteine hypothesis

In the late 1960s Dr. McCully, a Harvard pathologist, formulated the homocysteine hypothesis that attributed the metabolite homocysteine (or its derivatives) as *one* of the underlying causes of vascular disease.[23] This hypothesis emerged at a time when coronary heart disease (CHD) had been increasing steadily in the UK and USA, eventually reaching its peak around 1970.[24] Thereafter death rates from CHD declined year after year, for reasons that are a matter of debate.[24,25] Nonetheless, cardiovascular disease (CVD) remains the number one cause of death worldwide.[26]

The homocysteine hypothesis was formulated based on Dr. McCully's investigation and review of two cases—a child and an infant who had high urinary homocysteine, termed homocystinuria, and experienced premature atherothrombotic vascular events.[27] Autopsy findings revealed abnormalities in the vascular endothelium and atherosclerotic plaques similar to adult disease. One of the cases was deficient in the enzyme cystathionine synthase and the other in the enzyme methionine synthase. Based on family history, these enzyme deficiencies were presumed to be inherited inborn errors of metabolism. Findings from these two cases were corroborated by an independent case report of a child with similar findings including arterial abnormalities, homocystinuria, and elevated plasma homocysteine, but with deficiency in a third enzyme, methylenetetrahydrofolate reductase (MTHFR).[28]

Thus because an accumulation of homocysteine was the common feature between the homocystinuric cases, homocysteine was thought to be the factor contributing to the abnormal and premature development of ischemic disease. Homocystinuric patients treated with long-term homocysteine-lowering therapy have demonstrated reduced levels of plasma homocysteine and substantially lower

rates of atherothrombotic vascular events compared to the number of events expected in historical controls.[29]

2.1.2 Homocysteine metabolism

Homocysteine is an intermediate metabolite formed during the metabolism of the essential amino acid methionine (Figure 2.1). The precursor of homocysteine is an activated form of methionine that acts as a major donor of methyl groups for methylation reactions.[30] Homocysteine is metabolized via two main metabolic pathways: Firstly, homocysteine is irreversibly converted into the amino acid cysteine via trans-sulfuration by the enzyme cystathionine β-synthase, which requires Vitamin B_6 as a co-factor; and secondly, homocysteine is recycled back into methionine via re-methylation by the enzyme methionine synthase, which requires Vitamin B_{12} as a co-factor. Re-methylation 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).[30] This latter pathway inter-links the metabolism of homocysteine and folate.

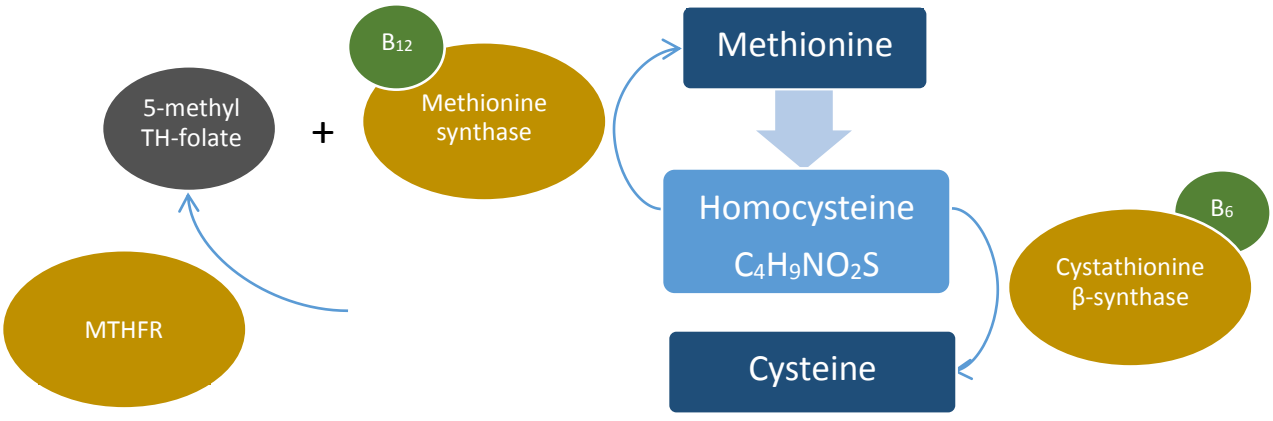

Figure 2.1 Homocysteine metabolism

2.1.3 Determinants of plasma homocysteine

A total blood serum homocysteine concentration less than 15 μmol/L is considered normal and a concentration between 16 and 60 μmol/L is considered moderately elevated. Moderately elevated homocysteine concentration is often referred to as "hyperhomocysteinemia". A homocysteine concentration greater than 60 to 100 μmol/L—as observed in cases of homocystinuria, is considered severely elevated.[31,32]

Elevated homocysteine is associated with polymorphisms in genes encoding the enzymes involved in homocysteine metabolism.[10] A relatively common single nucleotide polymorphism (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 elevated blood homocysteine concentration.[11,12]

During the early1990's, clinical trials of preconception supplementation with folic acid (a synthetic form of folate) demonstrated a reduced incidence and recurrence of neural tube defect (NTD)-affected pregnancies.[33] To raise population folate levels, folic acid fortification of food staples was mandated in numerous countries, particularly in North America.[34] In healthy individuals, higher folate concentrations were found to reduce the effect of the MTHFR 677C>T polymorphism on homocysteine concentrations.[12,13,35] For example, a summary of 70 surveys of 68,639 participants in population based studies conducted worldwide investigated the biochemical effects of MTHFR 677C>T on homocysteine concentration. Results were stratified according to mandatory folic acid-fortification defined by place and time (i.e., in some regions before and after folic acid-fortification in the mid-1990s). The places and time periods defined by mandatory folic acid-fortification showed correspondingly higher mean serum folate concentrations.[12] The TT (homozygous mutant) versus CC

(homozygous wild type) homocysteine difference was found to be 18% higher (95% CI 17%-19%), ranging from 25% higher (21%-30%) in non-folate-fortified East Asian populations to 7% higher (2%-13%) in folate-fortified North American (Canada and USA) and Australasian (Australia and New Zealand) populations.[12] Smaller studies in mildly folate-deficient populations have also found statistically significant interaction between MTHFR 677C>T genotype and serum folate in predicting homocysteine concentrations.[21,36]

Observational studies have identified nutritional, lifestyle, and biological factors associated with homocysteine concentration.[8,9,37] Data from the Framingham offspring cohort showed that homocysteine concentrations could be as much as 18% lower among users of B Vitamin supplements.[37] Homocysteine concentration was inversely associated with plasma Vitamin B_{12}, dietary folate (i.e., Vitamin B_9), and dietary Vitamin B_6,[8,37] and was positively associated with smoking status,[9,37] renal insufficiency,[38] and antihypertensive medication use.[37] In the Hordaland homocysteine study the determinants of *elevated* homocysteine were similar in a subgroup of 5883 women aged 40 to 42 years: cigarette smoking, coffee drinking, lower education, and less frequent use of vitamin supplements.[39] During pregnancy, homocysteine levels tend to decrease;[14,40] for this reason elevated homocysteine is often defined according to gestational age at the time blood samples are collected.

2.2 HOMOCYSTEINE AS A RISK FACTOR FOR VASCULAR DISEASE

Observational studies

Observational studies have established elevated plasma homocysteine as an independent risk factor for CHD, stroke, and dementia (i.e., cerebrovascular disease).[41–43] Overall, prospective studies of individuals with no history of cardiovascular disease indicate that an approximately 25% reduction in homocysteine is associated with an 11% reduction in CHD risk, adjusting for cardiovascular risk

factors and correcting for regression dilution bias.[41] The association was found to be stronger in case-control studies.[41,42,44] Additionally, a dose-response association between serum homocysteine and CHD has been demonstrated.[45]

RCTs

Meta-analyses have found no clear benefit of homocysteine-lowering interventions to reduce the risk of cardiovascular disease.[12,46–48] In B-Vitamin trials of folic acid alone or combined with Vitamins B_6 and/or B_{12}, a 25% reduction in plasma homocysteine maintained for an average of 5 years did not correspond to a reduced risk of CHD.[47] A meta-analysis of 8 large randomized placebo-controlled trials of folic acid allocation (with or without additional B Vitamins) included individual data on 37,485 participants with 9326 major vascular events and had greater than 99% power to detect a 10% reduction in events, as observed in cohort studies.[41,48] The pooled results showed an average reduction in homocysteine concentration by 25%, but no significant effect within 5 years on major coronary events (RR 1.03 [0.97-1.10]), and death due to CHD (RR 1.02 [0.90-1.16]).[48] As well, no differences in risk were found among predefined sub-groups, including pre-treatment serum folate, and mandatory folate fortification.

The results of RCTs are compatible with a 4% reduction in CHD risk from the lower limits of the confidence interval.[12,48] As well, the null results from RCTs are compatible with a reduction in primary CHD risk.[48] Many of the trials included patients with pre-existing cardiovascular disease and in trials reporting concurrent antiplatelet therapy; percentage use ranged from 22% to 100%.[47] Apart from the population under study, follow-up time may also be an issue. Atherosclerotic plaque development is a slow process that can span decades prior to a clinical event. Another consideration is the potential moderating effect of folate. Evidence from pooled genetic studies and RCTs conducted in regions with

mid to high folate intake suggest that moderate elevations in homocysteine levels may not be associated with cardiovascular disease risk in these regions.[12,13]

Gene-disease association studies

Using the principles of Mendelian inheritance as a 'natural experiment', pooled results from genetic studies suggest homocysteine may increase the risk of CHD.[12,47] A meta-analysis of 86 published case-control and nested case-control studies included 28,617 CHD cases and 41,857 controls. Aggregate data showed a 15% increase in CHD risk corresponding to an 18% higher concentration in homocysteine in TT (homozygous mutant) versus CC (homozygous wild-type) individuals (OR 1.15 [1.09-1.21]). When stratified by probable folate category, low folate regions in Asia with a 25% higher concentration of homocysteine in TT versus CC individuals had a correspondingly increased risk of CHD (OR 1.49 [1.29-1.73]), but not individuals in low-folate regions in Europe and mid to high-folate regions in Europe or elsewhere.

To investigate the possibility of publication bias the authors of these studies also analysed 19 unpublished large datasets (48,175 CHD cases, 67 961 controls). The findings suggested no evidence of increased CHD risk, either overall or within strata of probable folate status; for this reason, the authors speculated that publication bias could explain the findings of modest effect in the pooled genetic studies.[12] However, a dose-response effect was observed in 26 published genetic studies reporting homocysteine differences in TT versus CC individuals among controls. CHD risk increased significantly from the lowest tertile group of homocysteine difference (0 μmol/L, OR 0.86 [0.70-1.06]), to the highest tertile group (3.2 μmol/L, OR 1.51 [1.09-2.08]).[49] Thus the results from genetic studies in regions with low folate intake suggest that moderate elevations in homocysteine levels may be associated with an increased risk in CHD. Effect modification by folate intake or small study bias (i.e., publication bias) would explain the observed discrepancies.[12,13,47]

2.3 HOMOCYSTEINE AS A RISK FACTOR FOR PLACENTA-MEDIATED PREGNANCY COMPLICATIONS

2.3.1 Pregnancy as a "stress test"

Sattar and Greer were among the first to propose that pregnancy complications indicate future risk of cardio-metabolic and vascular disease.[50] Pregnancy is now viewed as a "stress test" that determines whether the maternal organ systems can meet increasing demands as gestation progresses.[51] This view is supported by large population based studies, which have demonstrated that delivering a low birth weight infant and maternal preeclampsia are associated with an increased risk of maternal death from cardiovascular disease.[52,53] Findings of elevated homocysteine in women with a history of pregnancy complications have also been reported:[39,54] In a systematic review and meta-analysis of non-classic cardiovascular biomarkers after hypertensive disorders of pregnancy, homocysteine was the only biomarker that was significantly higher in women with a history of hypertensive disorder in pregnancy (including preeclampsia and eclampsia).[54]

2.3.2 Epidemiology of placenta-mediated pregnancy complications

Preeclampsia, intrauterine growth restriction (IUGR), placental abruption, and fetal death are pregnancy complications that can have serious consequences for maternal and infant well-being.[1] These conditions are often an indication for pre-term birth, which can lead to additional challenges and adverse outcomes.[3] Placenta-mediated complications are considered a form of ischemic placental disease with common origins in the utero-placental vasculature.[3,4] The occurrence of placenta-mediated complications in nulliparous women is demonstrated to increase the risk of placenta-mediated complications in subsequent pregnancies.[55] Shared risk factors also suggest a common pathophysiology.[2]

Preeclampsia

Preeclampsia is defined as the development of hypertension with proteinuria induced by pregnancy, generally in the second half of gestation.[56] The syndrome affects around 5% of pregnancies world-wide and accounts for approximately 15% of maternal deaths.[1] It is the leading cause of both maternal and neonatal morbidity and mortality.[57] The only known "cure" for preeclampsia is delivery.

Preeclampsia is a particularly complex syndrome and thought to originate from poor placentation culminating in the maternal symptoms of hypertension and proteinuria.[58] Reduced placental perfusion from inadequate placentation leads to fluctuations in oxygen delivery, predisposing the vessels to oxidative stress.[59] Multisystem alterations including oligohydramnios (i.e., deficiency in amniotic fluid), which can lead to IUGR, are also observed alongside preeclampsia.[56] It is hypothesized that to overcome the consequences of reduced perfusion, one or more signal molecules are released form the fetal-placental unit. In the presence of reduced placental perfusion, IUGR can also be observed, absent the maternal syndrome that develops in preeclampsia. It is thought that risk factors for preeclampsia are predisposing factors leading to maternal intolerance of the fetal-placental signal.[58]

Placental abruption

Placental abruption is defined as premature separation of the placenta from the uterine side-wall. The specific cause of placental abruption is unknown, apart from physical trauma or premature rupture of membranes.[1] The condition affects one in 120 pregnancies in economically developed nations.[1]

Intrauterine growth restriction

IUGR is defined as a fetus that fails to reach growth potential. Growth restriction is commonly defined as small for gestational age (SGA) infant according to reference population standards for gestational age at delivery and sex. Growth restriction affects 4 to 8% of pregnancies.[1]

Pregnancy loss

The risk of pregnancy loss before 22 weeks (i.e., miscarriage or spontaneous abortion) after a clinically recognized pregnancy has been reported in different populations as 12% to 16%.[60–62] Chromosomal abnormalities are shown to account for approximately half of sporadic miscarriages.[63,64]

In Canada the rate of stillbirths (after 20 weeks or at least 500 grams) in 2017 was 4.2 per 1000 births.[65] The worldwide rate of stillbirths (after 22 weeks) in 2015 was estimated to be 18.4 per 1000 births.[66] In the year 2015 the majority of stillbirths occurred in low and middle income countries. Risk factors include preeclampsia/eclampsia, advanced maternal age (greater than 35 years), and obesity and non-communicable diseases (e.g., chronic hypertension, diabetes), for which the population attributable risk was 4.7%, 6.7% and 10% of stillbirths, respectively.

2.3.3 The utero-placental vasculature

The utero-placental vasculature in pregnancy consists of uterine arteries which form into spiral arteries that lead into the inter-villous space of the placenta (Figure 2.2). Maternal spiral arteries are remodelled in a process that includes trophoblast cell invasion (originating from the placenta), which dilates the distal ends of the spiral arteries.[58] Compromised utero-placental blood flow with inadequate trophoblast invasion and failed remodelling of the spiral arteries is the most common reason behind IUGR and is also implicated in placental abruption and preeclampsia.[1,58] In laboratory studies, the pathogenesis of

elevated homocysteine, but not folate (i.e., Vitamin B_9), on human trophoblast cells from the placenta has been demonstrated in vitro.[67,68]

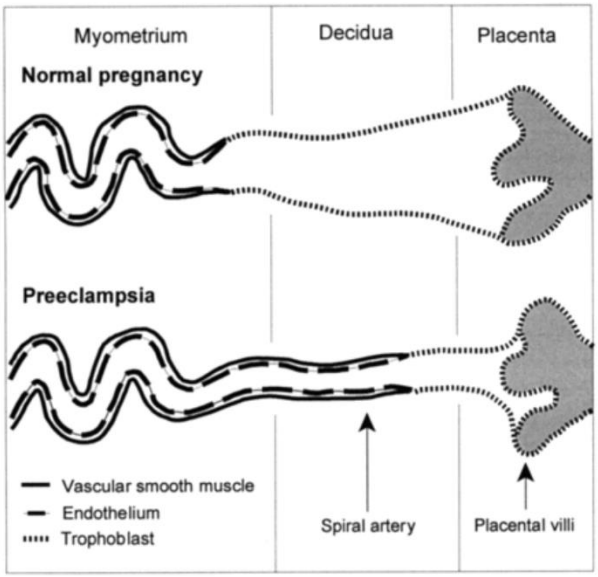

Figure 2.2 Normal versus abnormal remodelling of spiral arteries
Adapted from: Vascular function in preeclampsia
Cardiovasc Res. 2000;47(1):38-48. doi:10.1016/S0008-6363(00)00087-0
Cardiovasc Res | Copyright © 2000, European Society of Cardiology

2.3.4 The association of homocysteine with placenta-mediated complications

Prospective cohort studies

The association of maternal homocysteine with placenta-mediated complications has been investigated in prospective cohort studies. A Canadian birth cohort recruited participants from 2002 to 2005 and measured homocysteine levels before 20 weeks gestation. Analyses of 2119 women found that homocysteine concentration greater than the 90th percentile for gestational week was associated with an increased risk of preeclampsia (RR 2.7, 95% CI 1.4-5.0), and pregnancy loss (RR 2.1, 95% CI 1.2-3.6).[18] Similar analyses based on 2951 women recruited to the OaK birth cohort between 2002 and 2005 found no association with the risk of preeclampsia.[17] The Generation R Study of the Netherlands,

a cohort of 5805 pregnant women recruited between 2002 and 2006, examined the association of homocysteine, folic acid, and Vitamin B_{12} concentration with pregnancy complications.[19] The study found that homocysteine in the highest verus lowest quintile and folic acid in the lowest versus highest quintile, but not Vitamin B_{12}, were associated with an increased risk of SGA. Folic acid in the lowest quintile was associated with an increased risk of preeclampsia.

Systematic reviews and meta-analyses of observational studies suggest an association between elevated homocysteine and placenta-mediated complications. For the outcome preeclampsia, significant heterogeneity has been observed in pooled effect estimates:[15] A systematic review and meta-analysis published in 2005 investigated the association between homocysteine and preeclampsia. Six studies with a total of 1876 participants measured homocysteine concentration before the onset of preeclampsia and found a pooled mean difference in homocysteine concentration of 0.68 μmol/L (0.40-0.96, I^2=40%) in women who subsequently developed preeclampsia compared to those who did not. Meta-regression analyses found that dietary folate supplementation, gestational age at blood sample, and study quality did not explain the between-study heterogeneity. Furthermore, folate and Vitamin B_{12} levels measured in a subset of the studies were not significantly different between preeclampsia cases and controls. A larger systematic review and meta-analysis published in 2012 investigated the association between maternal homocysteine concentration and SGA infants.[16] The review included 21,326 participants from 19 studies that measured maternal homocysteine from pre-conception to delivery. Maternal homocysteine greater than the 90th percentile was associated with an increased risk of SGA (OR 1.25, 95% CI 1.09-1.44), with no obvious heterogeneity.

Gene-disease association studies

Further understanding of the role of homocysteine can be gained from studies investigating the risk of placenta-mediated complications due to the MTHFR 677C>T variant, which results in moderately elevated homocysteine. Pooled results from genetic studies suggest this variant can increase the risk of placenta-mediated complications. Maleki-Yazdi's 2013 systematic review (thesis, unpublished) included a meta-analysis of nine studies in which maternal homozygosity or carrier status for the MTHFR 677C>T genotype increased the risk of SGA infant (<10th percentile) (OR 1.18, 95% CI 1.03, 1.35), with no observed heterogeneity or evidence of publication bias.[69] A 2004 meta-analysis examined the association of the MTHFR 677C>T polymorphism with hypertension in pregnancy. The review included twenty-three study comparisons with 6213 participants. Twenty-two studies with preeclampsia cases demonstrated increased risk of preeclampsia in the MTHFR 677C>T carriers (i.e., TT or CT versus CC) (OR 1.23, 95% CI 1.02, 1.48), but with significant heterogeneity. Associations were stronger in earlier compared to later studies, meaning publication bias could not be excluded.[22] Similarly, a 2014 systematic review that included Chinese language databases found a significant association between the MTHFR 677C>T genotype and hypertension in pregnancy. Sub-group analyses revealed that the association was significant in East and South Asian populations but not in Caucasian populations and meta-regression confirmed that ethnicity was a source of heterogeneity. The authors speculated that the differences could arise from differences in allele frequencies across populations, lifestyle, and environmental factors; evidence of publication bias was not found.[70]

Systematic reviews and meta-analyses have also demonstrated an association between maternal MTHFR 677C>T genotype with recurrent pregnancy loss.[71,72] Wu et al.'s 2012 systematic review found that the TT homozygous genotype was associated with an increased risk of recurrent loss (mainly first or second trimester) (OR 1.57, 95% CI 1.163, 2.13).[71] The association of the MTHFR

genotype with placental abruption has been investigated,[73] but requires larger studies to synthesize results.[74]

CHAPTER 3. ANALYTIC DATA SET BASED ON OTTAWA AND KINGSTON (OAK) BIRTH COHORT

Chapter 3 presents an overview of the Ottawa and Kingston (OaK) Birth Cohort, which forms the basis of two investigations: the association of maternal homocysteine concentration in the early to mid-second trimester with placenta-mediated pregnancy complications (Chapter 4), and the determinants of maternal homocysteine in pregnancy (Chapter 6). The analytic data set inclusion and exclusion criteria are described, along with methods to handle missing data and imputation procedures.

3.1 Overview of the Ottawa and Kingston (OaK) Birth Cohort

Recruitment

The recruitment catchment areas of the OaK birth cohort were the cities of Ottawa and Kingston in South-Eastern Ontario. Women were eligible to participate if they were between 12 and 20 weeks gestation with a viable singleton or twin pregnancy and planned to deliver in the Ottawa area or at the Kingston General Hospital. The recruitment strategy was designed to obtain a sample that was representative, given that approximately 80% of women in Ontario seek antenatal care from an obstetrician and 98.4% deliver in hospital.[75] Of the 8085 women recruited, data were obtained on 7669 women (95%) with a recorded (i.e., non-missing) birth weight. Characteristics of the OaK cohort were comparable to provincial data on maternal age, smoking rates, infant sex, birth weight, and gestational age at delivery.[76]

Data collection at baseline

The baseline survey consisted of an interviewer-administered questionnaire, blood sample collection, and chart abstraction. The questionnaire collected maternal personal health information along with maternal and paternal demographic characteristics. To improve accuracy of the data, questionnaire responses were verified with information from medical records. Nine of 39 demographic variables relied solely on self-report. Research personnel also conducted brief telephone interviews to collect missing information.

Lab investigations

A 30 mL draw of maternal venous blood was collected by qualified research personnel at the time of recruitment. Where possible this was done with routine bloodwork to reduce venipuncture. Maternal blood samples were assayed for biologic and genetic exposures related to thrombophilias and folates.

Assays were performed by laboratory personnel blind to participant outcomes. Blood samples for the measurement of plasma homocysteine and serum folate, and MTHFR 677C>T genotype were collected and assayed following standard lab procedures. Maternal blood was collected in K_2EDTA Vacutainer tubes (Becton Dickinson, Lincoln Park, NJ) for homocysteine measurement and MTHFR 677C>T genotyping and in serum separator tubes (Becton Dickinson) for folate measurement.

Blood collected for homocysteine measurement was immediately placed on ice and within 30 minutes centrifuged in 4 °C at 3000 × *g* for 10 minutes. Blood plasma was aliquoted and stored at -20 °C. Plasma homocysteine (µmol/L) was measured in batches on the Abbott AxSYM II Immunoassay System (Abbott Laboratories, Abbott Park, IL) using fluorescence polarization immunoassay.

Blood collected for folate measurement was allowed to clot and then centrifuged at 3000 × *g* for 10 minutes. Blood serum was aliquoted and stored at -20 °C. Serum folate (ng/mL) was measured in batches on the Access 2 and UniCel® DxI 800 Immunoassay Systems using manufacturer's reagents (Beckman Coulter, Brea, CA).

DNA was extracted for MTHFR 677C>T genotyping using manual extraction and later switched to automated extraction. In manual extraction, blood collected for genotyping was centrifuged at 2500 × *g* for 10 minutes and DNA extracted from the buffy coat using the FlexiGene DNA Kit (QIAGEN, Hilden, Germany). In automated extraction, blood collected for genotyping was centrifuged at 1100 × *g* and DNA extracted using the BioRobot M48 and MagAttract DNA Blood Midi Kit (QIAGEN, Hilden, Germany). The MTHFR gene segment was amplified using polymerase chain reaction (PCR) and genotyped using the ABI 3130xl Genetic Analyzer and the ABI PRISM SNaPshot Multiplex Kit (Applied Biosystems, Waltham, MA).

Follow up procedures

Participants were followed until delivery. At post-partum, research staff completed the interviewer-administered questionnaire, with questions pertaining to folic acid and multivitamin supplementation during pregnancy. Research staff with clinical expertise abstracted pregnancy outcomes and infant data including indicators of neonatal health from hospital medical records into a structured form. Hospital records consisted of antenatal records, laboratory investigations, labour and delivery records, and perinatal records.

Pregnancy outcomes

Participant records were audited to validate the occurrence of preeclampsia and placental abruption if medical records suggested the diagnosis. A committee of medical experts blind to patient care and exposure status independently examined information abstracted from medical records to adjudicate the diagnoses. Outcomes were adjudicated by four OaK investigators with expertise in obstetrics, hematology, nursing practise, and clinical epidemiology. Disagreements were resolved by consensus in face-to-face meetings.

The investigators followed detailed definitions of diagnostic criteria as follows: Preeclampsia was defined as hypertension with proteinuria beyond 20 weeks gestation. Hypertension was a diastolic blood pressure reading greater than or equal to 90 mmHg and proteinuria was 2+ on a dipstick or proteinuria greater than 300 mg in a 24-hour urine collection, measured on two separate occasions of at least 6 hours apart. Placental abruption was defined as antepartum bleeding with evidence of a retro-placental thrombus. Evidence was gathered from reports on antenatal ultrasound, visual exam of the placenta by a medical practitioner, and placental pathology.

3.2 METHODS TO CONSTRUCT THE ANALYTIC DATA SET

Inclusion and exclusion criteria

The inclusion criteria for this study were women recruited in the early to mid-second trimester (between 12 and 20 weeks gestation) of a singleton pregnancy. Women were excluded if they withdrew from the cohort, were lost to follow-up, or if the pregnancy was terminated.

Data cleaning

Logic checks were performed for the variables of interest and the data were cleaned and re-categorized as necessary. Where appropriate categorical variables were re-categorized to avoid sparse distributions and continuous variables were transformed to normalize distributions or categorized.

Gestational age at recruitment was abstracted from hospital records. Cigarette dose, reported as the number of cigarettes smoked daily, was categorized. Smoking is demonstrated to have higher misclassification of exposure during pregnancy due to negative perceptions of prenatal smoking.[77] For this reason, smoking was defined as light/medium if less than 10 cigarettes/day and heavy if 10 or more cigarettes/day.[78]

The serum folate assay that was used on some of the samples had an analytical range of up to 45 nmol/L. Laboratory readings in approximately10% of the participants were therefore output as 'greater than' 45 nmol/L. These folate values were treated as missing and were handled separately in the multiple imputation procedure.

The serum folate distribution was skewed with some very high measurements. To normalize the folate distribution a ceiling was set to the 90th percentile, which ranged from 76 nmol/L at 12 weeks gestation

to 48 nmol/L at 20 weeks. The World Health Organization (WHO) sets the cut-off for serum folate deficiency at 10 nmol/L in a non-pregnant population.[79] Although a serum folate deficiency cut-off in pregnant women has not been established, we considered the 10% of values beyond these cut points as equally high and differentiating between them unnecessary.[80]

The outcomes SGA infant and pregnancy loss were determined as follows: SGA was an infant birth weight less than 10th percentile of sex and gestational age-adjusted population standards;[81] pregnancy loss was intrauterine death after enrollment or stillbirth.

Statistical software

The analytic dataset from the OaK Birth Cohort was constructed in SAS ® version 9.4. The cleaned dataset was then converted to an R data frame using the program Stat/Transfer version 13 (Circle systems Inc.). Descriptive statistics and imputation procedures were performed in R Studio version 0.99.892, R version 3.2.3.[82] Hardy-Weinberg equilibrium of the MTHFR 677C>T genotype was determined using the HardyWeinberg package in R.[83]

Multiple Imputation procedures

Rubin (1976) classified the types of missing data mechanisms into three categories: missing completely at random (MCAR), missing at random (MAR), and missing not at random (MNAR).[84,85] Missing data are considered MCAR if the cause of missingness is random (i.e., non-systematic) and unrelated to any observed or unobserved variables; for example, arising due to technical issues during data collection. Missing data are considered MAR if the cause of missingness is systematic, but related to other observed data—not the missing data; for example, a subgroup of participants defined by an observed variable such as age, may be more likely to leave a question unanswered. Missing data are considered

MNAR if the cause of missingness is systematic and related to the unknown values of the missing data or other unknown reasons; for example, participants with a lower or higher income may be less likely to report income.

MAR is the most commonly assumed mechanism and multiple imputation is the recommended approach to deal with MAR. In this approach, several values are drawn from a posterior predictive distribution of the missing data based on a specified model of the observed data and are used to replace missing values multiple times. Thus, multiple complete data sets are generated and analyses are performed on each. The estimates from the analyses are pooled, accounting for the model-generated uncertainty related to the missing data and variability in the complete dataset.[86,87]

The MAR assumption was examined by comparing the distribution of key baseline variables in participants with complete versus missing data.[88] Missing data patterns were visualized using the VIM package in R.[89] Multiple imputation was performed using the package mice, which stands for multivariate imputation by chained equations, also called fully conditional specification (FCS).[90] This approach uses sequential regression imputation to create multiple "complete" datasets. The sequential regression imputation procedure is very flexible and can be used to create multiple predictions for each missing value as a function of all the observed data (outcomes, confounders, risk factors and auxiliary variables), taking into account the type of variable (i.e., continuous, binary, categorical, ordinal). The default imputation method was used for each type of variable: continuous variables used predictive mean matching; binary used logistic regression; categorical used a multinomial logit model; and ordinal used an ordered logit model.

Within the mice function, a series of operators were used to tailor the imputation procedure. Firstly, to avoid collinearity we modified the predictor matrix, which specifies the variables that will be used in the imputation of each incomplete variable. The predictor matrix was modified to ensure that recoded variables would not predict the original variables from which they were derived. Secondly, in the case of derived variables like BMI (i.e., derived from the variables weight and height), the relationship between the variables was specified in a mathematical formula so that the imputation of these variables would remain synchronized. Thirdly, we ensured that the imputed values of certain variables were in the expected range. For example, in the case of the 10% of the serum folate values that had been set to missing because of a laboratory reading cut-off at 45 nmol/L, the range of the imputed values was set to be greater than 45.

In addition to the variables of interest, auxiliary variables were included in the multiple imputation procedure to improve estimates. Auxiliary variables were identified based on being associated with missingness in the variables of interest. For example, paternal education was included because it was associated with the missingness of maternal education and income. Also included were the variables alcohol use and infant birthweight.

The number of imputations was set to 10, with 200 iterations. About 10 to 20 imputations is considered adequate for the FCS method.[90] A rule of thumb based on simulation studies suggests a number of imputations that is equal to the percentage of incomplete cases.[91,92] Because the analytic data set had 37% incomplete observations, the number of imputations would have been at least 30 based on the above rule of thumb. However, Rubin's rule of up to 10 imputations was considered sufficient because the trade-off between a small decrease in error is off-set by the impracticality of a large number of

imputations.[93,94] Convergence of the imputation procedure was assessed on select variables by plotting the mean and standard deviation by iteration.

3.3 PARTICIPANT CHARACTERISTICS

Of the 8085 participants recruited to the OaK Birth Cohort, 498 (6%) were excluded because of withdrawn consent, ineligibility, loss to follow-up, twins or multiples, terminated pregnancy, and gestational age below 12 or greater than 20 weeks (Figure 3.1). The analytic data set comprised a total of 7587 women.

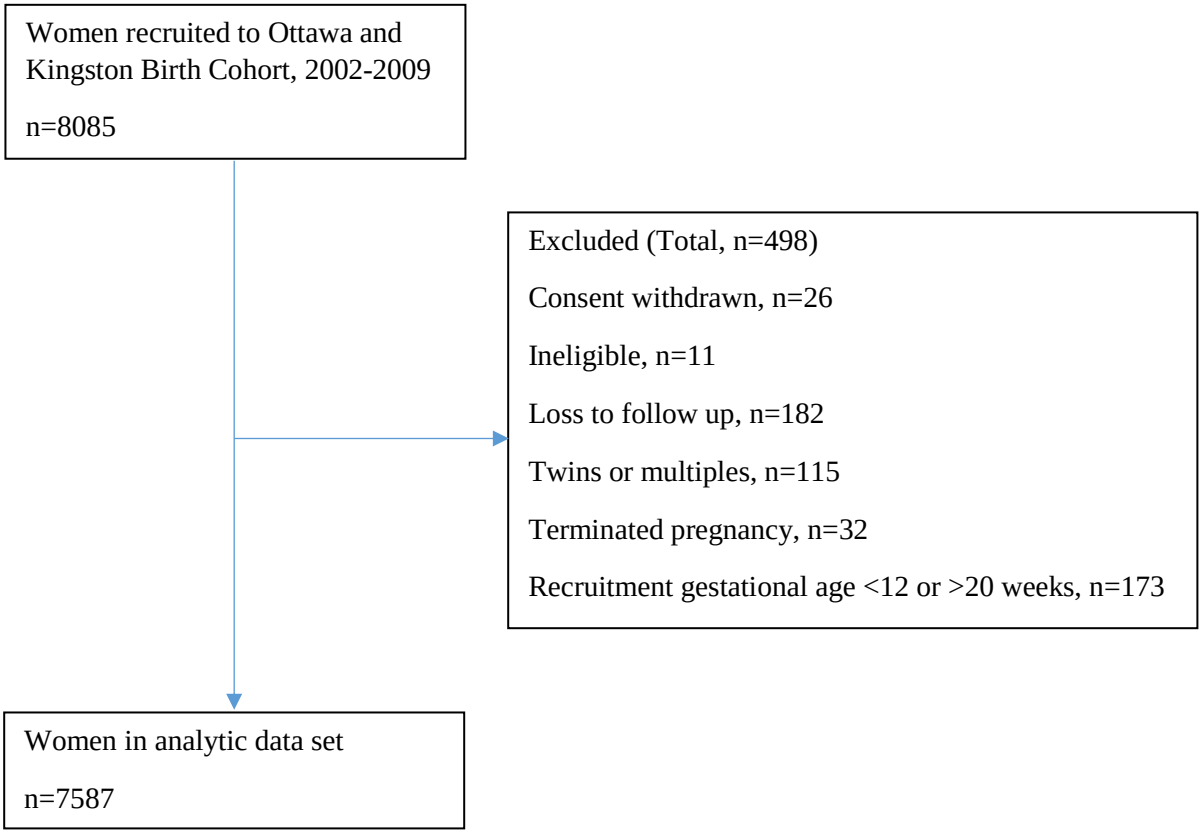

Figure 3.1 Participant flow diagram for the analytic dataset

The mean age of participants was 30 and most identified as Caucasian (Table 3.1). The majority of participants and their partners had completed college or university and as a household earned upwards of CAD $50,000 annually. The MTHFR 677C>T genotype was measured in approximately half of the

OaK participants. Close to 12% were homozygous for the TT mutant genotype (Table 3.2). A test for Hardy-Weinberg equilibrium confirmed that the genotype frequencies were not significantly different from Hardy-Weinberg equilibrium.[83]

Table 3.1 OaK Birth Cohort participant characteristics

Variable	Frequency n=7587
Age	
Mean (SD)	30.3 (5.06)
Race [a] (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 (1.73%)
BMI (missing N=136, 1.8%)	
Mean (SD)	24.9 (5.5)
Participant education (missing N=7, 0.09%)	
Grade school	153 (2.02%)
High school	962 (12.7%)
College/University not completed	754 (10.0%)
College/University completed	5711 (75.3%)
Paternal/partner education (missing N=95, 1.25%)	
Grade school	137 (1.83%)
High school	1519 (20.3%)
College/University not completed	590 (7.88%)
College/University completed	5246 (70.0%)
Household income (missing N=485, 6.4%)	
<25k	415 (5.84%)
25k - <50k	1188 (16.7%)
50k - <80k	2077 (29.2%)
>=80k	3422 (48.2%)
MTHFR genotype [b]	
CC (wild type)	1768 (44.1%)
CT (heterozygous)	1760 (43.9%)
TT (mutant)	478 (11.9%)

[a] Race was categorized similar to the U.S. Census, in which the categories are: White, Black or African American, American Indian or Alaska Native, Asian (Far-east and Indian subcontinent), and Hawaiian or Pacific Islander. Participants whose response suggested a Central/South American, Latino, Hispanic, or Aboriginal background were classified into the 'Other' category. This follows the U.S. Census Bureau's classification that: "People who identify their origin as Hispanic, Latino, or Spanish may be of any race." [95]

[b] Measured in a subset of participants (n=4006)

Most participants were recruited at the start of the second trimester, around 12 to 13 weeks gestation (Table 3.2). They had a mean homocysteine concentration of 5 µmol/L (SD 1), with measured values ranging from 1 to 34 µmol/L. Participants had a median serum folate concentration of 37 nmol/L, a distribution that was originally wide and skewed before ceiling values at the 90th percentile for gestational week at measurement. The homocysteine and folate distributions were similar to another Canadian cohort that recruited participants in early pregnancy during the same time period,[96] but distributions of homocysteine and folate were higher and lower, respectively, compared to a European cohort that recruited participants in early pregnancy.[19]

The majority of participants were non-smokers during pregnancy (less than 10% were smokers), had a normal to overweight BMI, were normotensive, non-diabetic, and reported taking folic acid supplements during pregnancy. Slightly less than half of the women were nulliparous (Table 3.2).

Table 3.2 OaK Birth Cohort independent variables of interest

Variable	**Frequency n=7587**	
Health indicators		
BMI (missing N=136, 1.8%)		
Mean (SD)	24.9 (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 (Preeclampsia, 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 multivitamin) (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 blood work (weeks)		
Mean (SD)	13.7 (2.09)	
Homocysteine (µmol/L) (missing N=87, 1.1%)		
Mean (SD)	4.8 (1.3)	
Range	1 - 34	
Serum folate (nmol/L) (missing N=987, 13.0% [a])		
Median (IQR)	37.4 (30.6 – 45.1)	
Range	3.70 – 79.6	

[a] Includes the 10% of folate values set to missing because of values higher than the analytical range of the assay (45 nmol/L)

About 10% of participants experienced at least one of the placenta-mediated complications. SGA was the most common complication (7%), followed by preeclampsia (3%), and placental abruption and pregnancy loss (both around 1%) (Table 3.3). We would expect the proportion of SGA cases to be around 10% based on the calculation of SGA as less than the 10th percentile for gestational age and sex-specific birthweight benchmarks for Canadian newborns.[81] The lower than expected percentage of SGA cases may have to do with higher education and household incomes that characterize Ottawa and Kingston's demographics (Table 3.1). As well, the population-based reference curves to calculate SGA were based on a publication from 2001 and likely do not reflect changing trends in overweight and obesity that have contributed to a rise in fetal macrosomia.

Table 3.3 OaK Birth Cohort pregnancy outcomes of interest

Variable	Frequency (n=7587)
Preeclampsia	
Yes	227 (2.99%)
No	7360 (97%)
Placental abruption	
Yes	68 (0.87%)
No	7519 (99%)
Small for gestational age (SGA) (missing N=79, 1.04%)	
Yes	512 (6.82%)
No	6996 (93%)
Pregnancy loss	
Yes	85 (1.12%)
No	7502 (99%)
Any PMC (Preeclampsia, placental abruption, SGA, pregnancy loss) (missing N=79, 1.04%)	
Yes	759 (10.0%)
No	6749 (90%)

3.4 MISSING DATA AND IMPUTATION PROCEDURES

Of the 7587 participants in the analytic dataset, 64% had complete data on all dependent and independent variables. With regard to participants with complete versus incomplete data, Table 3.4 shows similar distributions of select baseline characteristics, including maternal age, household income, and homocysteine concentration. No more than 13% of observations were missing from each of the dependent and independent variables of interest (Figure 3.2, Tables 3.1-3.3) and missing data appeared to form a relatively non-uniform pattern of missingness (Figure 3.2, right panel). We assumed that the data were missing at random (MAR), meaning that missingness could be related to one or more variables but not related to the values of missing observations, although missing not at random (MNAR) could not be ruled-out.

Table 3.4 OaK participant characteristics in complete and incomplete observations

Variable	Complete (n=4899)	Incomplete (n=2688)
	Frequency, n (%)	Frequency, n (%)
Age, Mean (SD)	30.7 (4.8)	29.7 (5.4)
Race: Caucasian	4267 (87%)	1983 (87%)
Education: Completed College/University	3765 (77%)	1946 (73%)
Household income: >=80k	2393 (49%)	1029 (47%)
Homocysteine, Mean (SD)	4.8 (1.25)	4.9 (1.32)
Gestational age at bloodwork, Mean (SD)	13.7 (2.1)	13.8 (2.1)
BMI, Mean (SD)	25 (5.6)	25 (5.3)

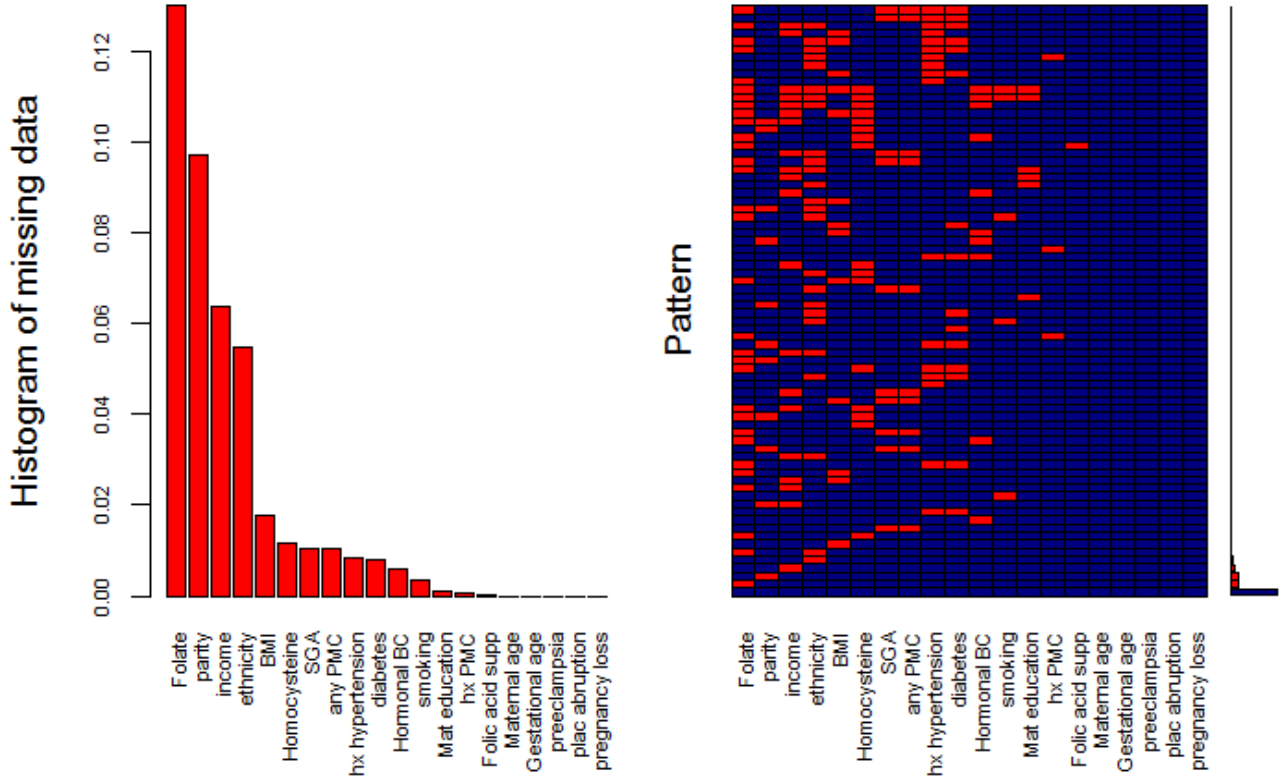

Figure 3.2 Histogram and pattern of missing data in the OAK Birth Cohort

Convergence of the multiple imputation procedure in the R package mice was assessed in select variables by plotting the variable mean and variance by iteration. The plots suggest convergence of the imputation procedure because of adequate mixing of the streams and lack of strong trends (Figure 3.3).[90]

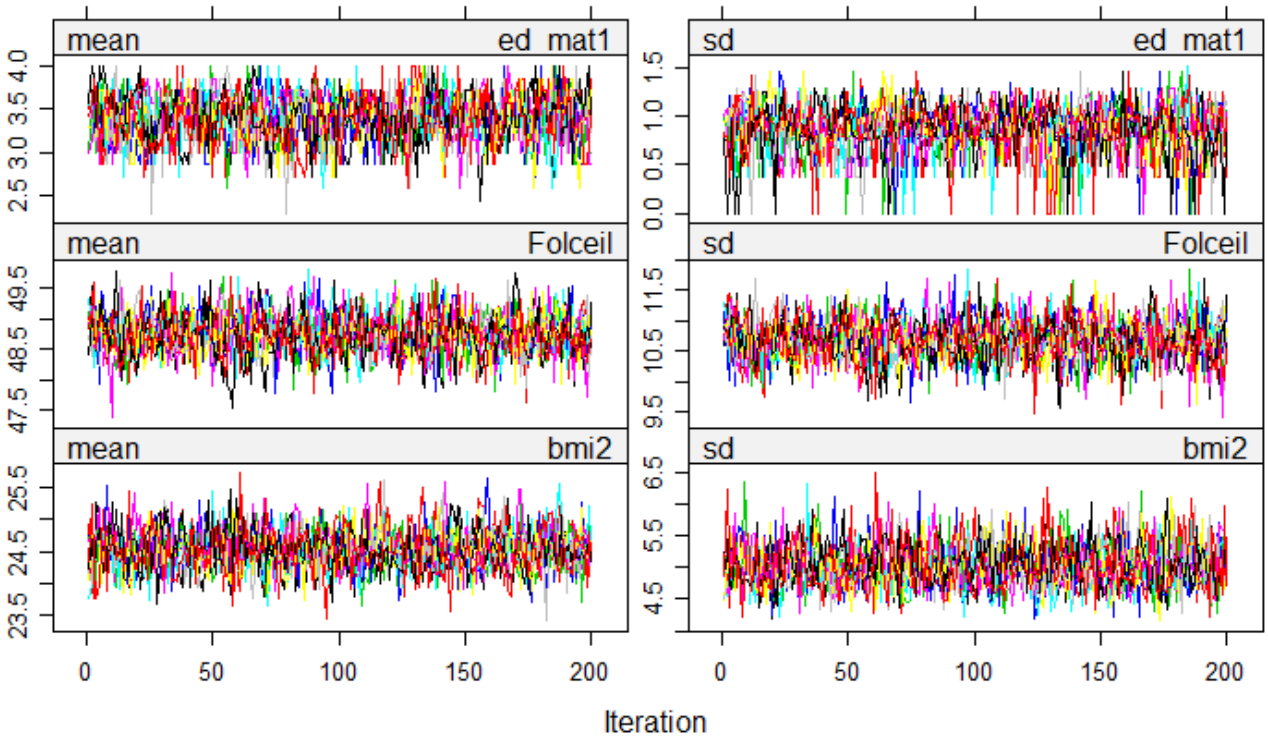

Figure 3.3 Plot of mean and variance (SD) by multiple imputation iteration for select variables: maternal education, serum folate, and BMI

CHAPTER 4. THE ROLE OF MATERNAL HOMOCYSTEINE CONCENTRATION IN PLACENTA-MEDIATED COMPLICATIONS: FINDINGS FROM THE OTTAWA AND KINGSTON BIRTH COHORT [MANUSCRIPT]

Chapter 4 presents the manuscript entitled, "*The role of maternal homocysteine concentration in placenta-mediated complications: findings from the Ottawa and Kingston birth cohort*". This manuscript was published in BMC (BioMed Central) Pregnancy and Childbirth. The research presented aligns with the first thesis objective. Using data from the OaK Birth Cohort, we investigated the associations of maternal homocysteine with placenta-mediated pregnancy complications, and investigated potential modifiers of the associations.

Chaudhry SH, Taljaard M, Macfarlane AJ, et al. The role of maternal homocysteine concentration in placenta-mediated complications: findings from the Ottawa and Kingston birth cohort. *BMC Pregnancy Childbirth*. 2019;19(75)

4.1 TITLE PAGE

The role of maternal homocysteine concentration in placenta-mediated complications: findings from the Ottawa and Kingston birth cohort

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Authors' contributions

SW, MW, RRW, GS, and MR are lead investigators of the OaK Birth Cohort. **SC**, SW, and MT designed the study. **SC** analyzed the patient data and wrote the draft manuscript. **SC**, SW, MT, and AM were involved in interpretation of the data. LG, MW, RRW, GS, and MR critically revised the manuscript and contributed to the final version. All authors read and approved the final manuscript.

4.2 ABSTRACT

Background: Homocysteine is an intermediate metabolite implicated in the risk of placenta-mediated complications, including preeclampsia, placental abruption, fetal growth restriction, and pregnancy loss. Large cohort and case-control studies have reported inconsistent associations between homocysteine and these complications. The purpose of this study was to investigate whether elevated maternal plasma homocysteine concentration in the early to mid-second trimester is associated with an increased risk of placenta-mediated complications. We examined the following potential moderating factors that may explain discrepancies among previous studies: high-risk pregnancy and the MTHFR 677C>T polymorphism.

Methods: We analyzed data from participants recruited to the Ottawa and Kingston (OaK) Birth Cohort from 2002 to 2009 in Ottawa and Kingston, Canada. The primary outcome was a composite of any placenta-mediated complication, defined as a composite of small for gestational age infant, preeclampsia, placental abruption, and pregnancy loss. Secondary outcomes were, individually: small for gestational age infant, preeclampsia, placental abruption, and pregnancy loss. We conducted multivariable logistic regression analyses with homocysteine as the primary continuous exposure, adjusting for gestational age at the time of bloodwork and explanatory maternal characteristics. The functional form, i.e., the shape of the homocysteine association with the outcome was examined using restricted cubic splines and information criteria (Akaike's/Bayesian Information Criterion statistics). Missing data were handled with multiple imputation.

Results: 7587 cohort participants were included in the study. Maternal plasma homocysteine concentration was significantly associated (linearly) with an increased risk of both the composite outcome of any placenta-mediated complication (p=0.0007), SGA (p=0.0010), severe SGA, and marginally with severe preeclampsia, but not preeclampsia, placental abruption and pregnancy loss. An increase in homocysteine concentration significantly increased the odds of any placenta-mediated

complication (odds ratio (OR) for a 5 µmol/L increase: 1.63, 95% Confidence Interval (CI) 1.23-2.16) and SGA (OR 1.76, 95% CI 1.25-2.46). Subgroup analyses indicated some potential for modifying effects of the MTHFR 677C>T genotype and high-risk pregnancy, although the interaction was not statistically significant (high-risk subgroup OR 2.37, 95% CI 1.24-4.53, p-value for interaction =0.14). Conclusions: Our results suggest an independent effect of early pregnancy elevated maternal homocysteine on placenta-mediated pregnancy complications.

Key words: homocysteine, hyperhomocysteinemia, pregnancy complication, placenta, preeclampsia, small for gestational age, placental abruption, pregnancy loss

4.3 BACKGROUND

Maternal plasma homocysteine concentration is proposed to be associated with certain pregnancy complications.[97] Based on evidence supporting the role of homocysteine in endothelial dysfunction and as a risk factor for cardiovascular disease, elevated maternal homocysteine is hypothesized to play a role in placenta-mediated pregnancy complications (PMCs), including preeclampsia, placental abruption, fetal growth restriction, and pregnancy loss.[23,27,47,49] All have been linked to abnormal placental vasculature, share a common placental pathophysiology, and have an increased risk to reoccur.[4,55]

Within the 1-carbon metabolic cycle, homocysteine is an intermediate metabolite formed in the methionine cycle. Homocysteine can be transmethylated to form methionine, which in turn is converted to S-adenosylmethionine, the main cellular methyl donor from which methyl groups can be transferred to multiple recipient molecules, including DNA and histones. The complex cycle involves key co-enzymes and co-factors including vitamins B_9 (folate), B_6 and B_{12}. Polymorphisms in genes related to 1-carbon metabolism, as well as various modifiable lifestyle and behavioural factors are associated with elevated homocysteine.[37,98]

Studies have reported inconsistent associations between maternal homocysteine, measured at different time points in pregnancy and placenta-mediated complications;[15,99] even among larger cohort and case-control studies measuring homocysteine from early pregnancy, the associations are inconsistent.[18–20,100–103] The discrepancies could be due to moderating factors like high-risk pregnancy and differences in population frequencies of the MTHFR 677C>T polymorphism that can lead to moderately elevated homocysteine.[11,12] Discrepancies could also arise from different percentile cut-offs used to define elevated homocysteine.[17,18,20]

The purpose of this study was to investigate whether elevated maternal plasma homocysteine concentration measured in the early to mid-second-trimester of pregnancy is associated with an increased risk of PMCs. Our analytic approach sought to explore potential non-linear effects of homocysteine concentration and other continuous factors so as to retain as much information about the association, which can otherwise be lost through categorizing continuous variables.[104] We also sought to determine whether the association is modified by the MTHFR 677C>T genotype and by high-risk pregnancy.

4.4 METHODS

Study design

Women attending prenatal appointments and planning to deliver at The Ottawa Hospital, the Ottawa region, or the Kingston General Hospital were recruited to the Ottawa and Kingston (OaK) Birth Cohort from 2002 to 2009. Participants were included if between 12 and 20 weeks gestation of a viable singleton or twin pregnancy. For the present study, participants were excluded from the analytic data set if non-singleton, recruited before 12 or after 20 weeks gestation, if they withdrew, were lost to follow-up, or if the pregnancy was terminated.

Details of the cohort study have been previously reported.[76] Briefly, the baseline survey and post-partum follow-up consisted of an interviewer-administered questionnaire and hospital record abstraction. Maternal blood samples were collected at baseline and laboratory personnel were blind to outcomes. Blood samples for homocysteine and MTHFR were collected in K_2EDTA Vacutainer tubes (Becton Dickinson, Lincoln Park, NJ) and for serum folate in serum separator tubes (Becton Dickinson). Samples for plasma were immediately placed on ice and within 30 minutes centrifuged in 4 °C at 3000 × *g* for 10 minutes, then aliquoted and stored at -20 °C. Plasma homocysteine (μmol/L) was measured on the Abbott AxSYM II Immunoassay System (Abbott Laboratories, Abbott Park, IL) using fluorescence polarization immunoassay. Blood samples for serum were centrifuged at 3000 × *g* for 10 minutes, then aliquoted and stored at −20 °C. Serum folate (nmol/L) was measured using the Beckman Coulter Access 2 and Unicel DxI 800 immunoassay analyzers using manufacturer's reagents (Beckman Coulter, Brea CA). Homocysteine and folate were measured within one month in batches.

Outcomes

The primary outcome was the composite of placenta-mediated complications (PMCs): small for gestational age (SGA), preeclampsia, placental abruption, and pregnancy loss. The secondary outcomes were individual components of the composite. Additionally we investigated severe SGA <5th percentile and severe preeclampsia.

A small for gestational age infant had a birth weight less than the 10th or 5th percentile of sex and gestational age-adjusted population standards.[81] Pregnancy loss was intrauterine death before 20 weeks or stillbirth. Preeclampsia was new onset hypertension with proteinuria. Hypertension was a diastolic blood pressure reading greater than or equal to 90 mmHg and proteinuria of 2+ on a dipstick or proteinuria greater than 300 mg in a 24-hour urine collection measured on two separate occasions of at least 6 hours apart. Preeclampsia with delivery prior to 35 weeks gestation was considered severe.[105] Placental abruption was antepartum bleeding with objective evidence either on ultrasound or inspection of the placenta at birth or a pathologic examination of a retro-placental clot.[106] A committee of medical experts blind to participant exposure status independently examined information abstracted from medical records to adjudicate preeclampsia and placental abruption.

Potential risk factors

Potential confounders, risk factors, and interactions were specified prior to multivariable modeling using knowledge of the subject matter, our analyses of homocysteine determinants in OaK participants (unpublished), and previous work investigating PMCs.[9,17–20] We considered the following factors: maternal age, race, education, income, nulliparity, smoking, diabetes, use of hormonal birth control prior to conception, chronic hypertension, and history of PMCs, folic acid supplementation, and serum folate. Gestational age at recruitment was abstracted from hospital records.

Statistical analyses

All statistical analyses were performed in SAS ® version 9.4 and RStudio version 0.99.892, R version 3.2.3[82] and statistical significance was assessed at the 5% level. A variable clustering algorithm was used to examine multicollinearity among independent variables. Missing data patterns were visualized and multiple imputation was performed using multivariate imputation by chained equations.[89,90] This approach uses sequential regression imputation to create multiple "complete" datasets, a procedure that is flexible and generates multiple predictions for each missing value as a function of all observed data (including auxiliary variables), taking into account the type of variable (i.e., continuous, binary, categorical, ordinal). The number of imputations was set to 10 which is considered an adequate number of imputations.[90,93,94]

Regression analyses

We conducted multivariable logistic regression analyses to examine the association of plasma homocysteine concentration (as the primary exposure of interest) with PMCs, while adjusting for the identified potential confounders and risk factors. We did not transform homocysteine because log-transformation did not substantially normalize the distribution (Additional file 1). Because serum folate is intermediate with respect to folic acid supplementation, analyses were conducted with and without serum folate to note any changes in effect estimates for folic acid supplementation. Changes were also noted for outliers in homocysteine concentration.

Multivariable model building was conducted as follows.[104] An initial model was fit including main effects for all independent variables. Continuous variables, i.e., homocysteine, gestational age, maternal age, BMI, and serum folate were modeled using restricted cubic splines with five knots set at the 5th, 27.5th, 50th, 72.5th, and 95th quantiles. Next, a plot of partial associations referred to as an ANOVA plot,

corrected for the number of degrees of freedom, was generated to visualize strong and weak partial associations. The strengths of association informed a decision of how many degrees of freedom to allocate to each variable: strong associations were modeled with greater complexity than weak associations. For example, weaker partial associations in continuous variables were reduced to fewer knots or a linear term, while categorical and ordinal variables were collapsed. Akaike's Information Criterion (AIC) and the Bayesian Information Criterion (BIC) were then used to confirm the allocations.[104]

The multivariable logistic regression models were fit using the *rms* (regression modelling strategies) package.[107] Odds Ratios for continuous variables modelled with restricted cubic splines were estimated comparing the 75th to 25th percentile. The logistic regression model was fit to each imputed dataset and results were combined across the 10 datasets using Rubin's method which computes imputation-adjusted variances and average betas.[90,108] Analyses of outcomes with a low frequency of events were conducted using penalized maximum likelihood. The best penalty factor was identified by tracing effective AIC for different penalties.[104,107]

Subgroup analyses

Subgroup analyses examined the modifying effect of the MTHFR 677C>T genotype and high-risk pregnancy, defined as chronic hypertension, diabetes, history of a PMC, or BMI greater than 35. This was done by including interaction terms with homocysteine and each potential moderator. Subgroup analyses were not conducted for placental abruption and pregnancy loss due to a low number of events.

4.5 RESULTS

Of the 8085 women recruited to the OaK Birth Cohort, 7587 were included in our study (Figure 4.1).

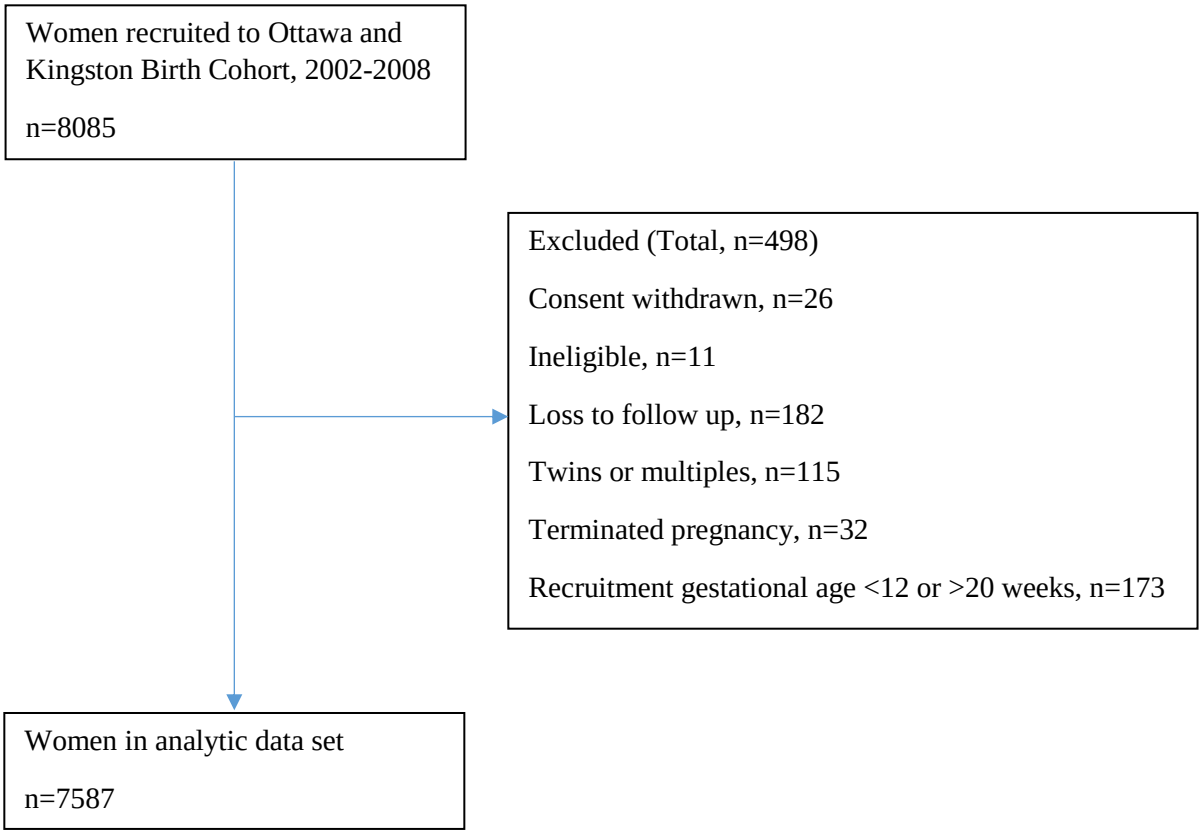

Figure 4.1 Participant flow diagram for the analytic dataset

Descriptive characteristics of participants are presented in Table 4.1. Maternal education and household income were highly correlated; income was dropped from multivariable analyses because education was more strongly associated with the outcomes. ANOVA plots of partial associations revealed relatively strong associations between homocysteine and the outcomes any placenta-mediated complication (PMC) and SGA (Additional file 2). Nevertheless, AIC and BIC values confirmed that for each outcome the models with homocysteine specified as a simple linear term provided the best fit to the data. The final models included both serum folate and folic acid supplementation because excluding folate did not substantially change effect estimates for folic acid supplementation. We identified two

outliers with homocysteine >20 µmol/L that were included in the final models because exclusion yielded similar results (data not shown).

Table 4.1 Participant characteristics

Variable	Frequency n=7587
Age	
Mean (SD)	30.3 (5.06)
Race [a] (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 (1.73%)
BMI (missing N=136, 1.8%)	
Mean (SD)	24.9 (5.5)
Range	14.7 – 61.3
Participant education (missing N=7, 0.09%)	
Grade school	153 (2.02%)
High school	962 (12.7%)
College/University not completed	754 (10.0%)
College/University completed	5711 (75.3%)
Paternal/partner education (missing N=95, 1.25%)	
Grade school	137 (1.83%)
High school	1519 (20.3%)
College/University not completed	590 (7.88%)
College/University completed	5246 (70.0%)
Household income (missing N=485, 6.4%)	
<25k	415 (5.84%)
25k - <50k	1188 (16.7%)
50k - <80k	2077 (29.2%)
>=80k	3422 (48.2%)
MTHFR genotype [b]	
CC (wild type)	1768 (44.1%)
CT (heterozygous)	1760 (43.9%)
TT (mutant)	478 (11.9%)

[a] Race was categorized similar to the U.S. Census, in which the categories are: White, Black or African American, American Indian or Alaska Native, Asian (Far-east and Indian subcontinent), and Hawaiian or Pacific Islander. Participants whose response suggested a Central/South American, Latino, Hispanic, or Aboriginal background were classified into the 'Other' category. This follows the U.S. Census Bureau's classification that: "People who identify their origin as Hispanic, Latino, or Spanish may be of any race." [95]

[b] Measured in a subset of participants (n=4006)

Effect of homocysteine

Homocysteine concentration was significantly higher in participants with the composite outcome of any PMC, small for gestational age (SGA) infants, and pregnancy loss (Table A, Additional file 1), also demonstrated visually by boxplots of the homocysteine distribution according to pregnancy outcome (Figure A, Additional file 1). In adjusted analyses, higher plasma homocysteine concentration was significantly associated with increased odds of any PMC (Table 4.2, p=0.0007), SGA (Table 4.3, p=0.0010), was marginally associated with preeclampsia (p=0.07), and was not associated with placental abruption, and pregnancy loss (Table 4.3, 0.99, and 0.16). Additionally, homocysteine was associated with severe SGA (<5th percentile) and marginally with severe preeclampsia (delivery <35 weeks gestation) (Table 4.3, 0.0012, and 0.0595). A 5 µmol/L change in homocysteine concentration, which is approximately 4 SDs of the homocysteine concentration, was associated with a 63% increased odds of any PMC (Odds Ratio (OR) 1.63, 95% Confidence Interval (CI) 1.23-2.16) and a 76% increased odds of SGA (OR 1.76, 95% CI 1.25-2.46). Serum folate and folic acid supplementation were not associated with the outcomes (Table 4.2 and Tables C.1-C.4, Additional file 3).

Table 4.2 Multivariable logistic regression analysis of the association between homocysteine and any placenta-mediated complication [a] (759 events [b]), n=7587

Variable	Odds ratio (95% CI)		p-value [c]
Homocysteine (linear)			0.0007
5 µmol/L increase	1.629	(1.227, 2.161)	
Age (restricted cubic spline, three knots)			0.0031
34 versus 27 years	1.187	(1.063, 1.325)	
Race			0.0002
Caucasian versus others	0.644	(0.509, 0.814)	
Education			0.0056
College/University completed versus less than completed	0.763	(0.630, 0.924)	
Nulliparous			<0.0001
Yes versus no	1.941	(1.636, 2.303)	
Smoking			<0.0001
No	Reference		
Second-hand	0705	(0.392, 1.268)	
Med/light smoker (<10 cigarettes per day)	1.631	(1.228, 2.166)	
Heavy smoker (≥10 cigarettes per day)	1.921	(1.348, 2.737)	
Diabetes			0.0336
Yes versus no	1.687	(1.041, 2.733)	
BMI (restricted cubic spline, four knots)			0.0499
27.3 versus 21.1 kg/m^2	1.057	(0.883, 1.265)	
Hormonal birth control prior to conception			0.3692
No	Reference		
Oral	0.927	(0.784, 1.096)	
Injection or IUD	0.732	(0.436, 1.227)	
Chronic hypertension			<0.0001
Yes versus no	2.750	(1.687, 4.483)	
History of PMC (Preeclampsia, placental abruption, IUGR, stillbirth, loss)			0.0110
Yes versus no	1.359	(1.073, 1.722)	
Folic acid supplementation			0.7328
Yes versus no supplementation	0.943	(0.674, 1.320)	
Serum folate (linear)			0.5326
45.1 versus 30.6 nmol/L	1.025	(0.949, 1.106)	
Gestational age at blood work (restricted cubic spline, four knots)			0.0004
13.7 versus 12.4 weeks	0.939	(0.804, 1.095)	

[a] Any placenta-mediated complication- composite of small for gestational age (SGA) <10th percentile, preeclampsia, placental abruption, and pregnancy loss

[b] 79 missing outcome values imputed

[c] Wald test of most meaningful hypotheses, pooled across multiple imputation datasets

Table 4.3 Summary of multivariable logistic regression analyses of the association between homocysteine and placenta-mediated complications, n=7587 [a]

Outcome variable	Odds ratio (95% CI) [b]		p-value [c]
Any placenta-mediated complication (759 events [d])			
Homocysteine (linear)			0.0007
5 µmol/L increase	1.629	(1.227, 2.161)	
SGA (512 events [d])			
Homocysteine (linear)			0.0010
5 µmol/L increase	1.756	(1.254, 2.458)	
SGA <5th percentile (221 events [d])			
Homocysteine (linear)			0.0012
5 µmol/L increase	2.022	(1.322, 3.092)	
Preeclampsia (227 events)			
Homocysteine (linear)			0.0736
5 µmol/L increase	1.546	(0.959, 2.491)	
Severe preeclampsia (43 events)			
Homocysteine (linear)			0.0595
5 µmol/L increase	1.762	(0.978, 3.177)	
Placental abruption (68 events)			
Homocysteine (linear)			0.9851
5 µmol/L increase	1.005	(0.590, 1.711)	
Pregnancy loss (85 events)			
Homocysteine (linear)			0.1586
5 µmol/L increase	1.392	(0.879, 2.206)	

[a] Complete results reported in Table 4.2: any placenta-mediated complication, Additional file 4, and Additional file 3: Table C.1: SGA, Table C.2: Preeclampsia, Table C.3: Placental abruption, and Table C.4: Pregnancy loss
[b] Models adjusted for maternal age, race, education, parity, smoking, diabetes, BMI, hormonal birth control prior to conception, chronic hypertension, history of a placenta mediated complication, folic acid supplementation, serum folate, and gestational age at blood work
[c] Wald test of most meaningful hypotheses, pooled across multiple imputation datasets
[d] 79 Missing outcome values imputed

Subgroup analyses

The results of the subgroup analyses are presented in Tables 4.4 and 4.5. In the subset of 4006 OaK participants with measured genotype, the interaction between MTHFR 677C>T and homocysteine was not statistically significant for preeclampsia (p=0.84) but there was some evidence of interaction for any PMC (p=0.12) and for SGA (p=0.07) with associations qualitatively different within each subgroup. Associations in the CC/CT subgroups were positive and significant, whereas in TT subgroup associations were negative and not significant, with wide confidence intervals. Figure 4.2 illustrates these trajectories for the composite outcome with homocysteine.

Table 4.4 Multivariable logistic regression analyses examining the moderating effect of MTHFR 677C>T genotype [a] on the association between homocysteine and placenta-mediated complications, n=4006

Outcome variable	Odds ratio (95% CI) for 5 µmol/L increase [b]		p-value [c]
	TT	CC/CT	
Any placenta-mediated complication (395 events [d])	0.712 (0.243, 2.083)	1.778 (1.159, 2.729)	0.1172
SGA (277 events [d])	0.639 (0.161, 2.536)	2.430 (1.450, 4.073)	0.0714
Preeclampsia (109 events)	1.174 (0.523, 2.633)	1.258 (0.682, 2.322)	0.8439

[a] Homocysteine*MTHFR 677C>T genotype (Factor + higher order factors)
[b] Model adjusted for maternal age, race, education, parity, smoking, diabetes, BMI, hormonal birth control, chronic hypertension, history of PMC, folic acid supplementation, serum folate, gestational age at blood work
[c] Wald test of most meaningful hypotheses, pooled across multiple imputation datasets
[d] 40 Missing outcome values imputed

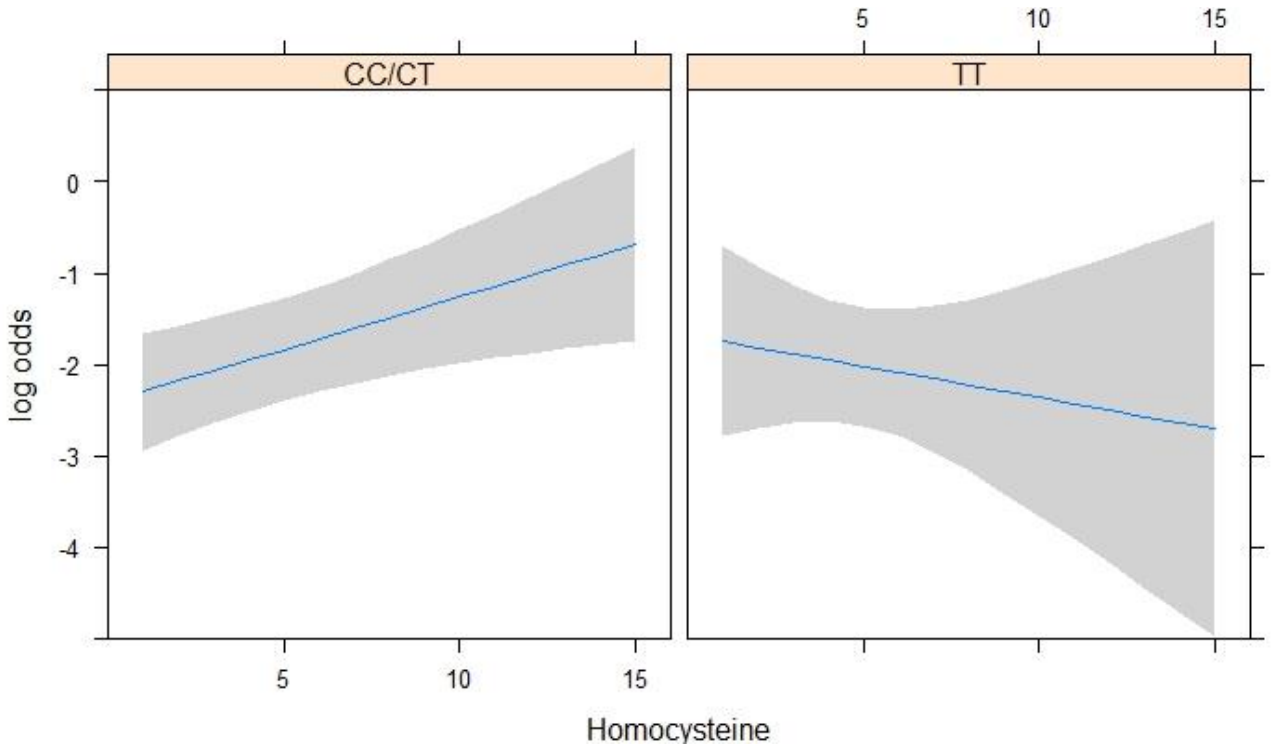

Figure 4.2 Modelled association between plasma homocysteine (linear) and any placenta-mediated complication, by MTHFR 677C>T genotype CC/CT (wild type and heterozygous) and TT (mutant). Shaded area represents 95% CI.

The interaction between high-risk pregnancy and homocysteine was not statistically significant for any PMC and SGA (p=0.27 and p=0.51 respectively), but there was some evidence of a moderating effect for preeclampsia (p=0.14, Table 4.5). While the interaction effect (and hence, the difference in association between the high and low-risk subgroups) was not statistically significant at the

conventional 5% level, the odds ratio was higher and statistically significant in the high-risk group (OR 2.84, 95% CI 1.19 to 6.79 for a 5 µmol/L change in homocysteine concentration) compared to a lower and non-statistically significant OR in the low-risk group (OR 1.31, 95% CI 0.74 to 2.30).

Table 4.5 Multivariable logistic regression analyses examining the moderating effect of high-risk pregnancy [a] on the association between homocysteine and placenta-mediated complications, n=7587

Outcome variable	Odds ratio (95% CI) for 5 µmol/L increase [b]		p-value [c]
	High-risk	Low-risk	
Any placenta-mediated complication (759 events [d])	2.368 (1.239, 4.525)	1.595 (1.176, 2.163)	0.2714
SGA (512 events [d])	2.474 (1.050, 5.828)	1.821 (1.276, 2.597)	0.5081
Preeclampsia (227 events)	2.839 (1.187, 6.792)	1.308 (0.743, 2.302)	0.1351

[a] Homocysteine*High-risk pregnancy (Factor + higher order factors)
[b] Model adjusted for maternal age, race, education, parity, smoking, hormonal birth control, folic acid supplementation, serum folate, gestational age at blood work
[c] Wald test of most meaningful hypotheses, pooled across multiple imputation datasets
[d] 79 Missing outcome values imputed

Effects of other factors

Different groups of risk factors were associated with SGA and preeclampsia (Tables 4.3, C.1-C.2). A high school or incomplete post-secondary education and smoking were associated with increased odds for any PMC and SGA. Chronic hypertension, diabetes, and history of experiencing a PMC were associated with increased odds for any PMC and preeclampsia.

4.6 DISCUSSION

Main Findings

Our analysis of 7587 participants from the Ottawa and Kingston (OaK) Birth cohort found that maternal homocysteine concentration in the early to mid-second trimester was associated with increased odds of any placenta-mediated complication (PMC): a composite of small for gestational age (SGA), preeclampsia, placental abruption, and pregnancy loss, and was associated with increased odds of SGA, severe SGA, and preeclampsia. In the high-risk subgroup homocysteine was associated with increased odds of preeclampsia.

Strengths and Limitations

To our knowledge, this is the largest cohort study to investigate the association between early to mid-second trimester maternal homocysteine concentration and the risk of placenta-mediated complications. We used multiple imputation to deal with missing values and conducted rigorous multivariable logistic regression analyses designed to explore flexible functional forms of association with homocysteine and other continuous factors, controlling for a wide range of potential confounders.

In most larger studies, homocysteine was dichotomized or grouped to investigate non-linearity and a threshold effect.[19,101] However, the Hordaland Homocysteine study of several thousand participants suggested that for most conditions including pregnancy complications and adverse pregnancy outcomes, homocysteine exhibits a continuous concentration-response relation.[9,97] Thus, one of the main strengths of our study is that we accounted for the functional form, i.e., shape of the association, of homocysteine and other continuous variables in relation to the outcomes of interest, which to the best of our knowledge has not been previously reported.

One of the main limitations of our study is that we did not examine the effect of Vitamins B_6 and B_{12}. In a survey of the Canadian population from 2007 to 2009, Vitamin B_{12} was the main determinant of elevated homocysteine concentration in the folate replete.[109] We would expect OaK participants to have adequate Vitamin B_{12} levels because 85 percent were supplementing with multivitamin or prenatal vitamin supplements, which likely contained Vitamin B_{12}. Caffeine or coffee consumption is another homocysteine determinant we did not examine,[98] though pregnant women tend to consume less caffeine during pregnancy.

Interpretation

Other studies have reported an increased risk of SGA associated with elevated homocysteine.[19,110] In Bergen et al.'s cohort of 5085 participants recruited in the Netherlands from 2002 to 2006, early second trimester homocysteine in the upper versus lower quintile was associated with an increased risk of SGA ($<5^{th}$ centile).[19] In Dodds et al.'s cohort of 2119 participants recruited in Nova Scotia, Canada from 2002 to 2005, a study time frame similar to our OaK birth cohort, a homocysteine concentration greater than the 90^{th} percentile was not associated with a risk of SGA.[18] The mean homocysteine concentration in the latter study was lower than Bergen et al.,[19] but comparable to our participants' mean homocysteine concentration of 4.8 µmol/L. In another study of 65 SGA cases and 358 controls recruited in a Chinese textile factory from 1996-1998, pre-conception homocysteine above the 90^{th} percentile was not associated with an increased risk of SGA.[101].These latter two studies dichotomized homocysteine concentration using cut-offs that are not necessarily physiological, which may limit comparability with our findings and those of Bergen et al.[19]

The MTHFR 677C>T genotype qualitatively modified the association of homocysteine with any PMC and SGA. However uncertainty around the negative effect of the TT genotype, likely due to fewer participants the TT subgroup, makes it difficult to interpret the modifying effect. Higher folate intake and status are known to mitigate but not eliminate the effect of this polymorphism on homocysteine concentration.[12,13,111] Although approximately 95% of our sample was consuming a folic acid supplement and white flour and other cereal products in Canada have been fortified with folic acid since the late 1990s, homocysteine concentration was associated with the MTHFR genotype in the OaK cohort. Given the variations in clinical testing for MTHFR genotype in current obstetric practice,[112] our study findings suggest testing may be warranted, particularly in high-risk subgroups, for example, those who exhibit comorbidities or a history of complications.

We found that elevated homocysteine was marginally associated with an increased risk of preeclampsia, and that the association was significant in the high-risk subgroup. Others have found no association[19,20]; for example, Kahn et al.'s nested case-control study with 113 preeclampsia cases and 443 controls recruited in Montreal from 1999 to 2004.[20] Studies of repeated homocysteine measurement during pregnancy have reported increasing homocysteine concentration during the course of preeclampsia.[40] In a longitudinal analysis of 252 women of whom 49 developed preeclampsia, homocysteine increased in the preeclampsia group independent of B-vitamin (B-6, B-12, and folate) and obesity status, while concentrations in the uncomplicated group remained steady.[113] Therefore early homocysteine measurement, as in our study, may pre-date onset of disease and changes in homocysteine. Our finding of a stronger association of homocysteine in severe preeclampsia, marginally significant with fewer outcome events, also suggests a role of homocysteine earlier in pregnancy when severity is increased.

Some studies have, however, reported an increased risk of preeclampsia associated with higher homocysteine concentrations.[18,114–117] Many of these studies were conducted during a time period before routine peri-conceptional folic acid supplementation and/or mandatory folic acid fortification (i.e., the early to mid-1990s),[114–116] or were conducted in countries without mandatory folic acid fortification.[117–119] This suggests that where folate intake is lower, homocysteine levels would tend to be higher and therefore homocysteine might play a greater role, earlier on, in the development of preeclampsia.

We report the association of the range of potential confounders with the composite and individual outcomes. Our results, although exploratory in this regard, demonstrated a greater role of diabetes and chronic hypertension in the development of preeclampsia compared to SGA. In a clinical opinion paper, Ness and Sibai[120] hypothesized that the maternal syndrome observed in preeclampsia develops in the presence of abnormal placentation that interacts with maternal metabolic syndrome, and that fetal growth restriction develops in the absence of metabolic syndrome. Our findings of increased odds of preeclampsia in the high-risk subgroup also lend support to this hypothesized role of developing endothelial dysfunction in preeclampsia.

In our study elevated homocysteine concentration was not associated with an increased risk of placental abruption and pregnancy loss, but a greater number of events would be necessary to confirm an association. Some studies have found no association of homocysteine with early pregnancy loss.[100,102,121] However, one-time early pregnancy losses are characteristically different from recurrent early pregnancy losses and losses throughout pregnancy.[99]

4.7 CONCLUSIONS

In summary, our results support an independent effect of early pregnancy elevated homocysteine on placenta-mediated pregnancy complications. Our findings are comparable with similar large studies; high-risk pregnancy and potentially the MTHFR 677C>T genotype may contribute to some of the observed differences between studies. As with ongoing investigations into the role of homocysteine in cardiovascular disease, large Mendelian randomization studies could further confirm the etiological role of homocysteine in placenta-mediated pregnancy complications.[122]

Additional files for Chapter 4 can be found in Appendix B

Additional file 1: Homocysteine distribution in the entire sample and by outcome

Additional file 2: ANOVA plots of partial associations from saturated model for each outcome

Additional file 3: Complete tables of results for the multivariable logistic regression analyses

Additional file 4: Modelled associations of restricted cubic spline functions

CHAPTER 5. THE ASSOCIATION OF MATERNAL HOMOCYSTEINE WITH PLACENTA-MEDIATED COMPLICATIONS, A SYSTEMATIC REVIEW [MANUSCRIPT]

Chapter 5 presents the manuscript entitled, "*The association of maternal homocysteine with placenta-mediated complications, A systematic review*". This manuscript will be submitted to the Journal Obstetrics & Gynecology. The research presented aligns with the first thesis objective. In a systematic review and meta-analysis of prospective studies, we investigated the association of maternal homocysteine with placenta-mediated pregnancy complications, and investigated potential modifiers of the associations.

5.1 TITLE PAGE

The association of maternal homocysteine with placenta-mediated complications

A systematic review

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Authors' contributions

SC, SW, and MT designed the study. **SC** and WS screened studies for the systematic review and extracted the data. **SC** analyzed the data and wrote the draft manuscript. **SC**, SW, MT, and AM were involved in interpretation of the data. SW, MT, and AM critically revised the manuscript and contributed to the final version. All authors read and approved the final manuscript.

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5.2 ABSTRACT

Objective: Elevated plasma homocysteine, a marker of cardiovascular disease risk, is associated with placenta-mediated pregnancy complications (small for gestational age (SGA), preeclampsia, placental abruption, pregnancy loss); however, inconsistencies in the associations limits the interpretability of previous findings. We conducted a systematic review and meta-analysis of prospective studies and investigated potential sources of heterogeneity: study design, study quality, probable folate status, study region, and whether analyses adjusted for important covariates and changes in homocysteine during pregnancy.

Data sources: Databases searched were MEDLINE, Embase and PubMed; supplementary sources included dissertations (until 16 April 2019).

Methods of study selection: Inclusion criteria were cohort, nested case-control, or case-cohort studies that measured maternal homocysteine concentration using samples obtained from pre-conception to the mid-second trimester of an index pregnancy. Non-English language studies were excluded. Two reviewers independently screened studies and extracted data. Quality was assessed using the Newcastle-Ottawa Scale.

Tabulation, Integration, and Results: Thirty prospective cohort or nested case-control studies were included. Twenty studies reported effect estimates using different cut-offs for elevated homocysteine and 24 reported homocysteine concentrations in cases versus controls. In 28 studies a random effects meta-analysis of pooled mean differences revealed significantly higher means for SGA: mean difference (SGA cases versus controls) 0.35 μmol/L (95% confidence interval (CI) 0.19 to 0.51, with moderate heterogeneity (I^2=33%); and for preeclampsia: 0.87 μmol/L (95% CI 0.52 to 1.21), with considerable heterogeneity (I^2=92%). A trend towards a significant difference was observed for pregnancy loss: 0.63 μmol/L (95% CI -0.02 to 1.27), with substantial heterogeneity (I^2=45%). Significant sources of heterogeneity were study region (SGA and preeclampsia), folate status

(preeclampsia—significant mean difference in low- and mid-folate subgroups), adjusting for covariates (preeclampsia), and severity (preeclampsia).

Conclusion: Our systematic review suggests that a slightly higher homocysteine concentration is associated with increased risk of SGA and in certain subgroups, preeclampsia.

PROSPERO registration number CRD42017080808.

5.3 INTRODUCTION

The homocysteine hypothesis advanced by Kilmer McCully proposed that moderately elevated concentration of the metabolite homocysteine could induce cardio- and cerebro-vascular damage as seen in cases of homocystinuria, which exhibit high homocysteine and disease progression at a young age. A large body of research has now established moderately elevated homocysteine as a risk factor for cardiovascular disease[47] and cognitive impairment.[123] Maternal homocysteine has also been linked to complications of the utero-placental vasculature, including small for gestational age (SGA), preeclampsia, placental abruption, and pregnancy loss,[39] but uncertainty remains regarding the role of homocysteine in the risk of placenta-mediated pregnancy complications.

Earlier systematic reviews and meta-analyses suggested that homocysteine, folate deficiency, and the methylenetetrahydrofolate (MTHFR) 677C>T variant were probable risks factors for placenta-mediated complications.[99,124–126] The most recent systematic reviews and meta-analyses of observational studies have demonstrated an association between homocysteine and the outcomes preeclampsia and SGA, but raised the issue of between-study heterogeneity and differences in the magnitude of effect sizes in studies with and without temporality of an association.[15,16] Recent large cohort studies have suggested homocysteine plays a role in pathogenesis; however, inconsistencies in the associations limits the interpretation of the evidence.[18,19,110,127]

Underlying the differences between studies are potential modifying effects of factors related to homocysteine concentration. The MTHFR 677C>T polymorphism, which varies in prevalence across populations, can result in moderately elevated homocysteine.[11] Serum folate concentration as an effect modifier might explain some of the observed inconsistencies in the associations because folate can

mitigate the effect of the MTHFR 677C>T polymorphism. Mandated folic acid fortification of food staples has been instituted in some regions and could reduce differences in homocysteine concentration according to MTHFR genotypes.[12,21,22] Differences are also possible due to fluctuations in homocysteine throughout pregnancy,[14] which are often unaccounted for in research syntheses.[15,16]

Our systematic review on the association of homocysteine with placenta-mediated complications was conducted to update previous reviews with results from larger cohort studies and to investigate sources of heterogeneity that might underlie inconsistencies in the association: probable folate status due to national folate-fortification policies, study region, study quality, and adjusting for important covariates and for the timing of homocysteine measurement during pregnancy. To maintain temporality of an association, we focused on prospective studies that had measured homocysteine until the mid-second trimester of an index pregnancy.

5.4 METHODS

SOURCES

Our systematic review sought to investigate the following question: Compared to women with lower plasma homocysteine concentration, are women with higher plasma homocysteine concentration at increased risk for placenta-mediated pregnancy complications? Each component of the question was defined as follows; **P**opulation: Pregnant women, **E**xposure: Higher maternal blood plasma homocysteine concentration (measured pre-pregnancy or until the mid-second trimester), **C**omparison: Lower maternal blood plasma homocysteine (measured pre-pregnancy or until the mid-second trimester), **O**utcome: 1) small for gestational age (SGA, birthweight < defined percentile for gestational age and sex), 2) preeclampsia, 3) placental abruption, and 4) pregnancy loss, and **T**ime: Pregnancy

Studies meeting the following criteria were included: English language study; Prospective or retrospective cohort study, nested case-control study, or case-cohort study; Women participating in a population-based or clinical study; Maternal blood plasma total homocysteine concentration measured from samples obtained from pre-conception to the mid-second trimester of pregnancy; At least one of the following placenta-mediated pregnancy complications: SGA, preeclampsia, placental abruption, pregnancy loss, or a composite outcome.

MEDLINE and Embase on the Ovid interface, and PubMed were searched without language restrictions using the search strategy shown in Supplementary file 1. A filter was applied to the PubMed search to retrieve only those citations that would not have been indexed in MEDLINE.[128]

The database search terms comprised key words and database-specific subject headings for the exposure and outcomes. Search terms for placenta-mediated complications were adapted from a search strategy developed by Rodger and colleagues.[129] Database searches included a hedge (i.e. filter) for observational studies that was developed by the Scottish Intercollegiate Guideline Network (SIGN).[130]

To supplement the database search, grey literature searches were conducted using two grey literature resources. Firstly, we used the University of Ottawa Health Sciences grey literature research guide.[131] Sources searched included the following: Web of Science Conference Proceedings Citation Index-Science (CPCI-S) --1990-present, Canadian electronic library, Canadian Research Index, and COS-Conference Papers Index, Health Reports (Statistics Canada), ClinicalTrials.gov, ProQuest Dissertations and Theses Global, DART-Europe E-theses Portal, and Open Access Theses and Dissertations. Secondly, we used the checklist developed by the Canadian Agency for Drugs and Technologies in Health (CADTH), 'Grey Matters: a practical search tool for evidence-based medicine'.[132] Relevant sources searched were: LILACS: Latin American and Caribbean Center on Health Sciences Information, TRIP: Trip Database - Clinical Search Engine, Centre for Reviews and Dissemination, National Institutes for Health Research –Dissemination Center Discover portal, PROSPERO, BMJ Clinical Evidence, and Public Health Agency of Canada: Reports and publications.

As a final step, we searched the bibliography of eligible studies (i.e., studies identified from the full-text screen). The search strategy was refined in consultation with information specialists who reviewed the search as per 'An evidence Based Checklist for the Peer Review of Electronic Search Strategies (PRESS EBC)'.[133] The search was last updated on 16 April 2019.

STUDY SELECTION

All citations retrieved from the database search and other sources were saved in Mendeley ® and duplicate citations removed. The citations were uploaded to Covidence ®, a web-based systematic review software. In the first screen, two reviewers (SC and WS) independently screened the title and abstract of all citations. In the second screen the two reviewers independently screened the full texts of articles included from the first screen. Screening procedures followed pre-specified eligibility criteria. Conflicts were resolved by consensus. Primary authors were contacted by email in the case of missing or insufficient information on gestational age of homocysteine measurement. The study flow diagram was generated according to the PRISMA statement (i.e., preferred reporting items for systematic reviews and meta-analyses).[134]

Data were independently extracted by the two reviewers; disagreements were resolved by consensus. Both reviewers pilot tested the data extraction form (Supplementary file 2) on a sample of eligible studies. The Newcastle-Ottawa Scale (NOS) was used to assess study quality (Supplementary file 3).[135] For the comparability component of the NOS, studies were given one star if adjusting (via study design or analysis) for gestational age at homocysteine measurement and one star if adjusting for any one of maternal BMI, serum folate/folic acid supplementation, smoking, or maternal age. The summary measures of interest were homocysteine concentration (and a measure of variance) in cases and controls, the risk ratio (RR), or odds ratio (OR) for placenta-mediated outcomes due to elevated maternal plasma homocysteine. Primary authors were contacted by email in the case of missing or insufficient information on the associations.

Data synthesis and meta-analysis methods

We conducted a meta-analysis of the mean difference in homocysteine concentration using the mean and standard deviation (SD) of homocysteine concentration in cases and controls. Where either the mean or SD were not reported in a given study they were calculated from available information including the sample size, median/mean (for SD), range, or interquartile range.[136,137] Data were analyzed in RStudio version 0.99.892, R version 3.2.3, with the packages meta and metafor.[138] If sufficient homogeneity was found overall or within pre-specified subgroups, then the mean difference for a given outcome was pooled using random effects methods. Heterogeneity was assessed using the I^2 method. Publication bias was assessed by visual inspection of the funnel plot.

Meta-regression was conducted if a sufficient number of studies were characterized by differences that included study design, study quality—from the total score of the Newcastle-Ottawa Scale (NOS), the comparability sub-component of the NOS, and outcome-specific factors; for example, severity of preeclampsia or timing of pregnancy loss. Another factor we examined was probable folate status of the sample as a surrogate measure for individual folate status.

Probable folate status was defined according to a 2012 publication by Clarke and colleagues, which categorized probable folate status based on national policies for mandatory folic acid fortification of food staples and a meta-analysis of mean serum folate concentrations from 81 surveys of 200,103 healthy participants.[12] The categories defined by Clarke et al. were: 1) Low folate, Asia, no fortification; 2) Low folate, Europe, pre-fortification (i.e., 1996); 3) Mid-folate, Europe, post-fortification; 4) Mid-folate, USA and Australasia, pre-fortification; 5) High folate, USA and Australasia, post-fortification. The survey did not include data for Canada, Asia, and South America post-fortification; because of mandated folate fortification in Canada, we assumed the high-folate

category for Canada, similar to the USA and mid-folate category for Asia and South America, similar to Europe post-fortification. We used three categories for probable folate status: low-, mid- and high-folate. A separate variable was included for study region.

Following the approach of Mignini et al.,[15] meta-regression was conducted separately (i.e., univariable analysis) for each factor to conserve power. This was followed by multivariable meta-regression for significant factors only. Subgroup meta-analyses were conducted for significant factors that accounted for most of the observed heterogeneity.

5.5 RESULTS

From 411 non-duplicate records we identified a total of 30 studies that measured maternal homocysteine from preconception to the mid-second trimester and reported on the placenta-mediated outcomes of interest (Figure 5.1). At the full text screen the most common reason for exclusion (82 studies) was maternal homocysteine measured during the 3rd trimester or during the post-partum period. Results from 28 studies were included in the meta-analysis.

Table 5.1 presents study characteristics, including study design, country, year of participant recruitment, and the type of effect estimates reported. All studies were either prospective cohort or nested case-control studies; the larger cohort studies included several thousand participants. The studies were conducted in diverse regions, and recruitment took place starting from the late 1980s in earlier publications. The majority of studies compared homocysteine concentration in cases versus controls and many also presented effect estimates with elevated homocysteine as a dichotomous exposure. Four of the five Authors who were contacted provided information on unreported homocysteine concentration in cases versus controls. Homocysteine was measured using different methods that included high performance liquid chromatography, fluorescence polarization immunoassay, enzymatic methods, and amino acid analyzer (one study). Among the included studies the outcome reported most frequently was preeclampsia (24 studies), followed by small for gestational age (SGA) (13 studies), pregnancy loss (6 studies; 2 studies within a composite outcome), placental abruption (2; 2 studies within a composite outcome), and a composite outcome (3 studies). Information about probable folate status was obtained mainly from external sources.[34,139]

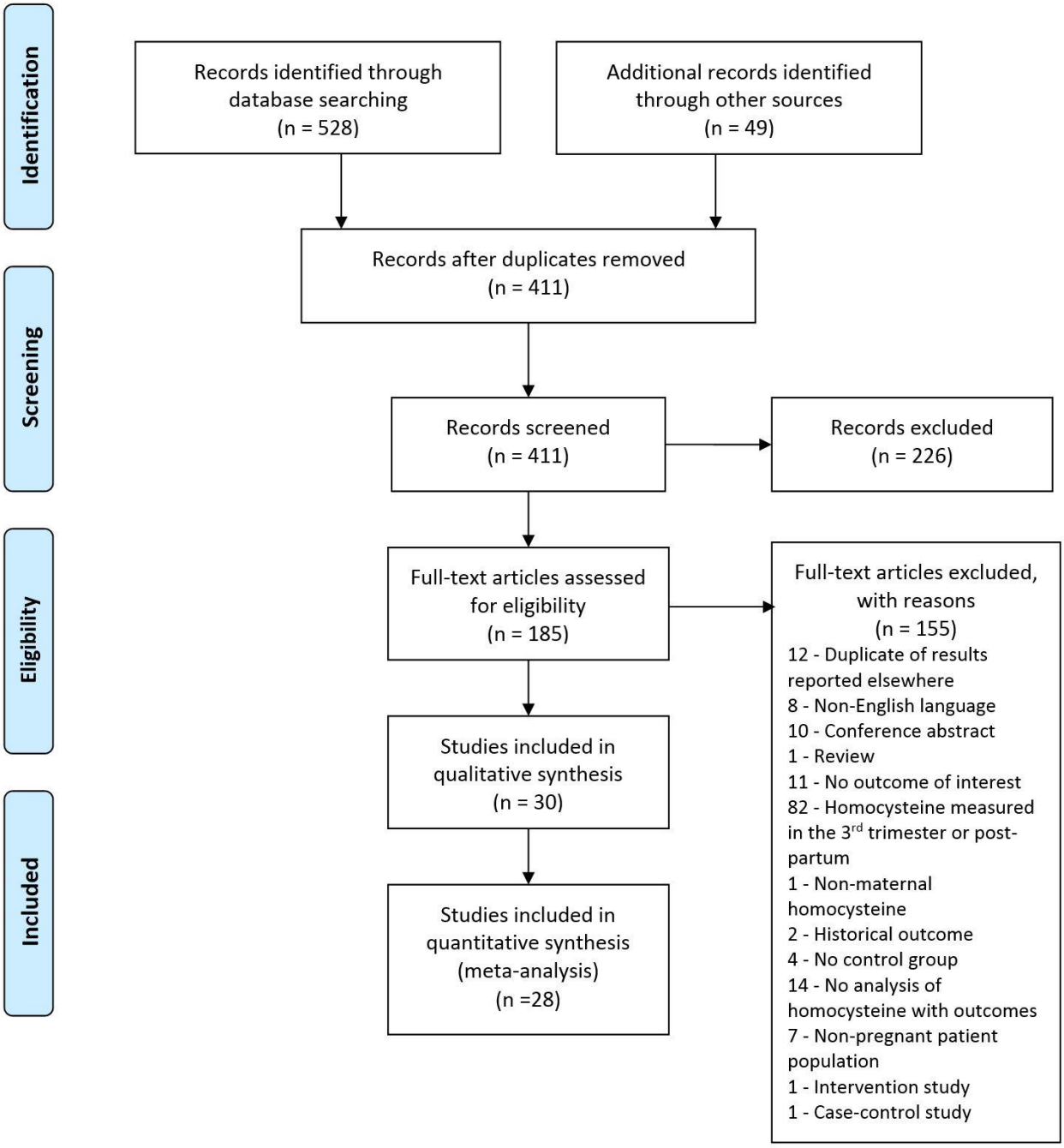

Figure 5.1 PRISMA Flow Diagram

Table 5.1 Characteristics of studies included in the systematic review (n=30)

Study author, year	Country, Ethnicity	Enroll-ment year(s)	Design	Participants		Outcomes of interest	GA of homocysteine measurement	Technique for measurement of homocysteine	Reporting of homocysteine		Folate status *
				N	Inclusion/ exclusion criteria				µmol/L cases vs. controls	Other	
Sorensen, 1999 [114]	USA (Washington), Black 11%	1993-1995	Nested case-control	108	Controls remained normotensive throughout pregnancy	preeclampsia	15-22 weeks	high performance liquid chromatography and electrochemical detection	yes	dichotomous exposure	Mid
Hogg, 2000 [126]	USA (Alabama), African American	1991-1993	Nested case-control	437	Healthy women, zinc levels <median	SGA, preeclampsia	26 weeks	high-performance liquid and chromatographic fluorescent detection	yes	no	Mid
Murakami, 2001 [140]	Japan, Japanese	1996-1998	Prosp-ective cohort	749	no prior significant medical illnesses, no intake of vitamins/ folic acid	SGA, preeclampsia, pregnancy loss	6-12 weeks	automatic amino acid analyzer	no	dichotomous exposure	-
Cotter, 2003 [116]	Ireland, Irish background (genetically homogeneous)	1986-1990	Nested case-control	213	Normotensive controls with uncomplicated pregnancies	non-severe preeclampsia	Mean ± SD in Cases/Controls: 15.9 ±3.6/ 15.6 ± 3.4	fluorescence polarization immunoassay	yes	dichotomous exposure	Low
Cotter, 2001 [115]	Ireland, Irish background (genetically homogeneous)	1986-1990	Nested case-control	168	Normotensive controls with uncomplicated pregnancies	severe preeclampsia	Mean ± SD in Cases/Controls: 15.3 ± 4/ 14.9 ± 3.4	fluorescence polarization immunoassay	yes	dichotomous exposure	Low
Ronnenberg, 2002 (AJCN) [101]	China, Chinese	1996-1998	Nested case-control	423	Married women working in textile factory,	SGA	Preconception	high-performance liquid chromatography	yes	dichotomous exposure	Low

					primigravid, non-smokers						
Ronnenberg, 2002 (OG) [102]	China, Chinese	1996-1998	Nested case-control	458	Married women working in textile factory, primigravid, non-smokers	Early pregnancy loss	Preconception	high-performance liquid chromatography	yes	dichotomous exposure	Low
Hietala, 2001 [141]	Finland, NR	1996	Nested case-control	917	Type 1 diabetes, multiples, spontaneous abortion excluded	preeclampsia	16 weeks	high-performance liquid chromatography and fluorescence detection	yes	dichotomous exposure	Low
D'Aniello, 2003 [142]	Italy, Italian origin	NR	Prosp-ective cohort	59	gestational hypertension, systemic diseases excluded: diabetes, renal disease, chronic hypertension	preeclampsia	24-26 weeks	fluorescent polarized immunoassay	yes	no	Mid
D'Anna, 2004 [143]	Italy, NR	2000-2001	Nested case-control	1874	Non-smoking controls without SGA and pregnancy complications	SGA, preeclampsia	16 weeks	bead chemilumine-scence	yes	no	Mid
Zeeman, 2003 [144]	USA (Texas), Hispanic 40%, Black 49%, and White 9%	2000-2002	Prosp-ective cohort	57	Women on antihyperten-sive therapy <20 weeks	preeclampsia	16-20 weeks	fluorescence polarization immunoassay	yes	dichotomous exposure	High
Polat, 2016 [145]	Turkey, homogenous ethnicity	2002-2004	Nested case-control	130	Multiples, chronic disease, drug intake, recurrent miscarriage, fetal demise, thromboem-bolic disease (including	preeclampsia	24-28 weeks	high performance liquid chromatography	yes	dichotomous exposure	Low

					family history) excluded						
Onalan, 2006 [118]	Turkey, NR	2004-2005	Nested case-control	406	Multiples, chronic disease, and high folate intake excluded	SGA, preeclampsia, composite outcome (including placental abruption, and stillbirth)	15-19 weeks	fluorescence polarization immunoassay	yes	dichotomous exposure	Low
Roes, 2006 [40]	The Netherlands, NR	2000-2001	Prosp-ective cohort	25	Medication use before study excluded	preeclampsia	Preconception	high performance liquid chromatography	yes	no	Low
Dodds, 2008 [18]	Canada, NR	2002-2005	Prosp-ective cohort	2119	Multiples, premature (<24 weeks) excluded	SGA, preeclampsia, pregnancy loss	4-20 weeks	fluorescence polarization immunoassay	no[†]	dichotomous exposure	-
Malek-khosravi, 2009 [146]	Iran, Ethnically homogeneous	1999-2004	Nested case-control	40	Overt diabetes or gestational, chronic hypertension, and renal failure excluded; controls normotensive throughout	preeclampsia	20-24 weeks	solid phase enzyme immunoassay	yes	no	Low
Kahn, 2009 [20]	Canada, 25%-29% born outside of Canada	1999-2003	Nested case-control	556	severe maternal chronic disease excluded	preeclampsia	24-26 weeks	fluorescence polarization immunoassay	yes	dichotomous exposure	High
Rodriguez-Guillen, 2009 [147]	Mexico, Mexican	2001-NR	Nested case-control	97	Non-lactating without history of chronic disease	pregnancy loss <20 weeks	Preconception	high performance liquid chromatography and fluorescent detection	yes	no	Mid

Furness, 2011 [110]	Australia, NR	2004-2006	Nested case-control	137	Multiples, fetal anomaly, and renal disorders excluded	SGA, preeclampsia	18-20 weeks	Fluorescence polarization immunoassay	yes	continuous exposure	High
Dwarkanath, 2011 (Thesis) [148]	India, South Asian	2000-2002	Prosp-ective cohort	637	Multiples, chronic disease excluded	SGA	11-13 weeks	gas chromatography –mass spectrometry	no†	dichotomous exposure	-
Bergen, 2012 [19]	The Netherlands, White-European 59%	2002-2006	Prosp-ective cohort	5805	Multiples, premature (<24 weeks) excluded	SGA, preeclampsia	Median (90% range): 13.4 (11.4–16.5)	immunoelectro-chemolumine-scence assay	no†	highest vs. lowest quintile	-
Mascarenhas, 2014 [149]	India, South Asian	2009-2011	Prosp-ective cohort	90	Diabetics and hypertensive treatment excluded	pregnancy loss	8 to 12 weeks	Chemilumine-scent immunoassay	yes	no	Mid
Wadhwani, 2016	India, South Asian	Unknown	Nested case-control	188	Multiples and most chronic diseases excluded	preeclampsia	16-20 weeks	Chemilumine-scent microparticle immunoassay	no	dichotomous exposure	-
Chaudhry, 2019 [150]	Canada, Caucasian 87%	2002-2009	Prosp-ective cohort	7587	Multiples, terminations excluded	SGA, preeclampsia, placental abruption, pregnancy loss, composite outcome	12-20 weeks	fluorescence polarization immunoassay	yes	continuous exposure	High
Lopez-Alarcon, 2015 [113]	Mexico, NR	2009-2011	Prosp-ective cohort	228	High-risk population; smoking, hypertension, diabetes excluded	preeclampsia	<20 weeks	high-performance liquid chromatography with fluorescence detector	yes	no	Mid
Cheng, 2016 [117]	Taiwan, NR	2007-2013	Nested case-control	284	Chronic disease and fetal chromosomal abnormalities excluded	preeclampsia	11-13 weeks	fluorescence polarization immunoassay	yes	no	Low

Choi, 2016 [151]	South Korea, NR	2012-2013	Prosp-ective cohort	278	Multiples excluded	SGA, preeclampsia	64 % in 1st- 2nd trimester	high-performance liquid chromatography with mass spectrometer	yes	no	Low
Cawley, 2019 [152]	Ireland, 75% born in Ireland	2014-2016	Prosp-ective cohort	498	Multiples, history of NTD, inability to understand English excluded	SGA	5-23 weeks	Enzymatic method	no†	continuous exposure; correlation	Mid
Maged, 2017 [103]	Egypt, NR	2015-2016	Prosp-ective cohort	453	Multiples, age >40, chronic disease, folic acid or folate antagonist drug intake excluded	SGA, preeclampsia, other complications (pregnancy loss and placental abruption)	15-19 weeks	Chemilumine-scence immunoassay	yes	no	Mid
Sun, 2017 [153]	China, NR	2016	Prosp-ective cohort	4565	Chronic disease, fetal abnormality, and stillbirth excluded; unexplained IUGR and placental abruption excluded as controls	Mild preeclampsia (excluding severe), severe preeclampsia (excluding mild)	11-13 weeks	Enzymatic cycling method	yes	continuous exposure	Mid

Abbreviations: GA, gestational age; SGA, small for gestational age; NR, not reported; OG, Obstetrics & Gynecology (Journal); AJCN, American Journal of Clinical Nutrition

* Probable folate status determined for studies included in the meta-analysis

† Data on homocysteine concentrations in cases versus controls were provided by the Author

In studies that reported effect estimates as an odds ratio or relative risk of the outcome due to elevated homocysteine, the homocysteine exposure was most often dichotomized using percentile-based cut-offs (e.g., 90th percentile of the homocysteine distribution in entire sample or in controls), or using definitions for elevated homocysteine from previous studies (Supplementary file 4, Tables D2, D4, D6, D8, D10). Because of the different cut-offs, these studies could not be combined statistically. However, some patterns were noted: for the outcome SGA, effects were significant and positive in larger studies, whereas in preeclampsia effects were significant and positive in smaller and earlier studies.

In studies that reported homocysteine concentration in cases versus controls, (Supplementary file 4, Tables D1, D3, D5, D7, and D9), the results could potentially be combined statistically for the outcomes SGA, preeclampsia, and pregnancy loss. For the outcome SGA, a random effects meta-analysis revealed a significant pooled mean difference in homocysteine between SGA cases and controls of 0.35 µmol/L (95% confidence interval (CI) 0.19 to 0.51), with moderate heterogeneity (I^2 = 33% [0%–66%]) (Figure 5.2). From the meta-regression analysis the factor that accounted for the heterogeneity was study region (Table 5.2).

For the outcome preeclampsia, a random effects meta-analysis revealed a significant pooled mean difference in homocysteine between cases and controls of 0.87 µmol/L (95% CI 0.52 to 1.21), with considerable heterogeneity (I^2 = 92% [89%–94%]) (Figure 5.3). From the meta-regression analysis the factors that accounted for 63% of the heterogeneity were study region, probable folate status, severity, and whether the study had adjusted for maternal factors in the design of the study (Table 5.2). Figure 5.3 shows that the difference in homocysteine concentration was larger and significant in the low- and mid-folate subgroups and smaller and non-significant in the high folate subgroup.

For the outcome pregnancy loss, a random effects meta-analysis of one low quality study (with a Newcastle-Ottawa Score of 5) and four high quality studies (Newcastle-Ottawa Score of 7) revealed a significant pooled mean difference in homocysteine of 1.24 μmol/L (95% CI 0.25 to 2.23), with high heterogeneity (I^2 = 92% [85%–96%]) (Figure 5.4). From the meta-regression analysis the factor that accounted for 66% of the heterogeneity was study quality (Table 5.2). In the four higher quality studies, a trend towards a significant difference was observed for pregnancy loss: 0.63 μmol/L (95% CI -0.02 to 1.27), with substantial heterogeneity (I^2 = 84% [61%–94%]).

Visual inspection of the funnel plots of the meta-analyses revealed symmetric plot for the outcomes SGA and some evidence of publication bias from one study for the outcome pregnancy loss (Figure 5.5). The funnel plot for the outcome preeclampsia showed asymmetry in the clustering of effects.

Table 5.2 Random-effects meta-regression analyses of studies reporting mean homocysteine concentration (µmol/L) for the outcomes small for gestational age (SGA), preeclampsia, and pregnancy loss

	SGA		Preeclampsia		Pregnancy loss	
Factor	I^2	R^{2*}	I^2	R^{2*}	I^2	R^{2*}
Study design	37.66%	0%	91.83%	0%	94.05%	0%
Probable folate status	43.93%	0%	80.94%	50.67%	95.35%	0%
Region	0%	100%	80.97%	49.66%	95.35%	0%
NOS total score †	38.92%	0%	91.97%	0%	84.28%	66.38%
NOS GA adjustment ‡	38.88%	0%	91.97%	0%	94.05%	0%
NOS Other factor adjustment ‡	39.22%	0%	88.79%	15.01%	NA §	
Outcome-specific	NA		Severity ‖: 91.38%	0.52%	Timing ¶: 94.05%	0%
Multivariable meta-regression with significant factors	NP		Probable folate status, Region, Severity, Other factor adjustment: 75.13%	63.65%	NP	

Abbreviations: SGA, small for gestational age; NOS, Newcastle-Ottawa Scale; GA, gestational age; NA, not applicable; NP, not performed

* R^2: Proportion of heterogeneity explained by the factor(s)

† Newcastle-Ottawa Scale, Score components: Selection (4 ✵), Comparability (2 ✵), Exposure (case-control study) or Outcome (cohort study) (3 ✵)

‡ Newcastle-Ottawa Scale, Comparability component: Comparability of most important factor gestational age (GA), 1✵; Comparability of other factors: BMI, serum folate/folic acid supplementation, smoking, or maternal age, 1✵

§ None of the studies adjusted for other factors

‖ Severe versus non-severe preeclampsia

¶ Loss before 20 weeks versus any time throughout pregnancy

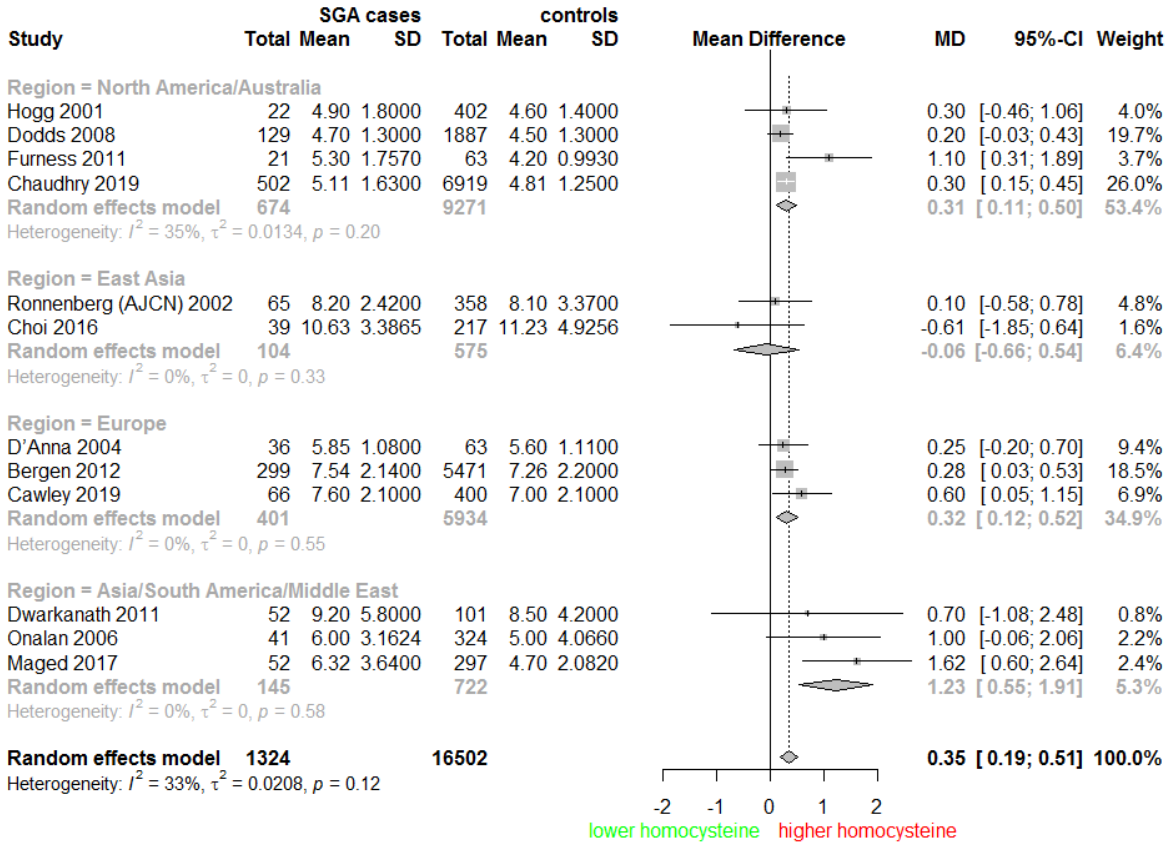

Figure 5.2 Forest plot of the random-effects meta-analysis for the outcome small for gestational age (SGA), from Table D1.
Results presented as mean difference in homocysteine concentration (µmol/L), with 95% confidence interval, between SGA cases and controls. The size of the square represents the weight contributed by each study.

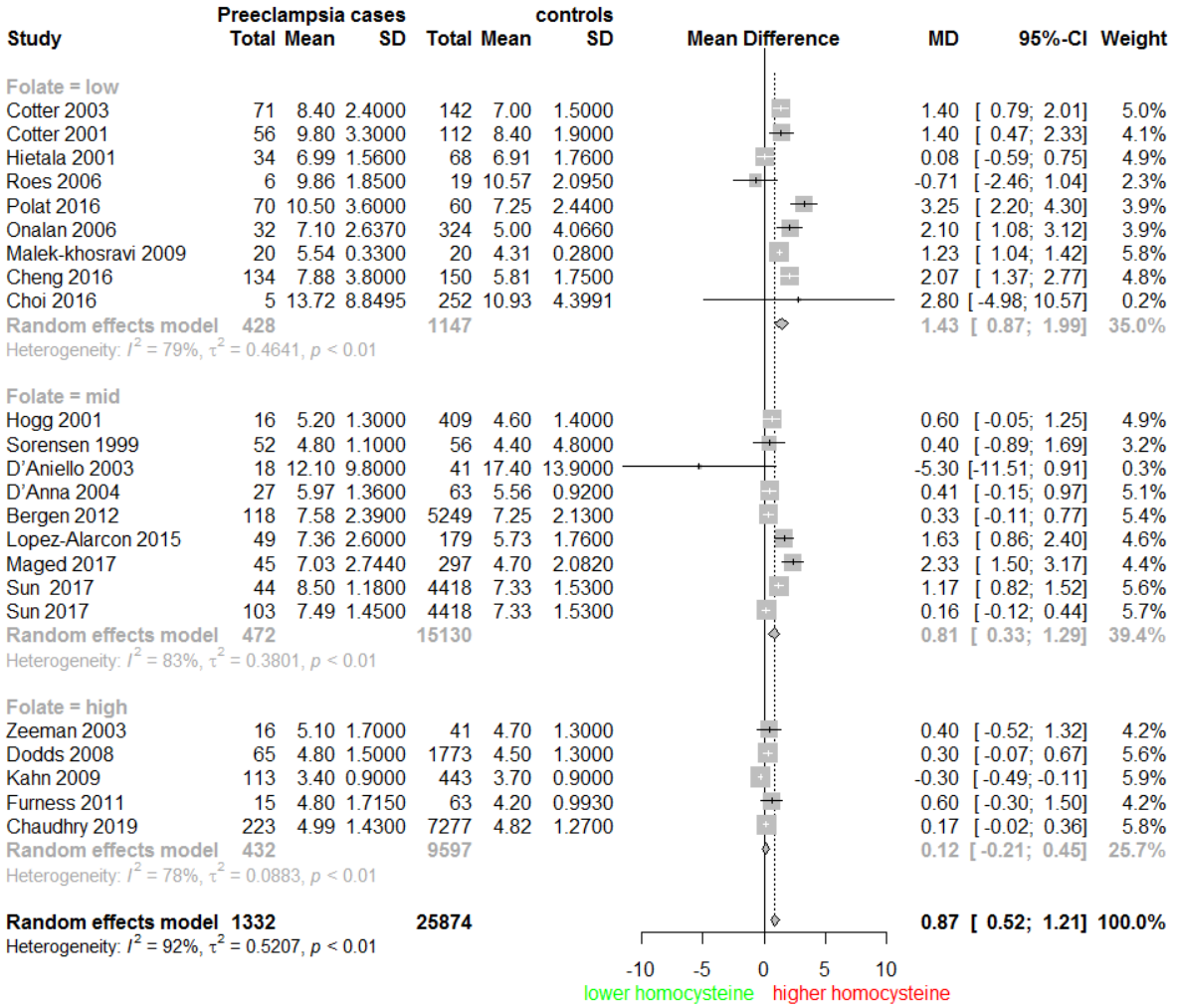

Figure 5.3 Forest plot of the random-effects meta-analysis for the outcome preeclampsia, from Table D3.
Results presented as mean difference in homocysteine concentration (µmol/L), with 95% confidence interval, between preeclampsia cases and controls. The size of the square represents the weight contributed by each study.

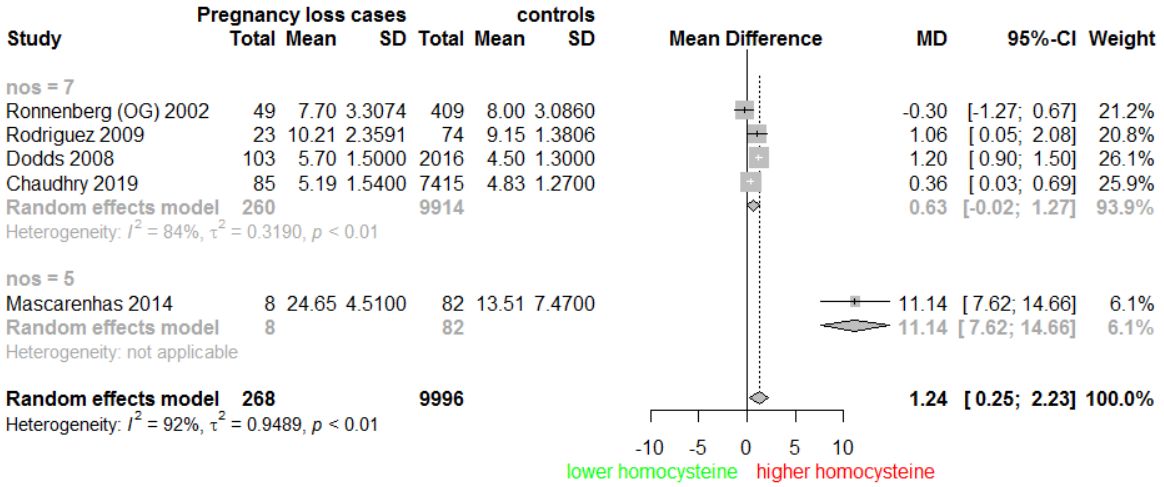

Figure 5.4 Forest plot of the random-effects meta-analysis for the outcome pregnancy loss, from Table D7.
Results presented as mean difference in homocysteine concentration (µmol/L), with 95% confidence interval, between pregnancy loss cases and controls. The size of the square represents the weight contributed by each study. NOS stands for Newcastle-Ottawa Scale.

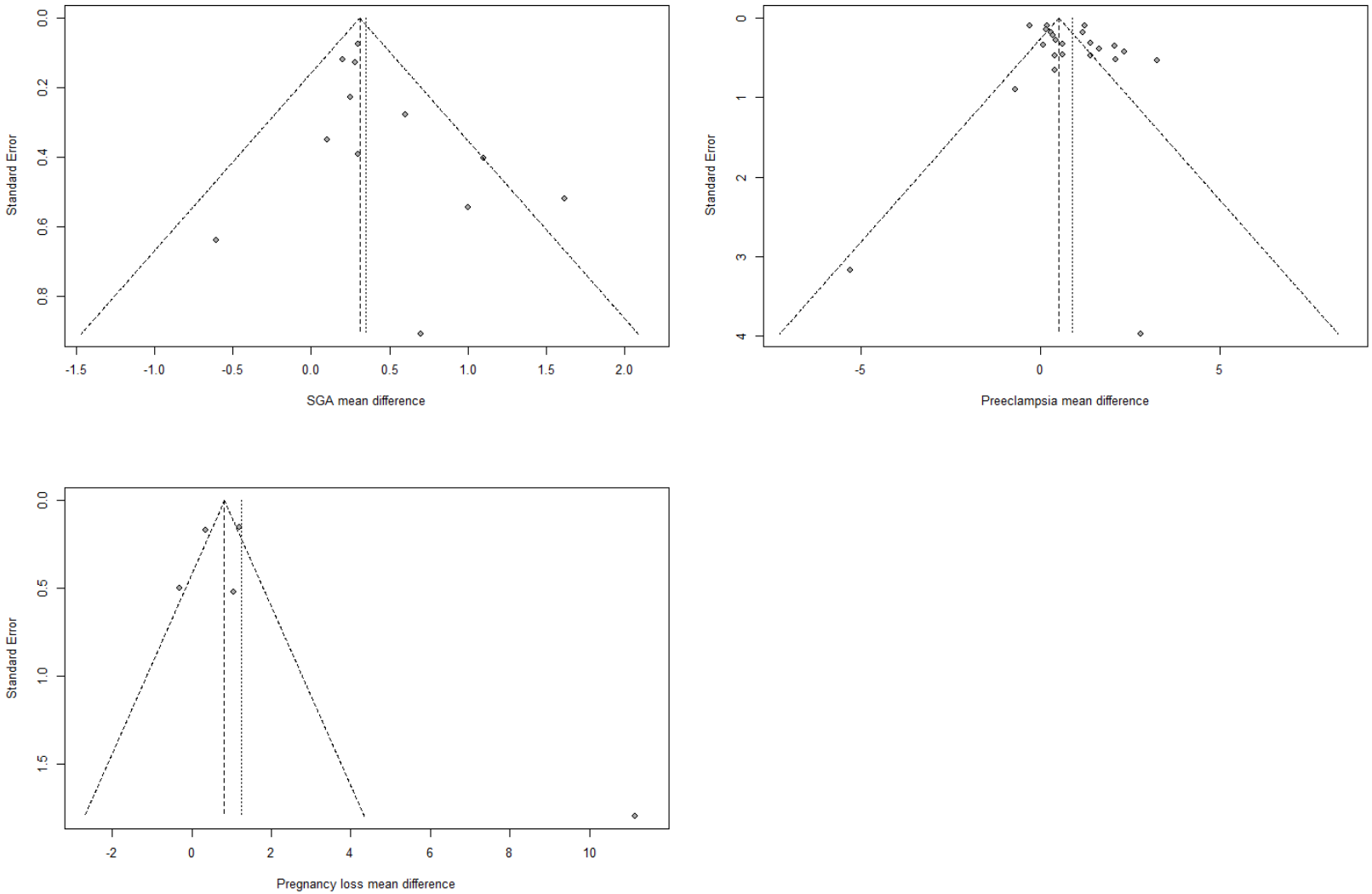

Figure 5.5 Funnel plots of the random-effects meta-analyses for the outcomes small for gestational age (SGA), preeclampsia, and pregnancy loss.

To investigate publication bias, each circle represents mean difference in homocysteine concentration (µmol/L) between cases and controls on the x-axis and the standard error on the y-axis.

5.6 DISCUSSION

Our systematic review investigated whether women with elevated plasma homocysteine concentration are at increased risk for placenta-mediated pregnancy complications. In our meta-analysis we have found that mean homocysteine concentration measured from preconception to the second trimester was significantly higher in study participants who subsequently delivered a small for gestational age (SGA) infant, with moderate heterogeneity, and was significantly higher in those who developed preeclampsia, with considerable heterogeneity. In good quality studies a trend towards higher homocysteine was observed in those who experienced pregnancy loss, with substantial heterogeneity. Investigating sources of heterogeneity revealed that for the outcome SGA study region (i.e., country of recruitment) accounted for the observed heterogeneity in the mean differences in homocysteine between case and control participants. For the outcome preeclampsia, study region, probable folate status, covariate adjustment, and severity of preeclampsia accounted for more than half of the observed heterogeneity, but high heterogeneity of approximately 75% remained unaccounted for.

Strengths and limitations

The main strength of this study is that temporality of the association was maintained by including only those studies that had measured homocysteine from preconception to the second trimester of an index pregnancy. Although homocysteine was measured using different methods, comparison studies have shown these methods to be concordant overall.[154–156] The design of included studies was prospective or retrospective cohort or nested case-control; as a result most scored well on the quality assessment scale. Additional strengths of the study were peer review of the search strategy using the PRESS standard[133] and the search of grey literature.

We were unable to statistically combine results from all studies because of differences in how effect estimates were reported. Different cut-offs for elevated homocysteine during pregnancy is problematic[157] and limited our ability to compare results across studies. By extension, conducting a meta-analysis of the mean difference in homocysteine meant that adjustment could by definition only have been for those factors that were matched on or restricted. In the meta-analysis of SGA and preeclampsia, roughly half of the studies had adjusted in this way for gestational age. Having matched or restricted on other important factors was less common, but many of the studies reported a comparable distribution of various factors between cases and controls. Nonetheless, a relatively crude analysis could lead to greater variability and less certainty in the observed effect of homocysteine. This was less of a concern for the outcome SGA, which exhibited less heterogeneity in pooled effects and none due to adjustment (or lack thereof) for relevant risk factors.

Although asymmetry in the funnel plot for the outcome preeclampsia is suggestive of publication bias, it is likely that heterogeneity gave rise to an apparent clustering of effects in the larger studies (i.e., with smaller standard errors). As the distribution of risk factors for preeclampsia can differ between study populations, their control or adjustment is particularly relevant to understand the role of homocysteine.

Interpretation

Results from previous systematic reviews and meta-analyses have suggested an association between homocysteine and placenta-mediated complications.[15,16] Hogeveen et al.'s systematic review and meta-analysis (published in 2012) investigated the association between maternal homocysteine concentration and SGA infant.[16] The review included 21,326 participants from 19

studies that measured maternal homocysteine from pre-conception to post-partum. Estimates that were reported in different ways were converted to an odds ratio (OR) (via a simulation study) for the effect of homocysteine greater than the 90th percentile. Maternal homocysteine greater than the 90th percentile was associated with an increased risk of SGA infant (OR 1.25, 95% CI 1.09-1.44, I^2=0%), with no observed heterogeneity. Comparably, our meta-analysis of twelve studies with over 17,000 participants also showed a slightly higher mean difference in maternal homocysteine between SGA cases and controls. We observed moderate heterogeneity, which was accounted for by study region.

Mignini et al.'s systematic review and meta-analysis (published in 2005) investigated the association between maternal homocysteine and preeclampsia and as in our study, pooled effects showed between-study variability.[15] In six studies with a total of 1876 participants, the pooled mean difference in homocysteine concentration measured before the onset of preeclampsia was 0.68 μmol/L higher (0.40-0.96, I^2=40%) in women who developed preeclampsia. Our meta-analysis of 23 studies with over 27,000 participants also showed a slightly higher mean difference in homocysteine between preeclampsia cases and controls, but with greater heterogeneity than Mignini et al.'s systematic review. The high heterogeneity in our study was likely due to the more recently published studies conducted in diverse regions—East and South Asia, South America, and the greater Middle East,[103,113,117,118,145,146,151,153] compared to earlier studies conducted in Europe and Scandinavia.[115,116,141,142]

The effect of homocysteine on placenta-mediated outcomes might vary across regions due to differences in genetics and dietary patterns. A single nucleotide polymorphism (SNP) C77C>T in the gene encoding the enzyme methylene tetrahydrofolate reductase (MTHFR) results in a

thermolabile enzyme with approximately 50% reduced activity;[11] it is associated with both higher homocysteine and lower folate status. An analysis of 7000 newborns worldwide showed ethnic and regional differences (including within ethnicities) in prevalence of the TT homozygous MTHFR genotype; the TT genotype ranged from 4-6% and 10-12% in parts of Europe to as high as 32% in Mexico.[158] MTHFR 677C>T- dependent elevation in homocysteine (i.e., penetrance) is influenced by study population factors that include age, race, and folic acid intake.[159] Meta-analyses have showed a 20% difference in homocysteine levels in TT versus CC individuals, ranging from 25% in non-folate-fortified East Asian populations to 7% in folate-fortified North American (Canada and USA) and Australasian (Australia and New Zealand) populations.[12] Thus in combination with population characteristics, probable folate status is likely to play a role in determining penetrance of the MTHFR 677C>T polymorphism.

Greater heterogeneity in pooled effects was observed for the outcome preeclampsia. The comparability component of the Newcastle-Ottawa Scale accounted for some of the observed heterogeneity; this component was used to assess whether a study had adjusted for important factors (i.e., BMI, folate/folic acid supplementation, smoking, maternal age). Risk factors for preeclampsia include maternal BMI and cardio-metabolic factors.[160] Given that a number of the included studies had excluded participants based on chronic disease (Table 5.1), the study populations included in our systematic review would likely have differed in the distribution of preeclampsia risk factors including BMI and chronic hypertension. This variability in risk factor distribution could be an important source of variability that was unaccounted for. For example, women with high BMI (i.e., overweight or obese) could particularly be susceptible to low folate, especially in non-fortified populations. Shen et al.[161] examined the association of BMI with serum and red blood cell (RBC) folate in the OaK birth cohort. In pregnant women, serum folate

was inversely correlated with BMI whereas RBC folate was positively correlated; these findings confirmed altered pharmacokinetics due to BMI, similar to the general population.

With regard to mandated folate fortification, the rate of preeclampsia did not significantly change after the introduction of folic acid fortification in Canada.[162] Post-folic acid fortification, serum folate was also not associated with the risk of preeclampsia in prospective cohorts of Canadian women.[150,163] However, in a non-fortified European sample, serum folate was associated with the risk of preeclampsia.[19]

As others have proposed,[16] the small but significant association of elevated homocysteine with the risk of placenta-mediated complications is unlikely to influence care in individual pregnancies. Initiatives to lower maternal homocysteine at the population level would be more conducive to favourable outcomes. One such intervention is promoting periconceptional folic acid supplementation. In a study of Irish women, McNulty et al. found a 1 μmol/L higher concentration in first trimester homocysteine when folic acid supplementation was initiated after 6 weeks gestation compared to an earlier start.[164] Moreover, in large population based cohort studies, preconception folic acid supplementation was shown to decrease the risk of preeclampsia and SGA.[165,166] Preconception uptake of folic acid supplementation varies among populations and tends to be sub-optimal.[167] Although most women from folate-fortified populations have adequate levels of folate, subgroups of women of child-bearing age continue to be identified with sub-optimal folate status.[168]

5.7 CONCLUSION

Our systematic review suggests that slightly higher maternal homocysteine is generally associated with placenta-mediated complications, particularly small for gestational age. In sub-groups with lower folate, higher homocysteine may increase the risk of preeclampsia. Higher homocysteine may also increase the risk of pregnancy loss, though more studies would be needed to confirm this association. Factors related to study region, including the distribution of risk factors and penetrance of the MTHFR 677C>T variant in relation to folate status, could be an important source of heterogeneity in the association of homocysteine with placenta-mediated complications. The risks associated with slight elevations in homocysteine could have important implications at the population level, with potential benefits from periconceptional folic acid supplementation, which tends to be suboptimal across populations.

Additional files for Chapter 5 can be found in Appendix C

Supplementary file 1: Systematic review search strategy

Supplementary file 2: Data extraction form

Supplementary file 3: Quality assessment scale

Supplementary file 4: Complete tables of results

CHAPTER 6. THE DETERMINANTS OF MATERNAL HOMOCYSTEINE IN PREGNANCY [MANUSCRIPT]

Chapter 6 presents the manuscript entitled, "The determinants of maternal homocysteine in pregnancy". This manuscript was submitted to the Journal Public Health Nutrition; the current status is revisions requested. The research presented aligns with the second thesis objective. Using data from the OaK Birth Cohort, we investigated the determinants of maternal homocysteine in the early to mid-second trimester of pregnancy, to inform practical efforts aimed at reducing maternal homocysteine concentrations.

6.1 TITLE PAGE

The determinants of maternal homocysteine in pregnancy

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Authors' contributions

SW, MW, RRW, GS, and MR are lead investigators of the OaK Birth Cohort. **SC**, SW, and MT designed the study. **SC** analyzed the patient data and wrote the draft manuscript. **SC**, SW, MT, and AM were involved in interpretation of the data. LG, MW, RRW, GS, and MR critically revised the manuscript and contributed to the final version. All authors read and approved the final manuscript.

6.2 ABSTRACT

Objective: Observational studies have linked elevated homocysteine to vascular conditions. Folate intake has been associated with lower homocysteine, although randomized 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.

Setting/Participants: Data were from the Ottawa and Kingston Birth Cohort with 8085 participants recruited in the early second trimester from 2002 to 2009.

Design: 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 status. Secondary analyses examined alternative approaches to account for gestational age-related changes in homocysteine; z-scores and dichotomizing at the 90^{th} percentile.

Results: In 7587 participants, factors significantly associated with higher homocysteine concentration were nulliparous, smoking, and chronic hypertension, while factors significantly associated with lower homocysteine 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 during pregnancy of >1 mg/day did not substantially increase folate concentration. In the subgroup analysis, MTHFR 677C>T modified the effect of folate status on homocysteine concentration. Secondary analyses showed some differences in the results of our analyses when the dependent variable was dichotomized.

Conclusions: We have identified determinants of maternal homocysteine relevant to the lowering of homocysteine in the post-folic acid fortification era, characterized by folate-replete populations.

Keywords: homocysteine, hyperhomocysteinemia, pregnancy, birth cohort, MTHFR

6.3 INTRODUCTION

Elevated plasma homocysteine is an independent risk factor for cardiovascular disease[41,47] and has also been associated with vascular-related complications in pregnancy.[18,19,39] These include preeclampsia, small for gestational age, and fetal demise.[15,18,19,169]

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.[9,18,19,37] Clinical trials have been inconclusive in determining the efficacy of folic acid supplementation to decrease the risk of vascular conditions, as a consequence of decreased homocysteine concentration.[48,170,171] This may be due to the relatively short duration of these trials[12,13,47,48] and due to the post-folic acid fortification era being characterized by folate-replete populations.[172]

Homocysteine is as 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.[173,174] A common genetic polymorphism in the enzyme methylenetetrahydrofolate reductase (MTHFR) 677C>T can result in moderately elevated homocysteine.[11]

In pregnancy, homocysteine concentrations decrease as early as the first trimester and are lowest during the second trimester.[175] Concentrations rise into the third trimester and do not reach pre-pregnancy values until post-partum.[14] It is therefore essential that any studies investigating homocysteine concentrations during pregnancy account for gestational age at the time of the

blood work. Methods used to account for gestational age-related changes in homocysteine concentration include multivariable regression adjustment for gestational age and dichotomizing the outcome relative to percentiles for gestational age at measurement.[18,19]

We aimed to identify factors associated with lower 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. We also compared different methodological approaches to account for gestational age at the time of homocysteine measurement.

6.4 SUBJECTS AND 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 below 12 or above 20 weeks gestation, carrying twins or multiples, and if they withdrew, were lost to follow-up, or if the pregnancy was terminated.

Ethics approval for the OaK Birth Cohort was obtained from 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 participating in the cohort study as well as banking maternal blood, banking 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).

The OaK Birth Cohort has been described previously.[76] 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.

Samples obtained for homocysteine measurement and MTHFR 677C>T genotyping (in a subset of participants due to logistics) were collected in K_2EDTA Vacutainer tubes (Becton Dickinson,

Lincoln Park, NJ). Samples for homocysteine measurement were immediately placed on ice and within 30 minutes centrifuged in 4 °C at 3000 × *g* for 10 minutes. Blood plasma was aliquoted and stored at -20 °C. Due to logistics MTHFR genotyping for the 677C>T variant was performed in a subset of participants.

Plasma homocysteine (µmol/L) was measured in batches on the Abbott AxSYM II Immunoassay System (Abbott Laboratories, Abbott Park, IL) using fluorescence polarization immunoassay. DNA was extracted using manual extraction and later switched to automated extraction. In manual extraction, blood samples were centrifuged at 2500 × *g* for 10 minutes 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 polymerase chain reaction (PCR) and genotyped using the ABI 3130xl Genetic Analyzer and the ABI PRISM SNaPshot Multiplex Kit (Applied Biosystems, Waltham, MA).

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 minutes. Serum was aliquoted and stored at -20 °C. Serum folate (nmol/L) was measured the Access 2 and UniCel® DxI 800 Immunoassay Systems using manufacturer's reagents (Beckman Coulter, Brea, CA). Samples for folate and homocysteine were measured within one month in batches.

Outcome and determinants of interest

The primary outcome in this study was maternal plasma homocysteine concentration in µmol/L. Factors of interest and interactions were pre-specified from a literature review of studies on homocysteine determinants,[8,9,18,19,37] and from studies investigating the role of folates and homocysteine in placenta-mediated (i.e., vascular-related) pregnancy complications.[9,18–20] We considered the following factors: gestational age at blood work (abstracted from medical records), maternal age, race, education, household income, parity, smoking during pregnancy, BMI (from measured height and weight), diabetes, use of hormonal birth control prior to conception, chronic hypertension, history of a placenta-mediated pregnancy complication (i.e., small for gestational age, preeclampsia, 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. We confirmed Hardy-Weinberg equilibrium of the MTHFR 677C>T genotype.[83]

Statistical analyses

Multivariable regression

In our primary approach, 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 blood work as a continuous variable. Prior to analysis, a variable clustering algorithm was used to rule out multicollinearity among the pre-specified 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.[90] 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 20 imputations are considered adequate for this imputation method.[90]

The regression modelling strategies (rms) package within R was used for the multivariable regression analyses.[107] Continuous variables were modeled with a restricted cubic spline function. Knots were set by default to the following quantiles; three knots at the 10^{th}, 50^{th}, and 90^{th} quantile, four knots at the 5^{th}, 35^{th}, 65^{th}, and 95^{th} quantile, and five knots at the 5^{th}, 27.5^{th}, 50^{th}, 72.5^{th}, and 95^{th} quantile.[104] To build the multivariable model, we first entered all pre-specified variables into the model.[104] Continuous variables were modeled with 5 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 the goodness of fit of the multivariable model by altering the number of knots for continuous variables and to examine the effect of collapsing categories in categorical variables. AIC and BIC penalize the log likelihood for complexity of the model (i.e., number of parameters) with the aim to avoid over-specifying the model.[104] 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 10 imputed datasets.[90,104,108]

Results are presented using regression coefficients (representing mean differences) with 95% confidence intervals. For continuous factors modelled with restricted cubic splines, effect estimates (mean differences) are presented for the 75^{th} versus 25^{th} percentiles. Plots of modeled

associations were generated to interpret the effects of continuous factors analyzed 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 were not self-selected 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.

Secondary analyses

In addition to the primary analysis adjusting for gestational age at blood work as a continuous covariate, we conducted two secondary analyses to examine alternative methods of accounting for gestational age at blood work. The first method used continuous normalized score (z-scores) as dependent variable. Z-scores were calculated for each participant by subtracting the mean and dividing by the standard deviation 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 90^{th} percentile at each gestational week at the time of bloodwork. The multivariable analysis was then repeated for each version of the outcome. Model building followed the same procedures as described for the primary approach.

6.5 RESULTS

Participant characteristics

We analyzed data from 7587 participants from the Ottawa and Kingston (OaK) Birth Cohort (**Figure 6.1**). Sixty-four percent had complete data on all dependent and independent variables.

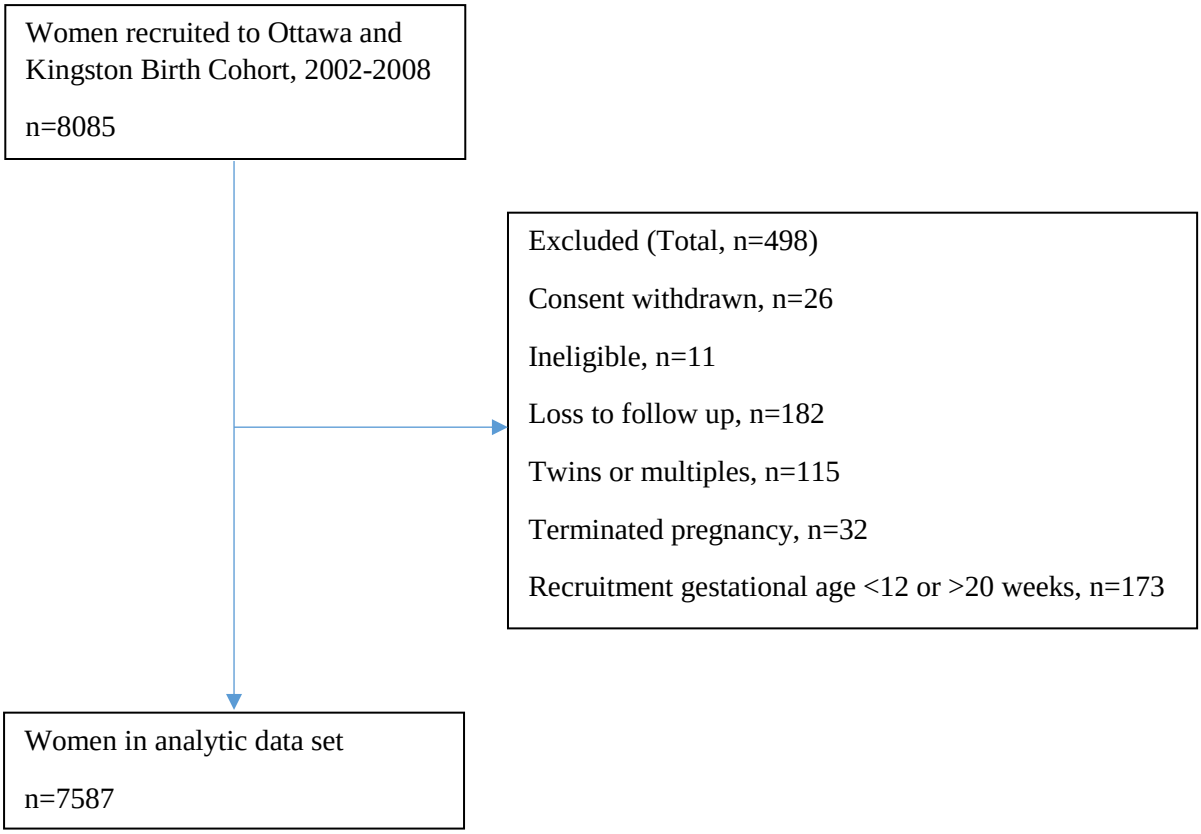

Figure 6.1 Participant flow diagram for the analytic dataset

Table 6.1 presents the distribution of participant demographic characteristics, health indicators, and factors associated with homocysteine metabolism. The majority of participants were non-smokers during pregnancy, had a normal to overweight BMI, were normotensive, non-diabetic and reported taking folic acid-containing supplements at the time of recruitment. Most participants were recruited around 12 to 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.[83] Around 12% of participants were homozygous for the TT mutant genotype.

Table 6.1 Participant characteristics and determinants of interest

Variable	**Frequency n=7587**	
Demographic characteristics		
Age		
Mean (SD)	30.3 (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 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 (SD)	24.9 (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 (Preeclampsia, 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 multivitamin) (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 blood work (weeks)		
Mean (SD)	13.7 (2.09)	
Homocysteine (µmol/L) (missing N=87, 1.1%)		
Mean (SD)	4.8 (1.3)	
Range	1 - 34	
Serum folate (nmol/L) (missing N=987, 13.0%)		
Median (IQR)	37.4 (30.6 – 45.1)	
Range	3.70 – 79.6	
MTHFR genotype [1]	(n=4006)	
CC (wild type)	1768	44.1%
CT (heterozygous)	1760	43.9%
TT (homozygous)	478	11.9%

[1] Measured in a subset of participants (n=4006)

Folic acid supplementation dose was categorized as 0 to 1 mg or greater than 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 30 nmol/L, above which serum folate was less responsive to increasing folic acid dose (**Figure 6.2**). The serum folate distribution was wide and

skewed; to normalize 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 World Health Organization (WHO) sets the cut-off for serum folate deficiency based on homocysteine concentration as a metabolic indicator at 10 nmol/L.[79] We therefore considered the 10% of values beyond the 90th percentile cut-points as equally high and differentiating between these high values as unnecessary.

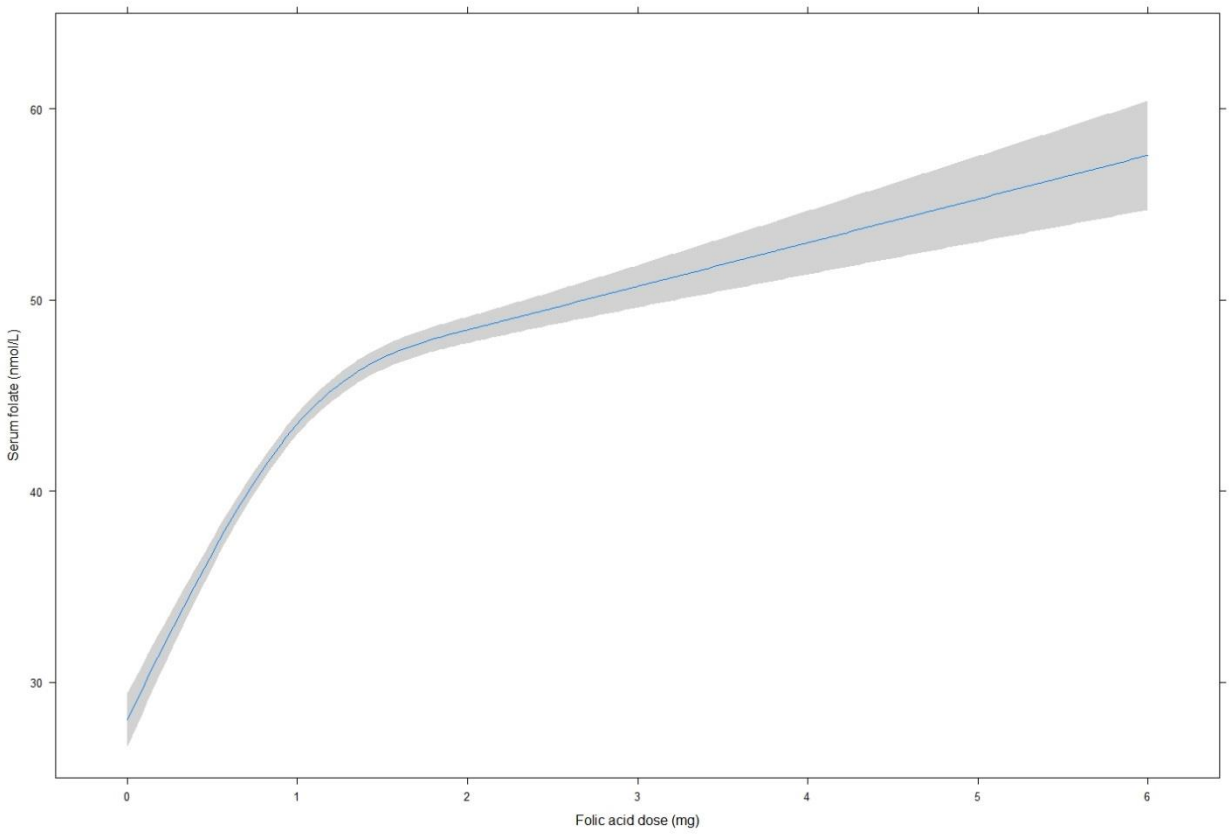

Figure 6.2 Modeled association between folic acid supplementation (restricted cubic spline with five knots) and serum folate, adjusting for gestational age at blood work and homocysteine.
Shaded area represents 95% CI.

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 6.2**. All factors were significantly associated with plasma homocysteine concentration, except for maternal education, diabetes, and hormonal birth control prior to conception (Table 6.2). 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 6.2). 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 6.3 shows plots of associations for continuous factors modeled using a restricted cubic spline function. For gestational age (five knots) the plot of the association demonstrated a peak in homocysteine concentration close to 13 weeks gestation and a decrease thereafter. Plots of the modeled 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 the modeled association demonstrated higher homocysteine concentration (i.e., a negative slope) at serum folate concentrations below approximately 30 nmol/L (Figure 6.3).

Table 6.2 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 [1]
Gestational age at blood work (restricted cubic spline (rcs), five knots)		<0.0001
13.7 versus 12.4 weeks	0.016 (-0.063, 0.095)	
Age (rcs, three knots)		0.0013
34 versus 27 years	-0.027 (-0.070, 0.016)	
Race		<0.0001
African	-0.370 (-0.561, -0.179)	
Middle eastern	-0.438 (-0.594, -0.282)	
Asian	-0.468 (-0.588, -0.349)	
Caucasian	Reference	
Other	-0.405 (-0.612, -0.197)	
Education		0.3987
College/University completed versus less than completed	-0.032 (-0.105, 0.042)	
Nulliparous		<0.0001
Yes versus no	0.140 (0.073, 0.207)	
Smoking		<0.0001
No	Reference	
Second-hand	0.298 (0.097, 0.499)	
Med/light smoker (<10 cigarettes per day)	0.418 (0.297, 0.539)	
Heavy smoker ($\geq$10 cigarettes per day)	0.864 (0.705, 1.022)	
Diabetes		0.6197
Yes versus no	-0.056 (-0.279, 0.166)	
BMI (rcs, three knots)		0.0103
27.3 versus 21.1 kg/m^2	-0.070 (-0.119, -0.022)	
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)	
Chronic hypertension		0.0001
Yes versus no	0.502 (0.250, 0.754)	
History of PMC (Preeclampsia, placental abruption, SGA, stillbirth/loss)		0.0078
Yes versus no	-0.124 (-0.215, -0.033)	
Folic acid supplementation		<0.0001
None	Reference	
>0 and $\leq$1 mg	-0.310 (-0.442, -0.178)	
>1 mg	-0.329 (-0.476, -0.181)	
Serum folate (rcs, 5 knots)		<0.0001
45.1 versus 30.6 nmol/L	0.137 (0.065, 0.210)	

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

Figure 6.3 shows plots of associations for continuous factors modeled using a restricted cubic spline function. For gestational age (five knots) the plot of the association demonstrated a peak in homocysteine concentration close to 13 weeks gestation and a decrease thereafter. Plots of the modeled 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 the modeled association demonstrated higher homocysteine concentration (i.e., a negative slope) at serum folate concentrations below approximately 30 nmol/L (Figure 6.3).

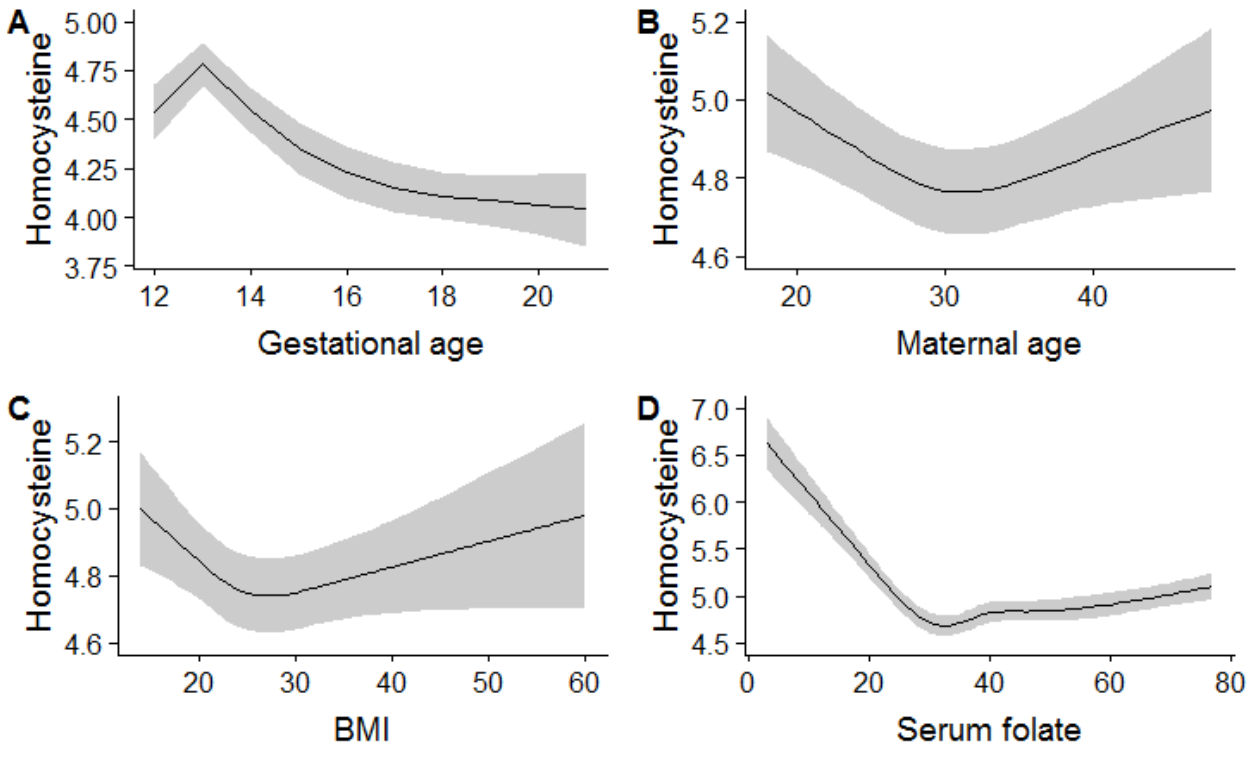

Figure 6.3 Association between homocysteine and continuous variables modeled as a restricted cubic spline.
A) gestational age at blood work with five knots at 12.1, 12.4, 12.8, 13.4, and 19; B) maternal age with three knots at 24, 30, and 37; C) BMI with three knots at 19.6, 23.5, and 32.2; and D) serum folate with five knots at 20.7, 32.3, 39.4, 45, and 74.1. Shaded area represents 95% CI.

Subgroup analysis

The subgroup analysis examining the interaction between MTHFR 677C>T genotype and serum folate is presented in **Supplementary Table S1**. In the subset of 4006 OaK participants with measured genotype the interaction between the MTHFR 677C>T genotype and serum folate was significant (p<0.0001). 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 leveled-off beyond a folate concentration of approximately 30 nmol/L (**Figure 6.4**).

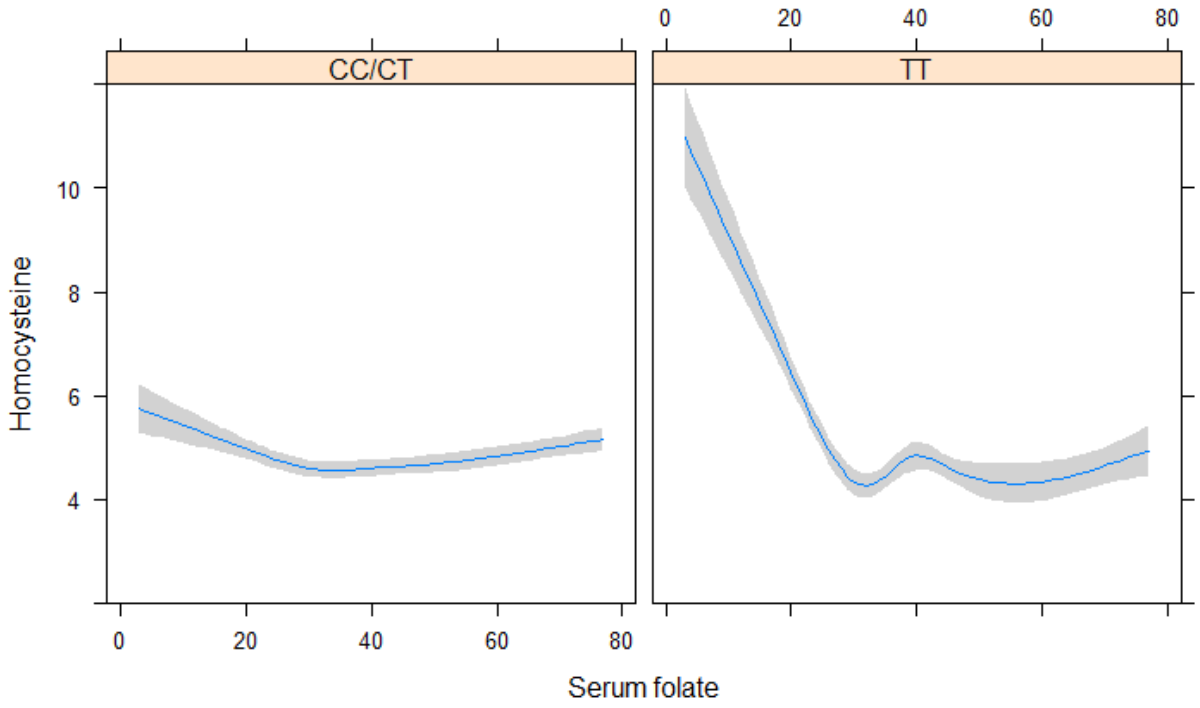

Figure 6.4 Modeled association between serum folate (restricted cubic spline) and homocysteine by MTHFR 677C>T genotype.
Panels by MTHFR genotype; CC (wild-type) or CT (heterozygous) and TT (homozygous mutant). Folate variable has five knots at 20.7, 32.3, 39.4, 45, and 74.1. Shaded area represents 95% CI.

Folic acid supplementation was not associated with homocysteine in the subgroup analysis (Supplementary Table S1). As well, African race (compared to Caucasian) and exposure to second-hand smoke were no longer associated with homocysteine concentration. Plots of the association of gestational age at blood work, maternal age, and BMI modeled using restricted cubic spline functions all demonstrated similar patterns of association with homocysteine concentration, as in the primary multivariable analysis (**Supplementary Figure 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 (**Supplementary Table S2**). In the analysis with the dependent variable specified as a dichotomous variable (homocysteine concentration greater than the 90^{th} percentile for each gestational week of recruitment), there were some differences in results, presented as odds ratios and 95% CI in Supplementary Table S3. Maternal age and history of a placenta-mediated pregnancy complication were no longer associated with homocysteine concentration. Gestational age at blood work was significant despite gestational week at blood work being factored into the outcome.

6.6 DISCUSSION

Using data on 7587 participants from the Ottawa and Kingston (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 age, race, BMI, chronic hypertension, history of a placenta-mediated pregnancy complication, smoking, folate, and folic acid supplementation. 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 has practical implications for the lowering of homocysteine in women of reproductive age.

The traditional approach of dichotomizing 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 analyzing homocysteine as a continuous variable. Categorizing continuous variables is often driven by simplicity and clinical interpretability,[157,176] but for analyses that involve hypothesis testing or estimation it is discouraged and the disadvantages associated with categorizing are well-documented.[104,177] As there is no agreed upon cut-off for elevated homocysteine during pregnancy, the use of data-derived cut-offs is common but additionally problematic because different distributions produce different cut-offs, limiting comparability between studies.[157]

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 analyzed 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 exercise and caffeine consumption, which are among other determinants of homocysteine identified in population-based studies and in studies of pregnant women.[98,178] 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.[179]

Interpretation

Our findings of nutritional, genetic, health and lifestyle determinants of plasma homocysteine in pregnant women agree with findings from previous studies.[37,179,180] The Hordaland Homocysteine study investigated the association of cardiovascular risk factors with non-fasting homocysteine and is the largest study in the general population on homocysteine determinants; approximately 8000 men and 8000 women ages 40 to 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.[180] 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.[19] 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.,[19] 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,[19] but not in the general population.[37,180] A u-shaped association for BMI may be linked to physical activity; in the Hordaland study, BMI modified the effect of exercise on homocysteine.[180]

Homocysteine and folate distributions in the OaK cohort were similar to another Canadian birth cohort that recruited participants in the same time period,[18] but the distributions of homocysteine and folate were lower and higher, respectively, than a European cohort.[19] 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.[12,13,159] Our subgroup analyses found that folic acid supplementation was no longer associated with homocysteine concentration; this finding is 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 above 1 mg/day, 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,[181] but folic acid supplementation during pregnancy has not demonstrated substantial benefit to reduce pregnancy outcomes like preterm birth, stillbirth, low birthweight, and neonatal death.[182] The multi-center 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 preeclampsia in high-risk women.[183] Folate

intake has, however, been identified among the major determinants of plasma homocysteine;[37,98] in the Hordaland study a folate intake score was validated against plasma folate in a subsample of study participants.

A number of studies conducted in the post-folate fortification era in Canada and the USA demonstrate folate-replete populations.[172,184–186] Based on current evidence WHO recommends population level red blood cell (RBC) folate concentrations above 906 nmol/L in women of childbearing age to protect against neural tube defects.[80] A comparison of mean RBC folate concentrations in women ages 15 to 44 years in NHANES III (1988-1994) and NHANES 1999 demonstrated a significant increase three years after mandatory folic acid fortification of cereal grains was introduced.[185] However, the Canadian Health Measures Survey (CHMC) cycle 2007-2009 found that although 1% of the population-based sample was folate deficient, 22% of women ages 15-45 years had suboptimal RBC folate status below 906 nmol/L.[168] In a more recent analysis of 1035 physician-ordered tests of RBC folate concentrations in Toronto, Canada, 7% of women ages 15 to 45 years had suboptimal folate status.[172]

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 six months of supplementation and three 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 per day.

Women taking hormonal contraceptives are another subgroup of women possibly susceptible to low folate status. A recent systematic review and meta-analysis found a significant red blood cell (RBC) and serum folate-lowering effect of oral contraceptive use.[187] However, we found that hormonal contraceptive use prior to conception was not a determinant of homocysteine in the early second trimester of pregnancy. Although in OaK participants homocysteine was measured in the second trimester, 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.[18,19] Thus improved folate intake in the preconception period could offset the folate-lowering effects of hormonal contraceptives.

In the Hordaland study, a six year follow up of participants found reductions in homocysteine concentration associated with increased folate and Vitamin B_{12} concentration, quitting smoking, and weight loss.[188] 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.[39,54] 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 child-bearing 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/day and approximately 22% with a dose of 4000 µg/day. However, 3 months after cessation the effects of the intervention were diminished. Similarly, in a trial of 27 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 discontinuing, homocysteine and folate returned to baseline levels.[189] 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 homocysteine concentration in the long term.[37,109] With or without additional changes, preconception folic acid intake has demonstrated short-term benefits; in a Norwegian population based-cohort study periconceptional folic acid intake shown to modify the effect of continued smoking during pregnancy on homocysteine concentration in the first trimester.[190]

Research is ongoing into the role of homocysteine in the development of cardiovascular disease and vascular-related pregnancy complications. Our findings of 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 characterized by folate-replete populations.

Additional file for Chapter 6 can be found in Appendix D

Supplementary data includes Tables S1-S3, and Figure S1

CHAPTER 7. DISCUSSION

In Chapter 7 we summarize the main findings from Chapters 4, 5 and 6 of the thesis, and discuss the interpretation and implications of these findings.

7.1 SUMMARY OF FINDINGS

We investigated the association of homocysteine with placenta-mediated outcomes in an analysis of data from the OaK Birth Cohort of approximately 8000 participants (Chapter 4) and a systematic review and meta-analysis of the mean difference in maternal homocysteine concentration between participants who developed placenta-mediated complications and those who did not (Chapter 5). The analytical results of the OaK Birth Cohort demonstrated that a slightly higher maternal homocysteine concentration was associated with an increased risk of a composite outcome of any placenta-mediated complication (SGA, preeclampsia, placental abruption, or pregnancy loss) and SGA infant, while the systematic review demonstrated an increased risk of SGA infant and preeclampsia, with moderate and high heterogeneity, respectively. Analysis of the OaK Birth Cohort also suggested a trend towards an association of homocysteine with the risk of pregnancy loss, which was confirmed by findings from the systematic review and meta-analysis.

With regard to modifying effects, in the analysis of the OaK Birth Cohort we found evidence of potential modifying effects of the MTHFR 677C>T polymorphism for SGA and high-risk pregnancy for preeclampsia (Chapter 4). The meta-analysis demonstrated modifying effects of study region for SGA and preeclampsia, probable folate status (based on mandated folic acid-fortification policy and time period) for preeclampsia, and risk factor adjustment and severity for preeclampsia (Chapter 5). The analytical results from the OaK Cohort relate to our findings from the meta-analysis, given that the distribution of high-risk characteristics, the MTHFR 677C>T polymorphism, as well as factors shown to influence penetrance of the MTHFR 677C>T

polymorphism (i.e., smoking, alcohol intake, age, and folate status),[159] likely differ among populations.

In Chapter 6 we investigated determinants of maternal homocysteine, measured in the early to mid-second trimester, in an analysis using data from the OaK Birth Cohort. Participants in the OaK Cohort were recruited in the post-folic acid-fortification era characterized by folate replete populations. We found that self-reported folic acid dosage was positively associated with serum folate, but the associations levelled-off at doses greater than 1 mg. This suggests that higher doses of folic acid during pregnancy, particularly in the early second trimester, may not substantially increase folate and lead to lowered homocysteine concentrations. Additionally, we found that the MTHFR 677C>T polymorphism modified the effect of folate on homocysteine; the TT homozygous mutant genotype resulted in higher homocysteine concentration at lower levels of folate. The overall pattern of results suggested that favourable health status and behaviors has potential to form an effective long-term approach to the lowering of homocysteine in reproductive age women. As well, in a folic acid-fortified population, particular sub-groups may remain susceptible to lower folate and higher homocysteine, including those with the TT variant of the MTHFR 677C>T polymorphism.

Another focus of this thesis was on methodological considerations regarding gestational age-related changes in maternal homocysteine concentration. In the meta-analysis, we found that whether or not a study restricted or matched on gestational age of homocysteine measurement (i.e., if measured in the first to second trimester) did not contribute to between-study

heterogeneity (Chapter 5). In analyses based on the OaK Birth Cohort (Chapters 4 and 6), homocysteine was analyzed as a continuous variable and gestational age of homocysteine measurement was a covariate in multivariable analyses. This was done to retain as much information on the associations while accounting for changes in homocysteine according to gestational age of measurement. In the analysis of homocysteine determinants based on the OaK Cohort (Chapter 6), we compared three different approaches to analyzing homocysteine: as a continuous variable while adjusting for gestational age in the multivariable model, the common approach of a dichotomous variable based on a 90^{th} percentile cut-off (relative to each gestational week of measurement), and as a z-score based on the mean homocysteine concentration and SD by gestational week of measurement. Results across the three analyses were similar except that the dichotomous outcome yielded less stable estimates, likely due to a loss of information.

7.2 STRENGTHS AND LIMITATIONS

Our analytic investigations were based on the OaK Birth Cohort and our systematic review included cohort or nested case-control studies that measured homocysteine prospectively before pregnancy complications were diagnosed. One of the strengths of this thesis, which lends credibility to our findings, is that we found evidence of a similar pattern of modifying effects in the individual patient data analysis based on the OaK Cohort and at the aggregate level in the sub-group meta-analysis and meta-regression analyses.

Given the known pathogenesis of homocysteine,[67,68] we investigated the association between homocysteine and placenta-mediated complications with the assumption that homocysteine is more than a biomarker. However, an overarching limitation of our approach relates to the fact that homocysteine is a metabolite and its concentration depends on a number of measured and unmeasured factors.

Comparison groups in observational studies can lack comparability and residual confounding will remain. Lack of comparability is particularly an issue when there is strong indication for exposure that is linked to prognosis.[191] If confounding by indication is not a major concern, instrumental variable methods are an analytic technique that can be used to achieve comparability between comparison groups in observational studies.[192,193] In section 7.3 we discuss applications of the instrumental variable method for investigating the association between homocysteine and placenta-mediated complications. Instrumental variable analyses have previously been conducted in the form of Mendelian randomization studies, or gene-disease association studies to investigate the association of homocysteine with cardiovascular disease.[12]

7.3 INTERPRETATION OF FINDINGS

Table 7.1 summarizes how the findings from each of the studies in this thesis inter-relate. Maternal homocysteine was associated with all independent variables of interest except for maternal education and diabetes (Chapter 6). We use the outcomes SGA and preeclampsia because these were the outcomes most frequently reported according to our systematic review (Chapter 5). Our findings suggest modifying effects of study region, the MTHFR 677C>T

variant, and high-risk pregnancy, which would likely represent the distribution of population characteristics and risk factors, and modifying effects of probable folate status (Chapters 4 and 5).

Table 7.1 Summary of determinants of maternal homocysteine and factors associated with preeclampsia and SGA

Independent variables	**Homocysteine association**	**SGA**		**Preeclampsia**		
		Association	**Modifying effect**	**Association**	**Modifying effect**	
Diabetes			Study region		Study region	Risk factor adjustment /high-risk (potential)
H_x hypertension						
H_x PMC						
BMI						
Race						
Parity						
SES						
Smoking						
Age						
MTHFR 677C>T			(potential)			
Folate status						
HBC						

Shaded cells represent an association or modifying effect.
Abbreviations: H_x, history of; SGA, small for gestational age; PMC, placenta-mediated complication; SES, socioeconomic status; BMI, body mass index; HBC, hormonal birth control (i.e., prior to conception)

Our findings also suggest that different sets of factors were the source of variability in the association of homocysteine with the outcomes SGA and preeclampsia. Findings from the meta-analysis showed that risk factor adjustment was a source of heterogeneity in the association of homocysteine with preeclampsia (Chapter 5). Analyses of the OaK Cohort (Chapter 4) similarly suggested that high-risk pregnancy, which was defined as the presence of one or more risk factors, could modify the association of homocysteine with preeclampsia. Thus, differences in the prevalence of risk factors predominantly associated with the risk of preeclampsia or SGA could introduce variability between studies investigating the association of homocysteine with these outcomes. In general, our findings suggest that folate status and the distribution of diabetes,

chronic hypertension, and BMI (i.e., factors listed on the top half of the Table 7.1) would likely contribute to variability in the association of homocysteine with preeclampsia, whereas maternal education (i.e., socioeconomic status), smoking, and covariate-dependent penetrance of the MTHFR 677C>T polymorphism (i.e., factors listed on the lower half of Table 7.1) would likely contribute to variability in the association of homocysteine with SGA.

Homocysteine as a causal factor

Rothman's "sufficient-component cause model" provides a basis for the concept of biological interaction and demonstrates how disease can be caused by more than one mechanism. In this framework, elevated homocysteine can be viewed as component cause among multiple causes sufficient for the development of placenta-mediated complications.[194] Within a sufficient cause containing elevated homocysteine as a component cause, it is possible that the potential modifying effects we have identified would comprise important component causes along with homocysteine. Using causal pies, Figure 7 shows how this might be the case. We found that in the study region of East Asia, homocysteine was not associated with SGA (Chapter 5). Low socioeconomic status (SES) is a risk factor for SGA and smoking has been shown to account for close to half of the risk of SGA due to low SES.[195] Moreover, the prevalence of smoking varies worldwide and is lower in East Asia compared to Europe and the Americas.[196] Therefore, if smoking rates are low in East Asia, two of three sufficient causes shown to involve homocysteine would be less likely to form a sufficient cause for the development of SGA (Figure 7).

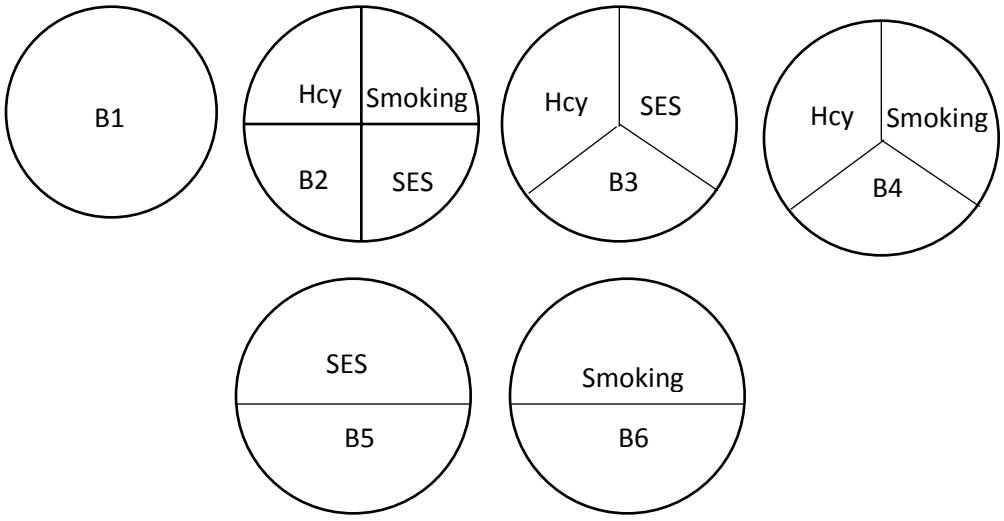

Figure 7 Homocysteine as a component cause among causes sufficient for the development of small for gestational age (SGA) infant.
Each circle represents a sufficient cause with identified component causes. Smoking and socioeconomic status (SES) modify the effect of homocysteine (Hcy) on SGA and exhibit independent effects. B1 to B6 represents a set of overlapping background factors.

Homocysteine is yet to be identified as a causal factor in the development of placenta-mediated complications. This thesis met two causal criteria suggested by Hill, namely temporality of an association, the only necessary criterion to establish causality, and consistency of an association. However, Hill's criteria were originally meant to be 'viewpoints' and not formal criteria to evaluate causation.[191,197]

In an integrative approach to causal inference, Bird (2011) posed the question: 'Under what circumstances, if any, can an observational study give us knowledge of a causal association between two variables?'[198] Our target hypothesis is that the exposure elevated homocysteine causes the outcome placenta-mediated complication. The homocysteine hypothesis for placenta-

mediated complications could be justified if evidence reliably eliminated plausible competing hypotheses. As formulated by Bird,[198] in both observational and experimental studies, the observed association could be accounted for by one of four hypotheses:

1. The target hypothesis (T): Elevated homocysteine or "hyperhomocysteinemia" (Hhcy) causes placenta-mediated complication (PMC);

2. The reverse cause hypothesis (R): PMC causes Hhcy;

3. The common cause hypothesis (C): Hhcy and PMC have a common cause; or

4. The null hypothesis (N): There is no causal relation between Hhcy and PMC—the association between Hhcy and PMC is pure chance.

Temporality of the association forms evidence that would eliminate (R).[198] Consistency of the association in a variety of populations was also demonstrated; where there were inconsistencies potential modifying factors were identified, which reduces the probability of a chance association (N).[198] Our finding of a potential linear association between homocysteine and placenta-mediated complications is suggestive of a dose-response relation, but a common cause (C) may exhibit effects on the same gradient. Observational studies can fail to eliminate all common causes (C) in a systematic way, whereas experimental studies can systematically eliminate (C). Observational studies that can justify comparability between comparison groups could rule out the common cause hypothesis (C).

A summary-level Mendelian randomization study could help to rule out the common cause hypothesis (C). The principles of Mendelian inheritance are the basis for combining genotype-

exposure and genotype-disease associations to investigate causality in the association between an exposure and disease outcome.[199,200] This type of study would be a multiple study synthesis of summary-level data on the MTHFR 677C>T genotype association with homocysteine and of the MTHFR 677C>T genotype association with placenta-mediated complications.[201] In an instrumental variable analysis restricted on factors that account for variability (i.e., study region), estimates from both associations could be used to obtain a Mendelian randomization estimate, interpreted as the effect of long term differences in homocysteine concentration on the development of placenta-mediated complications.[202] The effect of homocysteine could be compared to the estimates obtained from direct analyses of the association, as in our systematic review (Chapter 5). Additionally, the MTHFR 677C>T genotype association with a placenta-mediated complication could indicate a causal association between the exposure, elevated homocysteine, and the outcome, placenta-mediated complication, if the pooled estimate is away from the null.[202] A causal effect can also be visualized if a systematic review includes studies reporting both associations; the association of a placenta-mediated complication with each genotype is plotted against mean homocysteine concentration in each genotype (i.e., TT, CT, and CC). Doing so may illustrate a dose-response relation in the genotype-disease association corresponding to differences in the genotype-exposure association.[203]

7.4 IMPLICATIONS

Analysis of homocysteine concentration

Based on our findings, we strongly recommend that homocysteine be analyzed as a continuous variable. In our systematic review we found that many studies had investigated the effect of

elevated homocysteine, categorizing this variable based on percentile or other cut-offs. Although categorizing homocysteine may simplify the clinical interpretation of an analysis, categorization decreases precision and causes bias, especially in light of modifying effects, which may skew estimates of the true underlying association.[157,177] As our systematic review demonstrated, different cut-offs for categories can also limit comparability between studies, which precludes our ability to effectively combine estimates of effect across studies. As well, to compare across studies it will be necessary for future studies to account for modifying effects such as high-risk participants.

Interventions to reduce homocysteine concentration

In our analysis of homocysteine determinants, we found that healthy lifestyle changes could be the best way to promote optimal levels of homocysteine. Folic acid intake was associated with lower homocysteine in the OaK Birth Cohort, but we also found that reported doses greater than 1 mg did not correspond to a substantially higher serum folate concentration. Although these results were observational and based on self-reported folic acid dose, the recent international FACT (Folic acid Clinical Trial) study by OaK investigators was inconclusive about the benefits of a high dose of 5 mg folic acid during pregnancy to reduce the risk of preeclampsia, in women with one or more risk factors for preeclampsia.[183]

Periconceptional folic acid supplementation is strongly recommended to women of childbearing age to reduce the risk of neural tube defects (NTDs).[33] To raise population folate levels and

reduce the risk of NTDs, folic acid fortification of food staples is mandated in many countries. However, within folic acid fortified populations women of childbearing age remain deficient.[168]

Observational studies of periconceptional folic acid supplementation as a multivitamin or folic acid alone have consistently demonstrated a reduced risk of preeclampsia and SGA.[165,204–208] A trial currently underway in the Netherlands and Italy[209] is examining the impact of low versus high dose folic acid from preconception to early pregnancy on the risk of congenital anomalies and pregnancy complications, including SGA, preeclampsia, and placental abruption. The results of this study could inform whether periconceptional folic acid supplementation reduces the risk of placenta-mediated complications.

Individual testing for homocysteine concentration and the MTHFR 677C>T variant

Canadian guidelines issues by the Society of Obstetricians and Gynaecologists of Canada (SOGC) and Thrombosis Canada recognize associations of the MTHFR 677C>T genotype (SOGC) and homocysteine (Thrombosis Canada) with obstetric complications (Table 7.2). The Canadian College of Medical Geneticists has not issued guidelines on MTHFR or homocysteine testing. Both the SOGC and Thrombosis Canada guidelines state the significance of the association as unclear, and do not make specific recommendations for or against testing. On the other hand, American guidelines mainly discourage screening for homocysteine and the MTHFR 677C>T polymorphism. The American College of Obstetricians and Gynecologists (ACOG) (Bulletin No. 138, 2013) recommends against testing for MTHFR 677C>T and homocysteine, citing lack of evidence. The American College of Medical Genetics and Genomics (ACMG)

guideline states that ordering homocysteine testing for MTHFR homozygous mutant genotypes can help medical geneticists provide "accurate counselling", but notes that testing is meant to be informative and lacks clinical utility. Both ACMG and the American Heart Association (AHA) hold the position that testing would not change practice and emphasize following standard recommendations for women of childbearing age to take prenatal vitamins containing folic acid. The Canadian Consensus on Female Nutrition: Adolescence, Reproduction, Menopause, and Beyond (2016) recommends multivitamins containing Vitamin B_{12} to lower homocysteine. This relates to B_{12} as an important determinant of homocysteine in a folate fortified population, based on findings from the Canadian Health Measures Survey.[109] In practice, health care providers and/or patients may consider testing as necessary for recurrent pregnancy loss. Testing for other conditions would be considered exploratory or investigative.

Table 7.2 Current guidelines for testing homocysteine and MTHFR 677C>T genotype

Guideline issued by	Year	Title	Comments regarding homocysteine and/or MTHFR 677C>T
ACOG Practice Bulletin No. 138	2013	Inherited Thrombophilias [a] in Pregnancy	"Because of the lack of association between either heterozygosity or homozygosity for the MTHFR 677C>T polymorphism and any negative pregnancy outcomes, including any increased risk for venous thromboembolism (James et al. 2006, Dudding and Attia 2004), screening with either MTHFR mutation analyses or fasting homocysteine levels is not recommended." (p. 710)
ACMG	2013	ACMG Practice Guideline: lack of evidence for MTHFR polymorphism testing	"MTHFR polymorphism genotyping should not be ordered as part of the clinical evaluation for thrombophilia or re-current pregnancy loss" "If the patient is homozygous for the "thermolabile" variant c.665C→T, the geneticist

Guideline issued by	Year	Title	Comments regarding homocysteine and/or MTHFR 677C>T
		Scott	may order a fasting total plasma homocysteine, if not previously ordered, to provide more accurate counseling" "MTHFR status does not change the recommendation that women of childbearing age should take the standard dose of folic acid supplementation to reduce the risk of neural tube defects as per the general population guidelines (Zacho et al. 2011, De Stefano et al. 2000, Institute of Medicine. Food and Nutrition Board 1998, Vitamin Study Research Group 1991, Czeizel and Dudás 1992, CDC 1992, Toriello 2011)"
AHA Cadiology Patient Page	2015	Homocysteine and MTHFR Mutations	"I have had unexplained pregnancy losses, preeclampsia, or placental abruption and have been found to have MTHFR mutations. What does that mean for a future pregnancy?" It has no consequence. Like any other woman, the woman with MTHFR mutations should take a prenatal vitamin tablet containing folic acid (0.4 mg) every day throughout pregnancy." "I have had a DVT or PE – should I be tested for homocysteine or MTHFR?" No. Although elevated homocysteine is a marker for an increased risk for DVT and PE, finding elevated levels does not influence management. MTHFR mutations are not clotting disorders (thrombophilias)."
SOGC Clinical practice guideline No. 273	2012	Genetic Considerations for a Woman's Annual Gynaecological Examination	"Thrombophilias can be inherited or acquired (Sibai et al. 2007, James et al. 2007, Gibson et al. 2006, Spector et al. 2005). The most common inherited disorders are mutations of factor V Leiden, prothrombin gene (G20210A mutation), and MTHFR mutation or homozygosity to MTHFR 677C>T…" "In the presence of an inherited thrombophilia, the risk of developing preeclampsia has an

Guideline issued by	Year	Title	Comments regarding homocysteine and/or MTHFR 677C>T
			estimated frequency of … 8% to 24% with the MTHFR variant. As some studies show the risk as being similar to baseline and others show a significantly increased risk, it remains unclear whether this association is significant.
Thrombosis Canada	2017	Thrombophilia: Homocysteinemia and Methylene Tetrahydrofolate Reductase Gene Mutation	"Hyperhomocysteinemia has been associated with obstetric complications but the importance and significance of this association remain unclear."

Abbreviations: SOGC, Society of Obstetricians and Gynaecologists of Canada; ACOG, American College of Obstetricians and Gynecologists; ACMG, American College of Medical Genetics and Genomics

a- In the early research literature homocysteine was considered a thrombophilia. The Thrombosis Canada guidelines emphasize that MTHFR mutations are not clotting disorders. This point is re-iterated by the AHA guidelines with reference to Thrombosis Canada.

If further research continues to point to a causal role of homocysteine in placenta-mediated complications it is unlikely that clinical testing will be recommended for maternal homocysteine concentration or the MTHFR 677C>T genotype. Clinical trials may point to a benefit of preconception folic acid and/or multivitamin intake on the risk of placenta-mediated complications. In this case, a targeted approach towards high-risk or socially disadvantaged women may prove beneficial because of these factors being associated with higher homocysteine. Periconceptional uptake of folic acid is suboptimal worldwide including in Canada[164,167,210–215] - therefore promoting timely uptake in the wider population of reproductive age women is necessary, in addition to targeting at-risk groups.

7.5 CONCLUSION

The findings from this thesis suggest that in certain sub-groups there is an independent effect of homocysteine in the development of placenta-mediated complications. Mendelian randomization studies can further establish whether homocysteine plays a causal role in this association. A combination of periconceptional folic acid uptake and healthy behavioural changes could lead to lower homocysteine concentrations and a reduced risk of placenta-mediated pregnancy complications.

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APPENDIX A. ETHICS APPROVAL FOR SECONDARY ANALYSES OF OAK BIRTH COHORT

Ottawa Hospital Research Institute
Institut de recherche de l'Hôpital d'Ottawa

uOttawa

UNIVERSITY OF OTTAWA HEART INSTITUTE
INSTITUT DE CARDIOLOGIE DE L'UNIVERSITÉ D'OTTAWA

Ottawa Health Science Network Research Ethics Board/ Conseil d'éthique de la recherche du Réseau de science de la santé d'Ottawa
Civic Box 411 725 Parkdale Avenue, Ottawa, Ontario K1Y 4E9 613-798-5555 ext. 14902 Fax : 613-761-4311
http://www.ohri.ca/ohsn-reb

May 31, 2016

Dr. Shi Wu Wen
Ottawa Hospital - General Campus

[redacted]

Dear Dr. Wen:

Re: Protocol #	**20160163-01H**	**The association of hyperhomocysteinemia with preeclampsia and other placenta-mediated outcomes**
Protocol approval valid until -		**May 30, 2017**

I am pleased to inform you that this protocol underwent delegated review by the Ottawa Health Science Network Research Ethics Board (OHSN-REB) and is approved. No changes, amendments or addenda may be made to the protocol without the OHSN-REB's review and approval.

PLEASE NOTE: THE APPROVAL OF THIS PROTOCOL IS CONDITIONAL UPON A FULLY-SIGNED STUDY CONTRACT/AGREEMENT BETWEEN THE OTTAWA HOSPITAL RESEARCH INSTITUTE, THE PRINCIPAL INVESTIGATOR AND THE SPONSOR (OR AS OTHERWISE REQUIRED). YOU CANNOT START THE STUDY, OR BEGIN TO RECRUIT RESEARCH PARTICIPANTS INTO THE STUDY UNTIL THE STUDY CONTRACT/AGREEMENT HAS BEEN SIGNED BY ALL PARTIES, AND HAS BEEN RECEIVED BY THE OTTAWA HOSPITAL RESEARCH INSTITUTE'S CONTRACTS OFFICE. FOR FURTHER DETAILS, PLEASE CONTACT CONTRACTS ADMINISTRATION AT CONTRACTS@OHRI.CA OR AT 613-798-5555 EXT. 19843.

Approval is for the following:
- Thesis Proposal dated October 2015

If the study is to continue beyond the expiry date noted above, a Renewal Form should be submitted to the REB approximately six weeks prior to the current expiry date. If the study has been completed by this date, a Termination Report should be submitted.

The Ottawa Health Science Network Research Ethics Board (OHSN-REB) was created by the merger of both the Ottawa Hospital Research Ethics Board (OHREB) and the Human Research Ethics Board (HREB) for meetings held at the University of Ottawa Heart Institute.

.../2

The OHSN-REB complies with the membership requirements and operates in compliance with the Tri-Council Policy Statement: Ethical Conduct for Research Involving Humans; the International Conference on Harmonization - Good Clinical Practice: Consolidated Guideline and the provisions of the Personal Health Information Protection Act 2004.

Yours sincerely,

Francine F-A Sarazin, Ph.D., C.Psych.
Vice-Chairperson
Ottawa Health Science Network Research Ethics Board

FFAS/kd

APPENDIX B. ADDITIONAL FILES FOR CHAPTER 4

Additional file 1: Homocysteine distribution

Table A: Homocysteine concentration according to placenta-mediated complication

Homocysteine µmol/L	Outcome		P-value [a]
	Yes	No	
Entire cohort	n=7500 [b]		
Mean (SD)	4.83 (1.27)		
Median (Q1-Q3)	5 (4-5)		
Range	1-34		
Any placenta-mediated complication	n=745	n=6676	0.0001
Mean (SD)	5.03 (1.51)	4.81 (1.25)	
Median (Q1-Q3)	5 (4-6)	5 (4-5)	
Range	2-24	1-34	
Preeclampsia	n=223	n=7277	0.0880
Mean (SD)	4.99 (1.43)	4.82 (1.27)	
Median (Q1-Q3)	5 (4-6)	5 (4-5)	
Range	2-14	1-34	
Small for gestational age	n=502	n=6919	<0.0001
Mean (SD)	5.11 (1.63)	4.81 (1.25)	
Median (Q1-Q3)	5 (4-6)	5 (4-5)	
Range	2-24	1-34	
Placental abruption	n=65	n=7435	0.9238
Mean (SD)	4.82 (1.20)	4.83 (1.28)	
Median (Q1-Q3)	5 (4-5)	5 (4-5)	
Range	3-11	1-34	
Pregnancy loss	n=85	n=7415	0.0332
Mean (SD)	5.19 (1.54)	4.83 (1.27)	
Median (Q1-Q3)	5 (4-6)	5 (4-5)	
Range	3-14	1-34	

[a] Welch two sample t test

[b] 87 missing homocysteine measurement

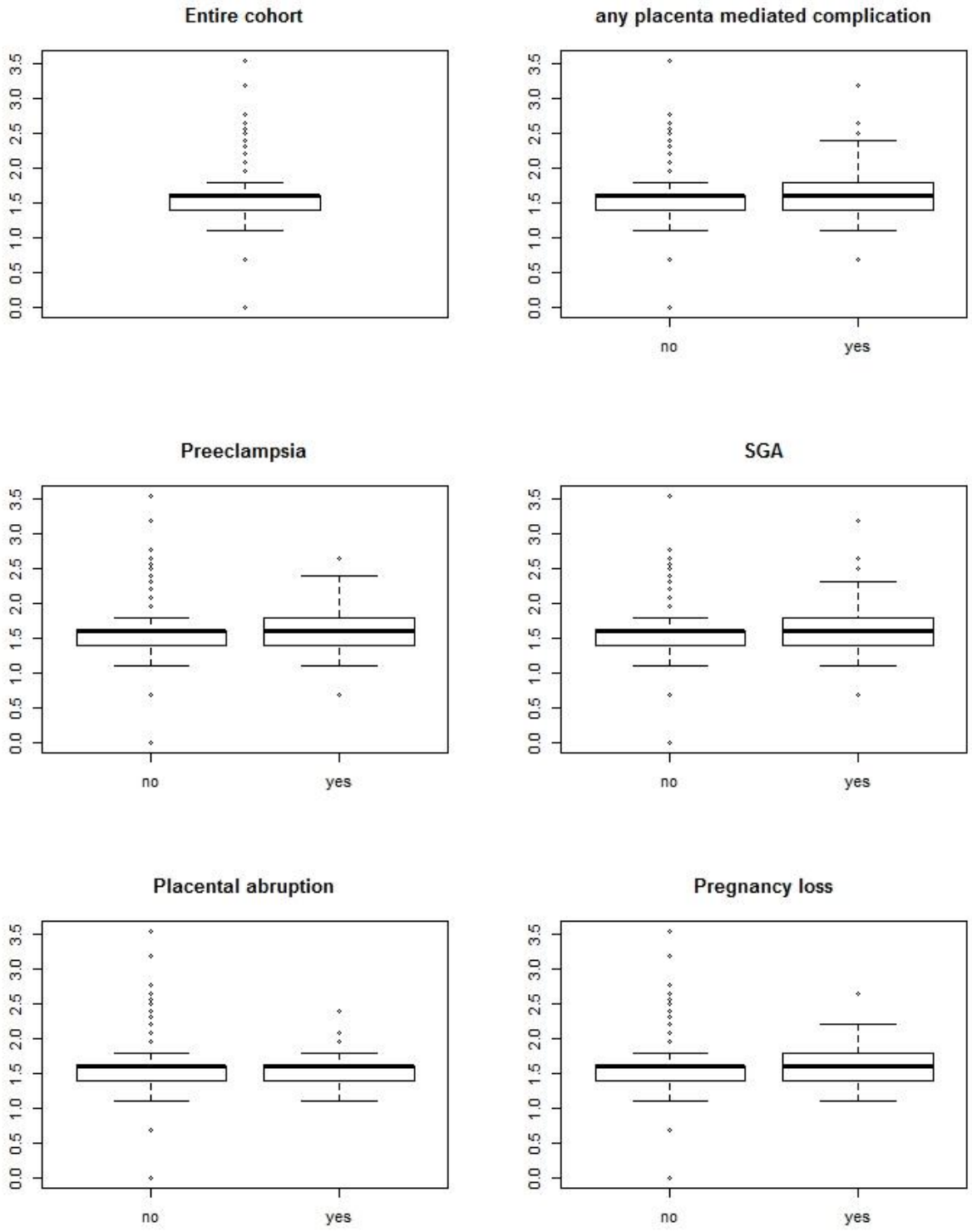

Figure A Boxplot of log-transformed plasma homocysteine concentration (µmol/L) in entire cohort and according to pregnancy outcome

Additional file 2: ANOVA plots of partial associations

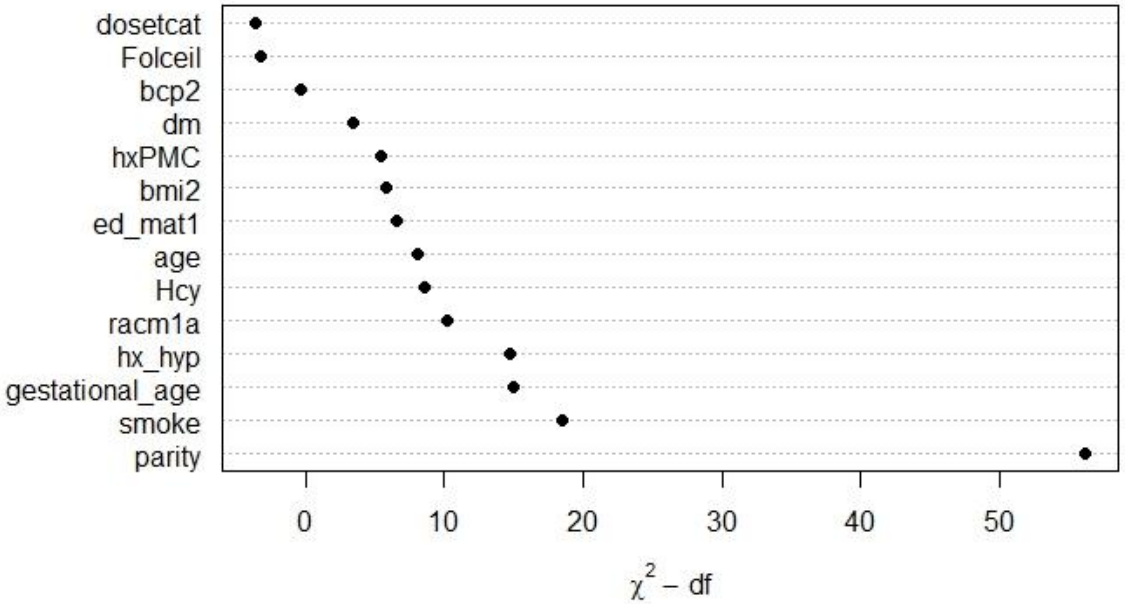

Figure B.1 ANOVA plot of partial associations from saturated model for any placenta-mediated complication (Table 4.3). Hcy: homocysteine.

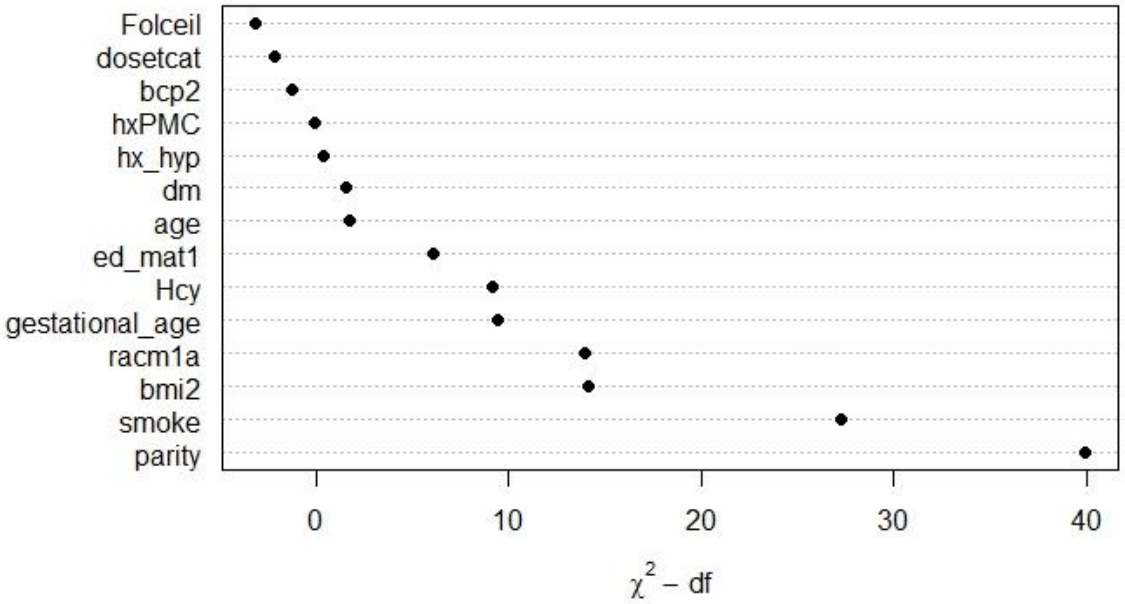

Figure B.2 ANOVA plot of partial associations from saturated model for small for gestational age (Table C.1). Hcy: homocysteine.

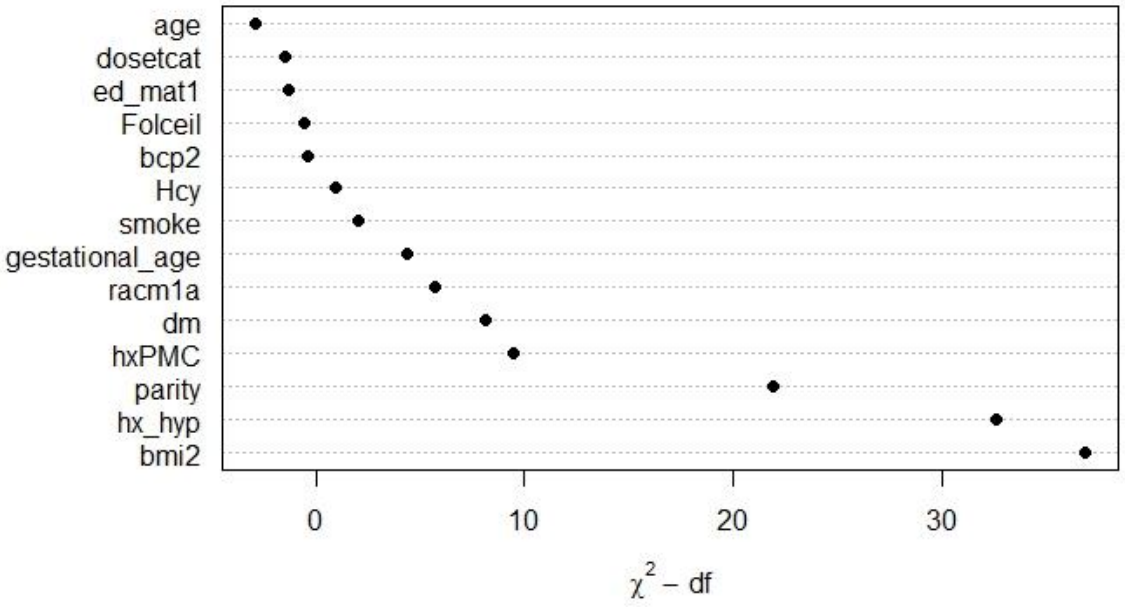

Figure B.3 ANOVA plot of partial associations from saturated model for preeclampsia (Table C.2). Hcy: homocysteine.

.

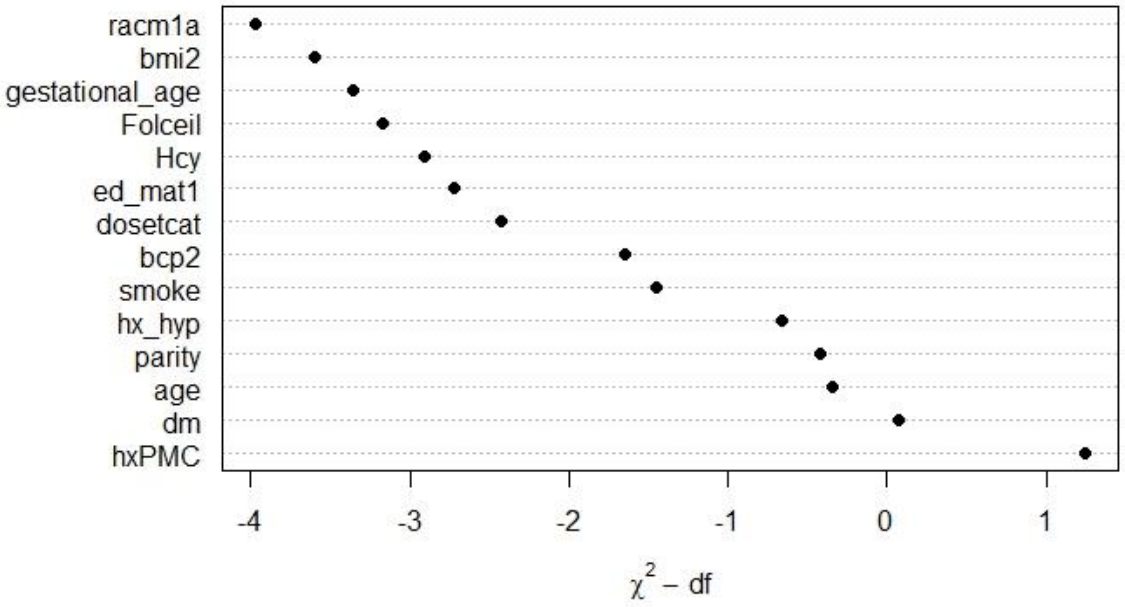

Figure B.4 ANOVA plot of partial associations from saturated model for placental abruption (Table C.3). Hcy: homocysteine.

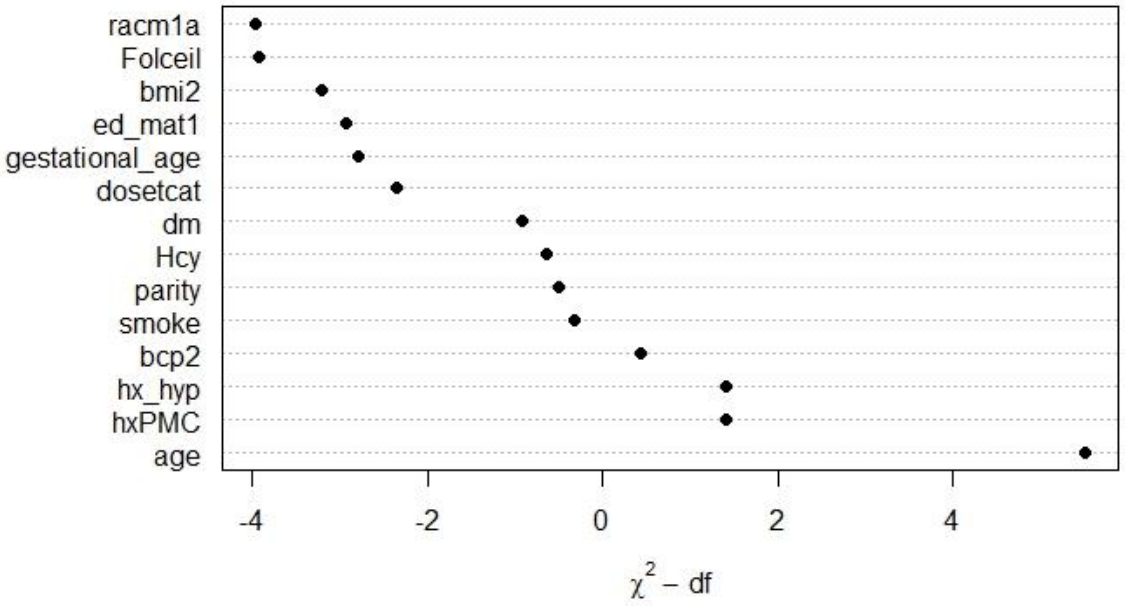

Figure B.5 ANOVA plot of partial associations from saturated model for pregnancy loss (Table C.4). Hcy: homocysteine.

Additional file 3: Complete results for multivariable logistic regression analyses

Table C.1 Multivariable logistic regression analysis of the association between homocysteine and SGA (512 events [a]), n=7587

Variable	Odds ratio (95% CI)		p-value [b]
Homocysteine (linear)			0.0010
5 µmol/L increase	1.756	(1.254, 2.458)	
Age (linear)			0.0538
34 versus 27 years	1.151	(0.998, 1.328)	
Race			0.0001
Caucasian versus others	0.578	(0.437, 0.766)	
Education			0.0038
College/University completed versus less than completed	0.714	(0.568, 0.897)	
Nulliparous			<0.0001
Yes versus no	2.063	(1.662, 2.560)	
Smoking			<0.0001
No	Reference		
Second-hand	0.845	(0.422, 1.694)	
Med/light smoker (<10 cigarettes per day)	2.205	(1.600, 3.038)	
Heavy smoker (≥10 cigarettes per day)	2.359	(1.554, 3.580)	
Diabetes			0.1085
Yes versus no	0.432	(0.155, 1.204)	
BMI (restricted cubic spline, 5 knots)			0.0004
27.3 versus 21.1 kg/m^2	0.803	(0.625, 1.031)	
Hormonal birth control prior to conception			0.6512
No	Reference		
Oral	0.956	(0.780, 1.173)	
Injection or IUD	0.752	(0.395, 1.431)	
Chronic hypertension			0.1981
Yes versus no	1.610	(0.780, 3.323)	
History of PMC (Preeclampsia, placental abruption, IUGR, stillbirth, loss)			0.3037
Yes versus no	1.179	(0.861, 1.614)	
Folic acid supplementation			0.7468
Yes versus no supplementation	1.074	(0.697, 1.654)	
Serum folate (linear)			0.4243
45.1 versus 30.6 nmol/L	1.039	(0.946, 1.140)	
Gestational age at blood work (restricted cubic spline, three knots)			0.0612
13.7 versus 12.4 weeks	0.816	(0.670, 0.994)	

[a] Additional 79 missing values imputed

[b] Wald test of most meaningful hypotheses, pooled across multiple imputation datasets

Table C.2 Multivariable logistic regression analysis of the association between homocysteine and preeclampsia (227 events), n=7587

Variable	Odds ratio (95% CI)		p-value [a]
Homocysteine (linear)			0.0736
5 µmol/L increase	1.546	(0.959, 2.491)	
Age (linear)			0.8614
34 versus 27 years	1.019	(0.829, 1.252)	
Race			0.0362
Caucasian versus others	0.627	(0.405, 0.970)	
Education			0.2625
College/University completed versus less than completed	0.826	(0.591, 1.154)	
Nulliparous			<0.0001
Yes versus no	2.353	(1.662, 3.332)	
Smoking			0.1693
No	Reference		
Second-hand	0.298	(0.072, 1.244)	
Med/light smoker (<10 cigarettes per day)	0.605	(0.300, 1.220)	
Heavy smoker (≥10 cigarettes per day)	1.175	(0.585, 2.358)	
Diabetes			0.0012
Yes versus no	2.809	(1.506, 5.239)	
BMI (restricted cubic spline, four knots)			<0.0001
27.3 versus 21.1 kg/m^2	2.428	(1.757, 3.356)	
Hormonal birth control prior to conception			0.3977
No	Reference		
Oral	1.114	(0.826, 1.504)	
Injection or IUD	0.515	(0.157, 1.695)	
Chronic hypertension			<0.0001
Yes versus no	5.824	(3.302, 10.27)	
History of PMC (Preeclampsia, placental abruption, IUGR, stillbirth, loss)			0.0007
Yes versus no	1.981	(1.333, 2.943)	
Folic acid supplementation			0.2458
Yes versus no supplementation	0.712	(0.401, 1.264)	
Serum folate (linear)			0.5877
45.1 versus 30.6 nmol/L	1.041	(0.900, 1.203)	
Gestational age at blood work (linear)			0.1720
13.7 versus 12.4 weeks	1.057	(0.976, 1.144)	

[a] Wald test of most meaningful hypotheses, pooled across multiple imputation datasets

Table C.3 Multivariable logistic regression analysis of the association between homocysteine and placental abruption (68 events), n=7587

Variable	Odds ratio (95% CI)		p-value [a]
Homocysteine (linear)			0.9851
5 µmol/L increase	1.005	(0.590, 1.711)	
Age (linear)			0.2044
34 versus 27 years	1.130	(0.935, 1.366)	
Race			0.9133
Caucasian versus others	1.026	(0.642, 1.640)	
Education			0.7271
College/University completed versus less than completed	1.059	(0.769, 1.457)	
Nulliparous			0.3963
Yes versus no	0.887	(0.673, 1.170)	
Smoking			0.6952
No	Reference		
Second-hand	1.049	(0.860, 1.280)	
Med/light smoker (<10 cigarettes per day)	1.090	(0.575, 2.067)	
Heavy smoker (≥10 cigarettes per day)	1.633	(0.732, 3.645)	
Diabetes			0.2926
Yes versus no	1.704	(0.632, 4.594)	
BMI (restricted cubic spline, four knots)			0.8112
27.3 versus 21.1 kg/m^2	0.982	(0.842, 1.144)	
Hormonal birth control prior to conception			0.8030
No	Reference		
Oral	0.931	(0.752, 1.151)	
Injection or IUD	0.968	(0.773, 1.213)	
Chronic hypertension			0.5191
Yes versus no	1.451	(0.468, 4.496)	
History of PMC (Preeclampsia, placental abruption, IUGR, stillbirth, loss)			0.1148
Yes versus no	1.401	(0.921, 2.129)	0.7765
Folic acid supplementation			
Yes versus no supplementation	1.092	(0.595, 2.002)	
Serum folate (linear)			0.4896
45.1 versus 30.6 nmol/L	0.954	(0.835, 1.090)	
Gestational age at blood work (linear)			0.5598
13.7 versus 12.4 weeks	1.025	(0.944, 1.112)	

[a] Wald test of most meaningful hypotheses, pooled across multiple imputation datasets

Table C.4 Multivariable logistic regression analysis of the association between homocysteine and pregnancy loss (85 events), n=7587

Variable	Odds ratio (95% CI)		p-value [a]
Homocysteine (linear)			0.1586
5 µmol/L increase	1.392	(0.879, 2.206)	
Age (linear)			0.0456
34 versus 27 years	1.265	(1.021, 1.567)	
Race			0.6982
Caucasian versus others	1.099	(0.681, 1.775)	
Education			0.7958
College/University completed versus less than completed	0.958	(0.691, 1.327)	
Nulliparous			0.4722
Yes versus no	0.900	(0.676, 1.199)	
Smoking			0.4109
No	Reference		
Second-hand	1.110	(0.901, 1.367)	
Med/light smoker (<10 cigarettes per day)	1.464	(0.800, 2.677)	
Heavy smoker (≥10 cigarettes per day)	1.716	(0.804, 3.664)	
Diabetes			0.7976
Yes versus no	1.149	(0.397, 3.321)	
BMI (restricted cubic spline, four knots)			0.4262
27.3 versus 21.1 kg/m^2	1.064	(0.913, 1.241)	
Hormonal birth control prior to conception			0.2841
No	Reference		
Oral	0.832	(0.663, 1.045)	
Injection or IUD	0.922	(0.721, 1.178)	
Chronic hypertension			0.1096
Yes versus no	2.229	(0.835, 5.950)	
History of PMC (Preeclampsia, placental abruption, IUGR, stillbirth, loss)			0.1192
Yes versus no	1.393	(0.918, 2.113)	
Folic acid supplementation			0.7994
Yes versus no supplementation	0.925	(0.508, 1.686)	
Serum folate (linear)			0.8072
45.3 versus 30.7 nmol/L	0.983	(0.860, 1.125)	
Gestational age at blood work (linear)			0.3440
13.7 versus 12.4 weeks	0.959	(0.879, 1.046)	

[a] Wald test of most meaningful hypotheses, pooled across multiple imputation datasets

Additional file 4: Modelled associations of restricted cubic spline functions

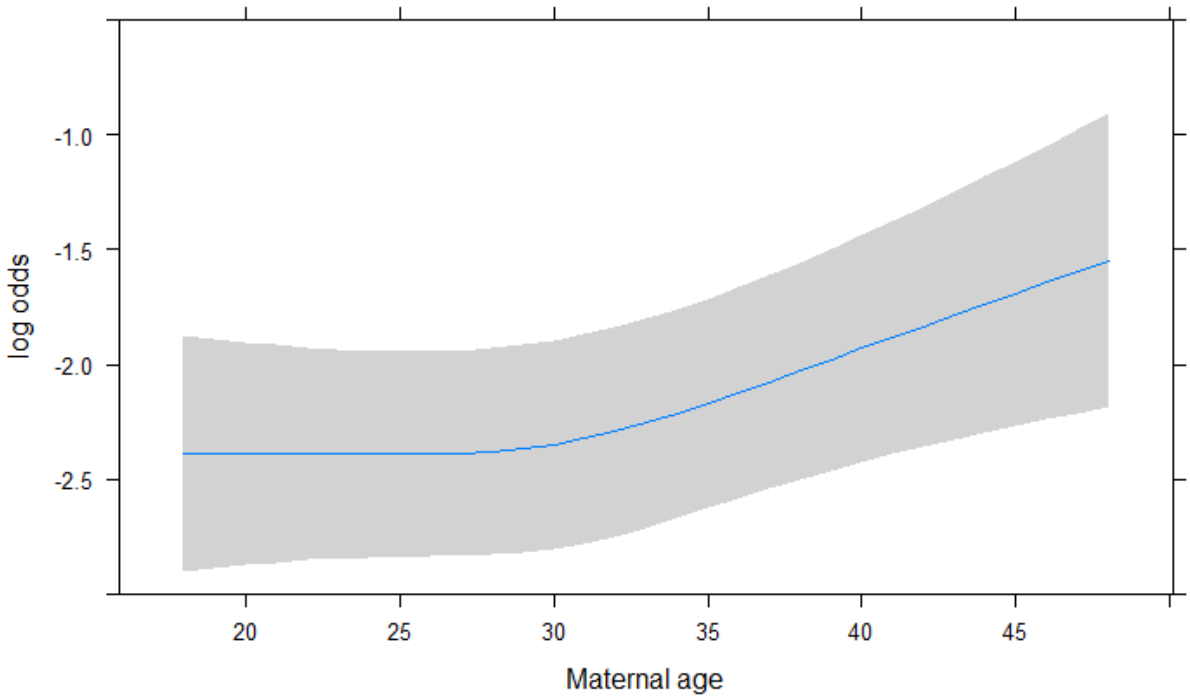

Figure D.1: Modelled association between maternal age (years) and any placenta-mediated complication, presented in Table 4.2. Restricted cubic spline with three knots at 24, 30, and 37. Shaded area represents 95% CI.

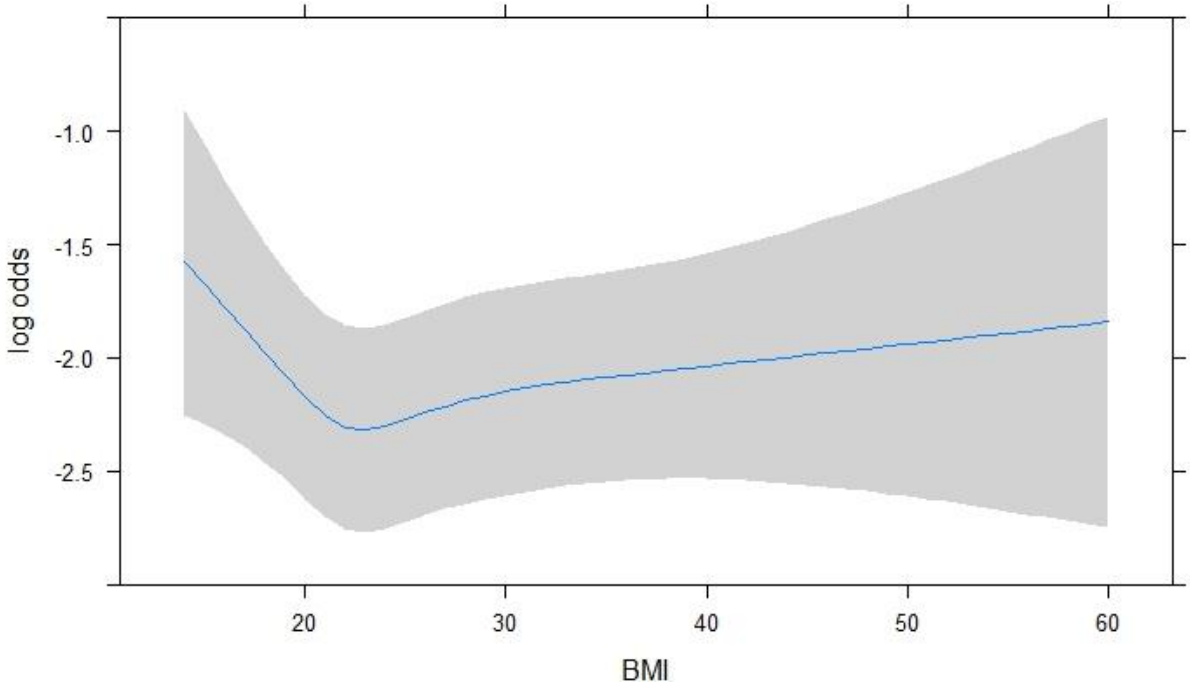

Figure D.2: Modelled association between BMI (kg/m^2) and any placenta-mediated complication, presented in Table 4.2. Restricted cubic spline with four knots at 19, 22, 25, and 36. Shaded area represents 95% CI.

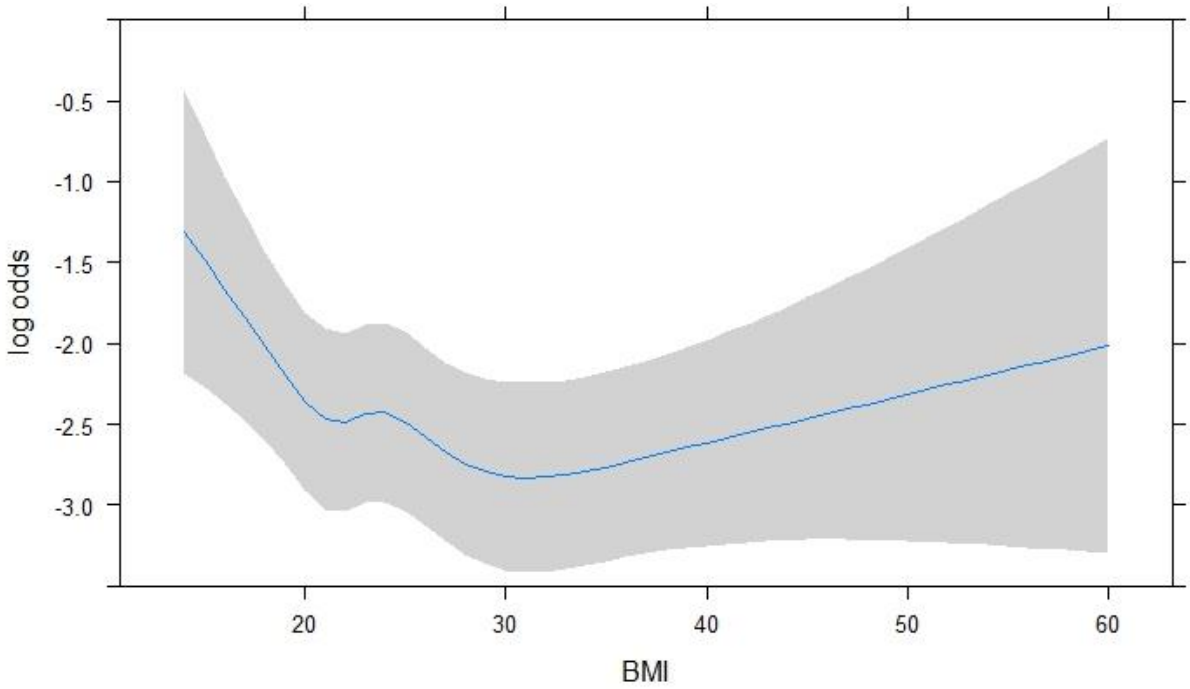

Figure D.3: Modelled association between BMI (kg/m^2) and SGA, presented in Table C.1. Restricted cubic spline with five knots at 19, 21, 23, 27, and 36. Shaded area represents 95% CI.

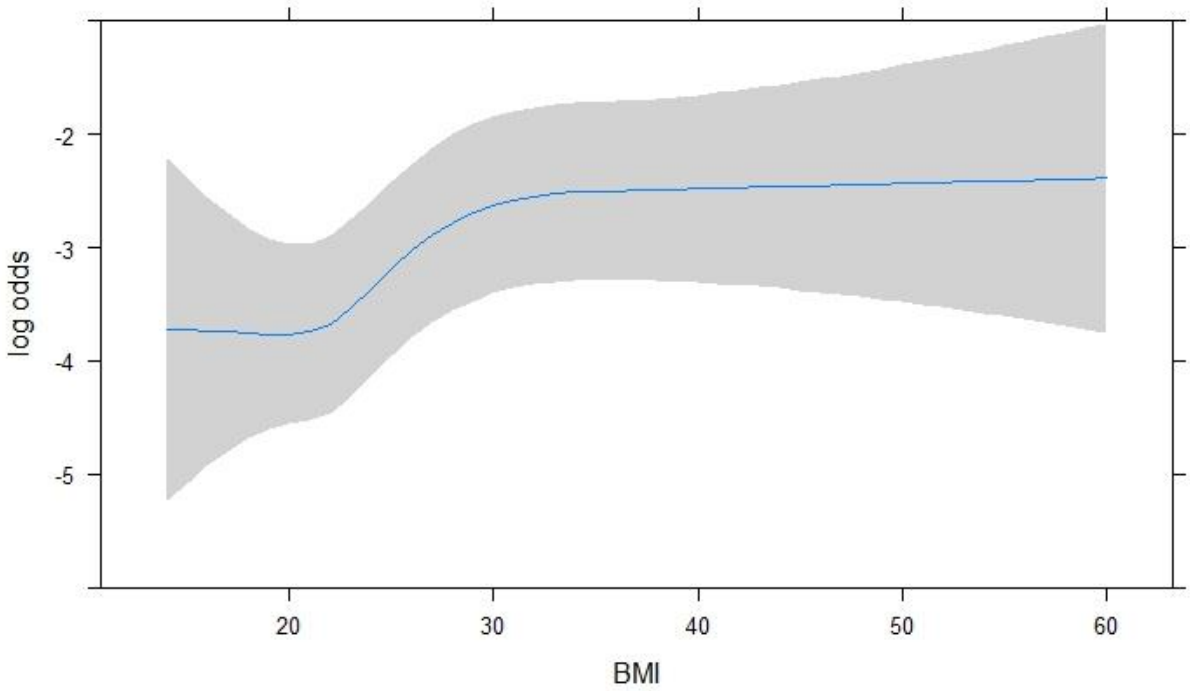

Figure D.4: Modelled association between BMI (kg/m^2) and preeclampsia, presented in Table C.2. Restricted cubic spline with four knots at 19, 22, 25, and 36. Shaded area represents 95% CI.

APPENDIX C. SUPPLEMENTARY DATA FOR CHAPTER 5

Supplementary file 1: Systematic review search strategy

Database 1: Ovid MEDLINE(R) In-Process & Other Non-Indexed Citations and Ovid MEDLINE(R) 1946 to 16 April 2019

1 exp Hypertension, Pregnancy-Induced/
2 (pregnan* adj5 hypertensi*).tw.
3 ((gestational or maternal) adj5 hypertens*).tw.
4 PIH.tw.
5 (((high* or rais* or elevat* or heighten* or increas*) adj3 (blood pressure or diastolic pressure or systolic pressure or pulse pressure)) and pregnan*).tw.
6 (((high* or rais* or elevat* or heighten* or increas*) adj3 (BP or DBP or SBP)) and pregnan*).tw.
7 (eclamp* or pre-eclamp* or preeclamp*).tw.
8 (EPH adj1 (Complex* or Gestos* or Toxemi* or Toxaemi* or Syndrome*)).tw.
9 ((Edema or oedema) and Proteinuria and Hypertension and Gestosis).tw.
10 ((pregnan* or gestational or gravidum or gravidarum) adj5 (toxemi* or toxaemi*)).tw.
11 HELLP.tw.
12 (Hemolysis and Elevated Liver and Lowered Platelet*).tw.
13 Infant, Small for Gestational Age/
14 "small for gestational age".tw.
15 SGA.tw.
16 Fetal Growth Retardation/
17 ((intrauterine or intra-uterine) adj2 growth restrict*).tw.
18 ((intrauterine or intra-uterine) adj2 growth retard*).tw.
19 ((fetal or foetal or fetus* or foetus*) adj2 growth restrict*).tw.
20 ((fetal or foetal or fetus* or foetus*) adj2 growth retard*).tw.
21 IUGR.tw.
22 exp Fetal Death/ or Stillbirth/
23 (stillbirth* or stillborn*).tw.
24 ((fetal or foetal or fetus* or foetus* or prenatal* or pre-natal* or perinatal* or peri-natal* or antepartum or ante-partum or antenatal* or ante-natal*) adj3 (loss* or death*)).tw.
25 exp Abortion, Spontaneous/
26 (abort* adj3 (spontaneous* or habitual* or frequen* or recur* or tubal)).tw.
27 (miscarriage* or miscarry or miscarries or miscarried or miscarrying).tw.
28 ((second trimester* or 2nd trimester* or third trimester* or 3rd trimester* or late pregnan* or advanced pregnan* or late intrauterine or late intra-uterine) adj3 (loss* or death*)).tw.
29 Placental Insufficiency/
30 ((placent* or uteroplacent* or utero-placenta*) adj3 (insufficien* or incompeten* or failure*)).tw.
31 Abruptio Placentae/

32 (placent* adj1 (abruptio* or ablation* or detachment* or separation* or solutio* or apoplexia*)).tw.
33 abruption*.tw.
34 (placent* and vascular and thrombos*).tw.
35 (("placenta-mediated pregnancy" or "placental-mediated pregnancy") adj3 (complicat* or problem* or difficult* or disorder*)).tw.
36 "Pregnancy Complications, Hematologic"/
37 exp Placenta/de
38 or/1-37

39 exp Homocysteine/
40 Homocysteine.tw.
41 Homocyst*ine.tw.
42 (2-amino-4-mercaptobutyric adj2 acid).tw.
43 (homocysteine adj2 l-isomer).tw.
44 (homocysteine adj2 l adj2 isomer).tw.
45 Hyperhomocysteinemia/
46 Hyperhomocysteinemia.tw.
47 (Hyper* adj2 homocystein*).tw.
48 (Hyper* adj2 homocyst*).tw.

49 or/39-48

50 38 and 49

51 Exp Animals/ not (Humans/ and exp Animals/)

52 50 not 51

-----SIGN Hedge for observational studies-----

53 Epidemiologic studies/
54 Exp case control studies/
55 Exp cohort studies/
56 Case control.tw.
57 (cohort adj (study or studies)).tw.
58 Cohort analy$.tw.
59 (Follow up adj (study or studies)).tw.
60 (observational adj (study or studies)).tw.
61 Longitudinal.tw.
62 Retrospective.tw.
63 Cross sectional.tw.
64 Cross-sectional studies/
65 Or/53-64

66 52 and 65

Database 2: EMBASE 1974 to 16 April 2019

1 maternal hypertension/
2 (pregnan* adj5 hypertensi*).tw.
3 (gestational adj5 hypertens*).tw.
4 (maternal adj5 hypertens*).tw.
5 PIH.tw.
6 (((high* or rais* or elevat* or heighten* or increas*) adj3 (blood pressure or diastolic pressure or systolic pressure or pulse pressure)) and pregnan*).tw.
7 (((high* or rais* or elevat* or heighten* or increas*) adj3 (BP or DBP or SBP)) and pregnan*).tw.
8 exp pregnancy toxemia/
9 (eclamp* or pre-eclamp* or preeclamp*).tw.
10 (EPH adj1 (Complex* or Gestos* or Toxemi* or Toxaemi* or Syndrome*)).tw.
11 ((Edema or oedema) and Proteinuria and Hypertension and Gestosis).tw.
12 ((pregnan* or gestational* or gravidum or gravidarum) adj5 (toxemi* or toxaemi* or toxicos*)).tw.
13 HELLP syndrome/
14 HELLP.tw.
15 (Hemolysis and Elevated Liver and Lowered Platelet*).tw.
16 exp intrauterine growth retardation/
17 "small for gestational age".tw.
18 SGA.tw.
19 ((intrauterine or intra-uterine) adj2 growth restrict*).tw.
20 ((intrauterine or intra-uterine) adj2 growth retard*).tw.
21 ((fetal or foetal or fetus* or foetus*) adj2 growth restrict*).tw.
22 ((fetal or foetal or fetus* or foetus*) adj2 growth retard*).tw.
23 IUGR.tw.
24 exp fetus death/
25 (stillbirth* or stillborn*).tw.
26 ((fetal or foetal or fetus* or foetus* or prenatal* or pre-natal* or perinatal* or peri-natal* or antepartum or ante-partum or antenatal* or ante-natal*) adj3 (loss* or death*)).tw.
27 spontaneous abortion/
28 (abort* adj3 (spontaneous* or habitual* or frequen* or recur* or tubal)).tw.
29 (miscarriage* or miscarry or miscarries or miscarried or miscarrying).tw.
30 ((second trimester* or 2nd trimester* or third trimester* or 3rd trimester* or late pregnan* or advanced pregnan* or late intrauterine or late intra-uterine) adj3 (loss* or death*)).tw.
31 placenta insufficiency/
32 ((placent* or uteroplacenta* or utero-placenta*) adj3 (insufficien* or incompeten* or failure*)).tw.
33 solutio placentae/
34 (placent* adj1 (abruptio* or ablation* or detachment* or separation* or solutio*)).tw.
35 abruption*.tw.

36 (placent* and vascular and thrombos*).tw.
37 (("placenta-mediated pregnancy" or "placental-mediated pregnancy") adj3 (complicat* or problem* or difficult* or disorder*)).tw.
38 (pregnan* and (hematolog* adj5 (complicat* or problem* or difficult* or disorder*))).tw.

39 Or/1-38

40 Homocysteine/
41 Homocysteine.tw.
42 (4,4 adj2 dithiobis*).tw.
43 (Homocyst*ine).tw.
44 (2-amino-4-mercaptobutyric adj2 acid).tw.
45 Betahomocystein.tw.
46 (dextro adj2 levo adj2 homocysteine).tw.
47 (dl adj2 homocysteine).tw.
48 (l* adj2 hoocysteine).tw.
49 Hyperhomocysteinemia/
50 Hyperhomocysteinemia.tw.
51 (Hyper* adj2 homocystein*).tw.
52 (Hyper* adj2 homocyst*).tw.

53 Or/40-52

54 39 and 53

55 exp animals/ or exp animal experimentation/ or exp models animal/ or exp animal experiment/ or nonhuman/ or exp vertebrate/
56 exp humans/ or exp human experimentation/ or exp human experiment/
57 55 not 56

58 54 not 57

-----SIGN hedge for observational studies-----

59 Clinical study/
60 Case control study/
61 Family study/
62 Longitudinal study/
63 Retrospective study/
64 Prospective study/
65 Randomized controlled trials/
66 64 not 65
67 Cohort analysis/
68 (Cohort adj (study or studies)).mp.

69 (Case control adj (study or studies)).tw.
70 (follow up adj (study or studies)).tw.
71 (observational adj (study or studies)).tw.
72 (epidemiologic$ adj (study or studies)).tw.
73 (cross sectional adj (study or studies)).tw.
74 Or/59-73

75 58 and 74

Database 3: PubMed (not MEDLINE)

1 "Hypertension, Pregnancy-Induced"[Mesh]
2 (pregnan*[tw] AND hypertensi*[tw])
3 ((gestational[tw] OR maternal[tw]) AND hypertens*[tw])
4 PIH[tw]
5 (((high*[tw] OR rais*[tw] OR elevat*[tw] OR heighten*[tw] OR increas*[tw]) AND (blood pressure[tw] OR diastolic pressure[tw] OR systolic pressure[tw] OR pulse pressure[tw])) AND pregnan*[tw])
6 (((high*[tw] OR rais*[tw] OR elevat*[tw] OR heighten*[tw] OR increas*[tw]) AND (BP[tw] OR DBP [tw] OR SBP [tw])) AND pregnan*[tw])
7 (eclamp*[tw] OR pre-eclamp*[tw] OR preeclamp*[tw])
8 (EPH[tw] AND (Complex*[tw] OR Gestos*[tw] OR Toxemi*[tw] OR Toxaemi*[tw] OR Syndrome*[tw]))
9 ((Edema[tw] OR oedema[tw]) AND Proteinuria[tw] AND Hypertension[tw] AND Gestosis[tw])
10 ((pregnan*[tw] OR gestational[tw] OR gravidum[tw] OR gravidarum[tw]) AND (toxemi*[tw] OR toxaemi*[tw]))
11 HELLP[tw]
12 (Hemolysis[tw] AND Elevated Liver[tw] AND Lowered Platelet*[tw])
13 Infant, Small for Gestational Age[mesh:noexp]
14 small for gestational age[tw]
15 SGA[tw]
16 Fetal Growth Retardation[mesh:noexp]
17 ((intrauterine[tw] OR intra-uterine[tw]) AND (growth restrict*[tw]))
18 ((intrauterine[tw] OR intra-uterine[tw]) AND (growth retard*[tw]))
19 ((fetal[tw] OR foetal[tw] OR fetus*[tw] OR foetus*[tw]) AND (growth restrict*[tw]))
20 ((fetal[tw] OR foetal[tw] OR fetus*[tw] OR foetus*[tw]) AND (growth retard*[tw]))
21 IUGR[tw]
22 “Fetal Death”[Mesh] OR Stillbirth[mesh:noexp]
23 (stillbirth*[tw] OR stillborn*[tw])
24 ((fetal[tw] OR foetal[tw] OR fetus*[tw] OR foetus*[tw] OR prenatal*[tw] OR pre-natal*[tw] OR perinatal*[tw] OR peri-natal*[tw] OR antepartum[tw] OR ante-partum[tw] OR antenatal*[tw] OR ante-natal*[tw]) AND (loss*[tw] OR death*[tw]))
25 “Abortion, Spontaneous”[Mesh]
26 (abort*[tw] AND (spontaneous*[tw] OR habitual*[tw] OR frequen*[tw] OR recur*[tw] OR tubal[tw]))
27 (miscarriage*[tw] OR miscarry[tw] OR miscarries[tw] OR miscarried[tw] OR miscarrying[tw])
28 ((second trimester*[tw] OR 2nd trimester*[tw] OR third trimester*[tw] OR 3rd trimester*[tw] OR late pregnan*[tw] OR advanced pregnan*[tw] OR late intrauterine[tw] OR late intra-uterine[tw]) AND (loss*[tw] OR death*[tw]))
29 Placental Insufficiency[mesh:noexp]

30 ((placent*[tw] OR uteroplacent*[tw] OR utero-placenta*[tw]) AND (insufficien*[tw] OR incompeten*[tw] OR failure*[tw]))
31 Abruptio Placentae[mesh:noexp]
32 (placent*[tw] AND (abruptio*[tw] OR ablation*[tw] OR detachment*[tw] OR separation*[tw] OR solutio*[tw] OR apoplexia*[tw]))
33 (abruption*[tw])
34 (placent*[tw] AND vascular[tw] AND thrombos*[tw])
35 ((placenta-mediated pregnancy[tw] OR placental-mediated pregnancy[tw]) AND (complicat*[tw] OR problem*[tw] OR difficult*[tw] OR disorder*[tw]))
36 Pregnancy Complications, Hematologic[mesh:noexp]
37 "Placenta/de"[Mesh]
38 #1 OR #2 OR #3 OR #4 OR #5 OR #6 OR #7 OR #8 OR #9 OR #10 OR #11 OR #12 OR #13 OR #14 OR #15 OR #16 OR #17 OR #18 OR #19 OR #20 OR #21 OR #22 OR #23 OR #24 OR #25 OR #26 OR #27 OR #28 OR #29 OR #30 OR #31 OR #32 OR #33 OR #34 OR #35 OR #36 OR #37

39 Homocysteine[Mesh]
40 Homocysteine[tw]
41 Homocyst*[tw]
42 (2-amino-4-mercaptobutyric[tw] AND acid[tw])
43 (homocysteine[tw] AND l-isomer[tw])
44 (homocysteine[tw] AND l[tw] AND isomer[tw])
45 Hyperhomocysteinemia[mesh:noexp]
46 Hyperhomocysteinemia[Mesh]
47 (Hyper*[tw] AND homocystein*[tw])
48 (Hyper*[tw] AND homocyst*[tw])

49 #39 OR #40 OR #41 OR #42 OR #43 OR #44 OR #45 OR #46 OR #47 OR #48

50 #38 AND #49

51 Animals[Mesh] NOT (Humans[mesh:noexp] AND Animals[Mesh)

52 #50 NOT #51

-----Search for observational studies-----

53 Epidemiologic studies[mesh:noexp]
54 "case control studies"[Mesh]
55 "cohort studies"[Mesh]
56 Case control[tw]
57 (cohort[tw] AND (study[tw] OR studies[tw]))
58 (Cohort analy*[tw])
59 (Follow up[tw] AND (study[tw] OR studies[tw]))

60 (observational[tw] AND (study[tw] OR studies[tw]))
61 Longitudinal[tw]
62 Retrospective
63 “Cross sectional”
64 Cross-sectional studies[mesh:noexp]
65 #53 OR #54 OR #55 OR #56 OR #57 OR #58 OR #59 OR #60 OR #61 OR #62 OR #63 OR #64

66 #52 AND #65

-----PubMed not MEDLINE*-----

67 publisher[sb] OR (("inprocess"[sb] OR medline[sb]) AND "2019/04/07" [EDAT]:"3000" [EDAT])

68 #66 AND #67

*date is one week prior to actual search date

Supplementary file 2: Data extraction form

Study characteristics		
Covidence study number		
Study title	The role of maternal homocysteine concentration in placenta-mediated complications: findings from the Ottawa and Kingston birth cohort	
Year published	2019	
Journal	BMC Pregnancy and Childbirth	
Corresponding author	Name Address Email	Shi Wu Wen
Type of paper	☒ ☐ ☐ ☐ ☐ ☐	Peer-reviewed published paper Dissertation Published abstract Conference proceeding Unpublished report Other (specify)
Study sponsor	Canadian Institutes of Health Research	
Source of search	☒ ☐ ☐ ☐	Database Review article Grey literature Other (specify)
Author contacted	☐ ☒	Yes (specify date) No
Author response	☒ ☐ ☐	NA (author not contacted) Yes (specify date) No
Data source *Check all that apply*	☒ ☐	Extracted from article Provided by Author(s)

Study design		
Prospective cohort	☒	Yes
	☐	No
	☐	Unclear
Retrospective cohort	☐	Yes
	☒	No
	☐	Unclear
Nested case-control	☐	Yes
	☒	No
	☐	Unclear
Case-cohort	☐	Yes
	☒	No
	☐	Unclear

Study population		
Study city, country/ countries	Ottawa and Kingston, Ontario, Canada	
Study setting/ recruitment	From 2002 to 2009, The Ottawa Hospital and Kingston General Hospital	
Ethnicity	Caucasian (80%)	
Study population	☒	General population
	☐	Other (specify sub-group)
Inclusion criteria	Eligible participants were between 12 and 20 weeks gestation of a viable singleton or twin pregnancy and provided written informed consent.	
Exclusion criteria	Participants were excluded if recruited before 12 or after 20 weeks gestation, if they withdrew from the cohort, were lost to follow-up, or if the pregnancy was terminated	
Co-morbid conditions *Check all that apply* ☐ Not assessed	☒	Cardiovascular disease
	☒	Diabetes (pre-gestation)
	☐	Renal disease
	☐	Other (specify)

Exposure measurement			
Maternal plasma homocysteine (specify testing)	Blood samples for homocysteine were collected in K_2EDTA Vacutainer tubes (Becton Dickinson, Lincoln Park, NJ). Blood samples for plasma were immediately placed on ice and within 30 minutes centrifuged in 4 °C at 3000 × *g* for 10 minutes, then aliquoted and stored at -20 °C. Plasma homocysteine (µmol/L) was measured in batches on the Abbott AxSYM II Immunoassay System (Abbott Laboratories, Abbott Park, IL) using fluorescence polarization immunoassay.	☒ ☐ ☐	Yes No Unclear
Folate measure (specify)	Blood samples for serum folate were collected in serum separator tubes (Becton Dickinson). Blood samples for serum were centrifuged at 3000 × *g* for 10 minutes, then aliquoted and stored at −20 °C. Serum folate (nmol/L) was measured using the Beckman Coulter Access 2 and Unicel DxI 800 immunoassay analyzers using manufacturer's reagents (Beckman Coulter, Brea CA).	☒ ☐ ☐	Yes No Unclear
Other measure of interest (specify)	Blood samples for MTHFR were collected in K_2EDTA Vacutainer tubes (Becton Dickinson, Lincoln Park, NJ). Blood samples for plasma were immediately placed on ice and within 30 minutes centrifuged in 4 °C at 3000 × *g* for 10 minutes, then aliquoted and stored at -20 °C.	☒ ☐ ☐	Yes No Unclear

Disease outcomes measurement				
Primary outcome (describe)		Composite: Any placenta-mediated complication		
Secondary outcome (describe)		SGA, preeclampsia, placental abruption, pregnancy loss		
SR Outcome definitions	Preeclampsia	Preeclampsia was defined as hypertension with proteinuria beyond 20 weeks gestation. Hypertension was a diastolic blood pressure reading greater than or equal to 90 mmHg and proteinuria of 2+ on a dipstick or proteinuria greater than 300 mg in a 24-hour urine collection, measured on two separate occasions of at least 6 hours apart		
	IUGR/SGA/FGR	A small for gestational age (SGA) infant was defined as an infant with birth weight less than the 10^{th} percentile of sex and gestational age-adjusted population standards		
	Placental abruption	Placental abruption was defined as antepartum bleeding with evidence of a retro-placental thrombus.		
	Fetal death	Pregnancy loss was defined as intrauterine death before 20 weeks or stillbirth		
Outcome adjudication procedure *Check all that apply*		☒	Expert adjudication committee (specify outcome(s))	Preeclampsia and placental abruption: A committee of medical experts blind to patient exposure status independently examined information abstracted from medical records to adjudicate the diagnosis of preeclampsia, and placental abruption
		☒	Expert individual (specify outcome(s))	Pregnancy loss
		☒	Other (specify method and outcome(s))	Calculation: SGA
		☐	Unclear (specify outcome(s))	
Outcome assessor blind to exposure status? *Check all that apply*		☒	Yes (specify outcome(s))	All
		☐	No (specify outcome(s))	
		☐	Unclear (specify outcome(s))	

Participant flow			
Participant category	**Entire study**	**Exposed**	**Unexposed**
Identified	8085		
Excluded/ lost to follow-up	498		
Included	7587		

All participants accounted for?	☒	Yes
	☐	No (explain)

Baseline parameters					
	Units	Overall	cases	controls	Significance
Gestational age at blood sampling	weeks	12-20 weeks			
Maternal age	Mean (SD) µmol/L	30.3 (5.06)			
Other (specify):					
BMI	Mean (SD)	24.9 (5.50)			
Homocysteine distribution	Mean (SD) µmol/L	4.83 (1.27)			
Folate distribution					
Other distribution					
Other distribution					

Outcome (specify): Any PMC											
Outcome (describe): Any placenta-mediated complication											
Exposure (describe): Homocysteine µmol/L											
	Unadjusted							Adjusted			
Exposure	Outcome, n; %			Effect estimate				Effect estimate			
Exposure level	Yes	No	Total								
Mean (SD) OR: 5 µmol/L increase in Hcy	5.03 (1.51)	4.81 (1.25)		☐ OR ☐ RR ☒ Oth	 0.0001	☐ LCI ☐ UCI ☐ SE		☒ OR ☐ RR ☒ Oth	1.629 0.0007	☒ LCI ☒ UCI ☐ SE	1.227 2.161
				☐ OR ☐ RR ☐ Oth		☐ LCI ☐ UCI ☐ SE		☐ OR ☐ RR ☐ Oth		☐ LCI ☐ UCI ☐ SE	
				☐ OR ☐ RR ☐ Oth		☐ LCI ☐ UCI ☐ SE		☐ OR ☐ RR ☐ Oth		☐ LCI ☐ UCI ☐ SE	
				☐ OR ☐ RR ☐ Oth		☐ LCI ☐ UCI ☐ SE		☐ OR ☐ RR ☐ Oth		☐ LCI ☐ UCI ☐ SE	
				☐ OR ☐ RR ☐ Oth		☐ LCI ☐ UCI ☐ SE		☐ OR ☐ RR ☐ Oth		☐ LCI ☐ UCI ☐ SE	
Total				☐ OR ☐ RR ☐ Oth		☐ LCI ☐ UCI ☐ SE		☐ OR ☐ RR ☐ Oth		☐ LCI ☐ UCI ☐ SE	
Total	745	6676									

If Other effect estimate (specify):	p-value for adjusted and unadjusted		
Relevant confounders described?	☒ ☐	Yes No	
Confounders considered:	maternal age, race, education, income, nulliparity, smoking, diabetes, use of hormonal birth control prior to conception, chronic hypertension, history of a placenta-mediated complication, folic acid supplementation, and serum folate.		
Method to identify confounders described?	☒	Yes (describe):	knowledge of the subject matter, analyses of homocysteine determinants in OaK participants (unpublished), and studies investigating the association of homocysteine with placenta-mediated complications
	☐	No	
Method used to control for confounding?	☐	Matching (describe variables):	
	☐	Stratification	
	☒	Multivariable regression (describe):	Multivariable Logistic regression. Results combined across 10 multiply imputed data sets. Initial model was fit including main effects for all independent variables. Continuous variables homocysteine, gestational age, maternal age, BMI, and serum folate were modeled using restricted cubic splines with five knots set at the 5th, 27.5th, 50th, 72.5th, and 95th quantile. Next, an ANOVA plot of partial

			associations, corrected for the number of degrees of freedom, was generated to identify strong and weak partial associations. Strengths of associations were used to decide how many degrees of freedom to allocate to each variable in the final model. Akaike's Information Criterion (AIC) and the Bayesian Information Criterion (BIC) were used to confirm goodness of fit with different numbers of knots
	☐	Multivariate regression (describe outcomes)	
	☐	Propensity score (matching)	
	☐	Propensity score (regression)	
	☐	Other (specify)	
Newcastle-Ottawa Scale, Comparability subcomponent	☒	a)	Gestational age
	☒	b)	BMI OR serum folate/folic acid supplementation OR smoking OR maternal age
	Total score:	9	

Supplementary file 3: Quality assessment scale

NEWCASTLE - OTTAWA QUALITY ASSESSMENT SCALE
CASE CONTROL STUDIES

Note: A study can be awarded a maximum of one star for each numbered item within the Selection and Exposure categories. A maximum of two stars can be given for Comparability.

Selection

1) Is the case definition adequate?
   a) yes, with independent validation ✸
   b) yes, eg record linkage or based on self reports
   c) no description

2) Representativeness of the cases
   a) consecutive or obviously representative series of cases ✸
   b) potential for selection biases or not stated

3) Selection of Controls
   a) community controls ✸
   b) hospital controls
   c) no description

4) Definition of Controls
   a) no history of disease (endpoint) ✸
   b) no description of source

Comparability

1) Comparability of cases and controls on the basis of the design or analysis
   a) study controls for _gestational age_ (Select the most important factor.) ✸
   b) study controls for any additional factor ✸ (This criteria could be modified to indicate specific control for a second important factor.)

Exposure

1) Ascertainment of exposure
   a) secure record (eg surgical records) ✸
   b) structured interview where blind to case/control status ✸
   c) interview not blinded to case/control status
   d) written self report or medical record only
   e) no description

2) Same method of ascertainment for cases and controls
   a) yes ✸
   b) no

3) Non-Response rate
   a) same rate for both groups ✸
   b) non respondents described
   c) rate different and no designation

COHORT STUDIES

Selection

1) Representativeness of the exposed cohort
   a) truly representative of the average _____ (describe) in the community ✵
   b) somewhat representative of the average _____ in the community ✵
   c) selected group of users eg nurses, volunteers
   d) no description of the derivation of the cohort

2) Selection of the non exposed cohort
   a) drawn from the same community as the exposed cohort ✵
   b) drawn from a different source
   c) no description of the derivation of the non exposed cohort

3) Ascertainment of exposure
   a) secure record (eg surgical records) ✵
   b) structured interview ✵
   c) written self report
   d) no description

4) Demonstration that outcome of interest was not present at start of study
   a) yes ✵
   b) no

Comparability

1) Comparability of cohorts on the basis of the design or analysis
   a) study controls for _gestational age_ (select the most important factor) ✵
   b) study controls for any additional factor ✵ (This criteria could be modified to indicate specific control for a second important factor.)

Outcome

1) Assessment of outcome
   a) independent blind assessment ✵
   b) record linkage ✵
   c) self report
   d) no description

2) Was follow-up long enough for outcomes to occur
   a) yes (select an adequate follow up period for outcome of interest) ✵
   b) no

3) Adequacy of follow up of cohorts
   a) complete follow up - all subjects accounted for ✵
b) subjects lost to follow up unlikely to introduce bias - small number lost - > _% (select an adequate %) follow up, or description provided of those lost) ✵
c) follow up rate < ____% (select an adequate %) and no description of those lost
d) no statement

Supplementary file 4: Tables of complete results for systematic review

Studies that investigated the outcome small for gestational age (SGA)

Table D1: Studies reporting homocysteine concentration in small for gestational age (SGA) cases versus comparison

Study (Author, year)	Country	Enrollment	Design	Definition of SGA	Cases		Controls		p- value	Newcastle-Ottawa Scale *	
					n	Mean ± SD homocysteine µmol/L	n	Mean ± SD homocysteine µmol/L		Total score (9 ✵ max)	Comparability score: GA (1 ✵) and other factors (1 ✵) †
Hogg, 2000	USA	1991-1993	Nested case-control	<10th percentile	22	4.9 ± 1.8	402	4.6 ± 1.4	0.48	8 ✵	GA ✵
Ronnenberg, 2002 (AJCN)	China	1996-1998	Nested case-control	<10th percentile	65	8.2 ± 2.42	358	8.1 ± 3.37	>0.05	7 ✵	GA ✵
D'Anna, 2004	Italy	2000-2001	Nested case-control	<10th percentile	36	5.85 ± 1.08	63	5.6 ± 1.11	0.20	8 ✵	GA ✵ Other ✵
Onalan, 2006	Turkey	2004-2005	Nested case-control	<5th percentile (isolated IUGR)	41	6 (range: 2.3-16)	324	5 (range: 1.8-25.3)	<0.001	7 ✵	
Furness, 2011	Australia	2004-2006	Nested case-control	<10th percentile	21	5.3 ± 1.757	63	4.2 ± 0.993	0.20	8 ✵	GA ✵
Chaudhry, 2019	Canada	2002-2009	Prospective cohort	<10th percentile	502	5.11 ± 1.63	6919	4.81 ± 1.25	<0.0001	7 ✵	
Choi, 2016	South Korea,	2012-2013	Prospective cohort	<10th percentile	39	Median: 10.2 (range: 8.6-13)	217	Median: 10.6 (range: 8.2-14.8)	0.62	6 ✵	
Cawley, 2019	Ireland	2014-2016	Prospective cohort	<10th percentile	66	7.6 (2.1)	400	7.0 (2.1)	0.167	6 ✵	
Maged, 2017	Egypt	2015-2016	Prospective cohort	<10th percentile	52	6.32 ± 3.64	297	4.70 ± 2.082	<0.05	7 ✵	

Abbreviations: SGA, small for gestational age; GA, gestational age; NR, not reported; AJCN, American Journal of Clinical Nutrition

* Newcastle-Ottawa Scale, Score components: Selection (4 ✵), Comparability (2 ✵), Exposure (case-control study) or Outcome (cohort study) (3 ✵)

† Newcastle-Ottawa Scale, Comparability component: Comparability of most important factor gestational age (GA), 1✵; Comparability of other factors: BMI, serum folate/folic acid supplementation, smoking, or maternal age, 1✵

Table D2: Studies reporting effect estimates for the association of homocysteine with small for gestational age (SGA)

Study (Author, year)	Country	Enrollment	Design	Definition of SGA	Cases	Controls	Threshold homocysteine	OR (95% CI)	Newcastle-Ottawa Scale *	
									Total score (9 ✸ max)	Comparability score: GA (1 ✸) and other factors (1 ✸) †
Murakami, 2001	Japan	1996-1998	Prospective cohort	gestational age birth weight standard for Japanese population	27	722	≥ mean + 1.96 standard deviation	0.78 (0.10, 5.9)	7 ✸	
Ronnenberg, 2002 (AJCN)	China	1996-1998	Nested case-control	<10th percentile	65	358	≥90th percentile in women with adequate folate and B_{12} (≥12.4 µmol/L)	1.0 (0.4, 2.3)	8 ✸	GA ✸ Other ✸
Onalan, 2006	Turkey	2004-2005	Nested case-control	<5th percentile (isolated)	41	324	≥95th percentile in controls (6.3 µmol/L)	12.6 (5.2, 30.7)	8 ✸	Other ✸
Dwarkanath, 2011	India	2000-2002	Prospective cohort	<10th percentile	52	101	≥10 µmol/L	1.61 (0.777, 3.33)	8 ✸	GA ✸
Furness, 2011	Australia	2004-2006	Nested case-control	<10th percentile	21	63	1 µmol/L increase	1.556 (0.798, 3.033)	9 ✸	GA ✸ Other ✸
Dodds, 2008	Canada	2002-2005	Prospective cohort	<10th percentile	129	1707	≥90th percentile in normotensive controls with live-born infants not SGA (cut-off determined per bi-weekly GA interval)	Relative risk: 1.5 (0.9, 2.6)	9 ✸	GA ✸ Other ✸

Bergen, 2012	The Netherlands	2002-2006	Prospective cohort	<5th percentile	299	5506	Highest versus lowest quintile (≥8.3 vs. ≤5.8 µmol/L	1.68 (1.16, 2.43)	9 ✵	GA ✵ Other ✵
Chaudhry, 2019	Canada	2002-2009	Prospective cohort	<10th percentile	502	6919	5 µmol/L increase	1.756 (1.254, 2.458)	9 ✵	GA ✵ Other ✵

Abbreviations: SGA, small for gestational age; GA, gestational age; NR, not reported; AJCN, American Journal of Clinical Nutrition

* Newcastle-Ottawa Scale, Score components: Selection (4 ✵), Comparability (2 ✵), Exposure (case-control study) or Outcome (cohort study) (3 ✵)

† Newcastle-Ottawa Scale, Comparability component: Comparability of most important factor gestational age (GA), 1✵; Comparability of other factors: BMI, serum folate/folic acid supplementation, smoking, or maternal age, 1✵

Studies that investigated the outcome Preeclampsia

Table D3: Studies reporting homocysteine concentration in Preeclampsia cases versus comparison

Study (Author, year)	Country	Enroll-ment	Design	Definition of Preeclampsia as threshold for blood pressure and proteinuria	Cases		Controls		p-value	Newcastle-Ottawa Scale *	
					n	Mean ± SD homocysteine µmol/L	n	Mean ± SD homocysteine µmol/L		Total score (9 ✵ max)	Comparability score: GA (1 ✵) and other factors (1 ✵) †
Cotter, 2003	Ireland	1986-1990	Nested case-control	140/90; 0.3 g/24 hr or +1/+2	71	8.4 ± 2.4	142	7.0 ± 1.5	<0.0001	8 ✵	GA ✵ Other ✵
Cotter, 2001	Ireland	1986-1990	Nested case-control	160-180/110; ≥5 g/24 hr or +3/+4	56	9.8 ± 3.3	112	8.4 ± 1.9	<0.0001	8 ✵	GA ✵
Hogg, 2000	USA (Alabama)	1991-1993	Nested case-control	NT /90; 0.3 g/24 hr or ≥ +1	16	5.2 ± 1.3	409	4.6 ± 1.4	0.11	8 ✵	GA ✵
Sorensen, 1999	USA (Washington)	1993-1995	Nested case-control	140/90 or Δ30/Δ15; 0.1 g/l	52	4.8 ± 1.1	56	4.4 ± 4.8	0.08	7 ✵	
Hietala, 2001	Finland	1996	Nested case-control	140/90 or Δ30/Δ15; 0.3 g	34	6.99 ± 1.56	68	6.91 ± 1.76	0.83	8 ✵	GA ✵
D'Aniello, 2003	Italy	NR	Prospective cohort	140/90; 0.3 g/24 hr or 0.1 g	18	12.1 ± 9.8	41	17.4 ± 13.9	NS	7 ✵	GA ✵
Roes, 2006	The Netherlands	2000-2001	Prospective cohort	140/90; 0.3 g	6	9.86 ± 1.85	19	10.57 ± 2.095	>0.05	8 ✵	GA ✵
D'Anna, 2004	Italy	2000-2001	Nested case-control	140/90; ≥0.5 g	27	5.97 ± 1.36	63	5.56 ± 0.92	0.11	8 ✵	GA ✵ Other ✵
Zeeman, 2003	USA (Texas)	2000-2002	Prospective cohort	150/100; ≥0.3 g/24 hr	16	5.1 ± 1.7	41	4.7 ± 1.3	0.564	6 ✵	
Kahn, 2009	Canada	1999-2003	Nested case-control	NT/90; 0.3 g/24 hr or +2	113	3.4 ± 0.9	443	3.7 ± 0.9	0.002	8 ✵	GA ✵
Malek-khosravi, 2009	Iran	1999-2004	Nested case-control	140/90; ≥0.3 g/24 hr or ≥ +2	20	5.54 ± 0.33	20	4.31 ± 0.28	0.03	8 ✵	GA ✵ Other ✵
Polat, 2016	Turkey	2002-2004	Nested case-control	140/90;	70	10.5 ± 3.6	60	7.25 ± 2.44	<0.001	7 ✵	

				≥0.3 g/24 hr or ≥ +2							
Onalan, 2006	Turkey	2004-2005	Nested case-control	110/90; 0.3 g/24 hr or +2	32	7.1 (range: 2.3-13.2)	324	5 (range: 1.8-25.3)	<0.001	7 ✵	
Furness, 2011	Australia	2004-2006	Nested case-control	140/90; ≥0.3 g/24 hr	15	4.8 ± 1.715	63	4.2 ± 0.993	NS	8 ✵	GA ✵
Chaudhry, 2019	Canada	2002-2009	Prospective cohort	NT/90; 0.3 g/24 hr or +2	223	4.99 ± 1.43	7277	4.82 ± 1.27	0.0880	7 ✵	
Lopez-Alarcon, 2015	Mexico	2009-2011	Prospective cohort	140/90; ≥0.3mg/dl	49	7.36 ± 2.6	179	5.73 ± 1.76	<0.05	7 ✵	
Cheng, 2016	Taiwan	2007-2013	Nested case-control	140/90; 0.3 g/24 hr or +1	134	7.88 ± 3.8	150	5.81 ± 1.75	<0.0001	8 ✵	GA ✵
Choi, 2016	South Korea	2012-2013	Prospective cohort	140/90; ≥ 0.3 g/24 hr	5	Median: 11.7 (IQR: 9.9-18.7)	252	Median: 10.4 (IQR: 8.2-14.1)	0.40	6 ✵	
Maged, 2017	Egypt	2015-2016	Prospective cohort	140/90; ≥ 0.3 g/24 hr	45	7.033 ± 2.744	297	4.701 ± 2.082	<0.05	7 ✵	
Sun, 2017	China	2016	Prospective cohort	140/90; ≥ 0.3 g/24 hr or ≥ +1 or >30 mg/dL; Severe defined as one or more of: 160/110 mm Hg or ≥ 2 g/24 hr or ≥ +3 or serum creatinine >1.1 mg/dL, cerebral or visual disturbances, pulmonary edema, epigastric or right-upper-quadrant pain, impaired liver function, thrombocytopeni	44	8.50 ± 1.18	4418	7.33 ± 1.53	<0.001	7 ✵	

				a, and fetal growth restriction.							
Sun, 2017	China	2016	Prospective cohort	140/90; ≥ 0.3 g/24 hr or ≥ +1 or >30 mg/dL (Mild preeclampsia, excludes severe)	103	7.49 ± 1.45	4418	7.33 ± 1.53	0.301	7 ✵	

Abbreviations: GA, gestational age; NT, no threshold; NR, not reported; IQR, inter-quartile range

* Newcastle-Ottawa Scale, Score components: Selection (4 ✵), Comparability (2 ✵), Exposure (case-control study) or Outcome (cohort study) (3 ✵)

† Newcastle-Ottawa Scale, Comparability component: Comparability of most important factor gestational age (GA), 1✵; Comparability of other factors: BMI, serum folate/folic acid supplementation, smoking, or maternal age, 1✵

Table D4: Studies reporting effect estimates for the association of homocysteine with Preeclampsia

Study (Author, year)	Country	Enrollment	Design	Definition of Preeclampsia as threshold for blood pressure and proteinuria	Cases	Controls	Threshold homocysteine	OR (95% CI)	Newcastle-Ottawa Scale *	
									Total score (9 ✸ max)	Comparability score: GA (1 ✸) and other factors (1 ✸) †
Cotter, 2003	Ireland	1986-1990	Nested case-control	140/90; 0.3 g/24 hr or +1/+2	71	142	≥upper third (7.8 µmol/L)	4.1 (1.4, 12.6)	8 ✸	GA ✸ Other ✸
Cotter, 2001	Ireland	1986-1990	Nested case-control	160-180/110; ≥5 g/24 hr or +3/+4	56	112	≥upper quartile (10 µmol/L)	2.84 (1.37, 5.88)	9 ✸	GA ✸ Other ✸
Sorensen, 1999	USA (Washington)	1993-1995	Nested case-control	140/90 or Δ30/Δ15; 0.1 g/l	52	56	≥90th percentile (5.5 µmol/L)	3.2 (1.1, 9.2)	8 ✸	Other ✸
Hietala, 2001	Finland	1996	Nested case-control	Δ30/Δ15 or 140/90; 0.3 g	34	68	>75th percentile in controls (7.7 µmol/L)	1.59 (0.57, 4.43)	8 ✸	GA ✸
Murakami, 2001	Japan	1996-1998	Prospective cohort	Criteria of the Japan Society of Obstetrics and Gynecology	7	742	≥ mean + 1.96 standard deviation	8.6 (1.61, 45)	7 ✸	
Kahn, 2009	Canada	1999-2003	Nested case-control	NT [c] /90; 0.3 g/24 hr or +2	113	443	≥75th percentile in controls (11 µmol/L)	0.8 (0.4, 1.4)	9 ✸	GA ✸ Other ✸
Zeeman, 2003	USA (Texas)	2000-2002	Prospective cohort	150/100; ≥0.3 g/24 hr	16	41	>95th percentile (6.9 µmol/L)	2.786 (0.358, 21.7)	6 ✸	
Polat, 2016	Turkey	2002-2004	Nested case-control	140/90; ≥0.3 g/24 hr or ≥ +2	70	60	>95th percentile in controls (10.97 µmol/L)	7.6 (2.13, 27)	7 ✸	

Dodds, 2008	Canada	2002-2005	Prospective cohort	NT/90; 0.3 g/24 hr or ≥+1	65	1836	≥90th percentile in normotensive controls with live-born infants not SGA (cut-off determined per 2-week GA interval)	Relative risk: 2.7 (1.4, 5.0)	9 ✵	GA ✵ Other ✵
Bergen, 2012	The Netherlands	2002-2006	Prospective cohort	140/90; 0.3 g/24 hr or ≥+1	118	5687	Highest versus lowest quintile (≥8.3 vs. ≤5.8 µmol/L	1.60 (0.88, 2.90)	9 ✵	GA ✵ Other ✵
Onalan, 2006	Turkey	2004-2005	Nested case-control	110/90; 0.3 g/24 hr or +2	32	324	≥95th percentile in controls (6.3 µmol/L)	36.7 (12.4, 108.7)	8 ✵	Other ✵
Wadhwani, 2016	India	NR	Nested case-control	140/90; >0.3 g/24 hr or +1	61	124	>10 µmol/L	1.6702 (0.432, 6.4567)	6 ✵	
Chaudhry, 2019	Canada	2002-2009	Prospective cohort	NT/90; 0.3 g/24 hr or +2	223	7277	5 µmol/L increase	1.546 (0.96, 2.49)	9 ✵	GA ✵ Other ✵
Sun, 2017	China	2016	Prospective cohort	140/90; ≥ 0.3 g/24 hr or ≥ +1 or >30 mg/dL Severe defined as one or more of: 160/110 mm Hg or ≥ 2 g/24 hr or ≥ +3 or serum creatinine >1.1 mg/dL, cerebral or visual disturbances, pulmonary edema, epigastric or right-upper-	44	4418	1 µmol/L increase	1.12 (1.06, 1.20)	8 ✵	Other ✵

				quadrant pain, impaired liver function, thrombocytopenia, and fetal growth restriction.						
Sun, 2017	China	2016	Prospective cohort	140/90; ≥ 0.3 g/24 hr or ≥ +1 or >30 mg/dL (Mild preeclampsia, excludes severe)	103	4418	1 µmol/L increase	1.05 (0.95, 1.15)	8 ✵	Other ✵

Abbreviations: GA, gestational age; NT, no threshold, NR, not reported

* Newcastle-Ottawa Scale, Score components: Selection (4 ✵), Comparability (2 ✵), Exposure (case-control study) or Outcome (cohort study) (3 ✵)

† Newcastle-Ottawa Scale, Comparability component: Comparability of most important factor gestational age (GA), 1✵; Comparability of other factors: BMI, serum folate/folic acid supplementation, smoking, or maternal age, 1✵

Studies that investigated the outcome placental abruption

Table D5: Studies reporting homocysteine concentration in placental abruption cases versus comparison

Study (Author, year)	Country	Enrollment	Design	Definition of placental abruption	Cases		Controls		p- value	Newcastle-Ottawa Scale *	
					n	Mean ± SD homocysteine µmol/L	n	Mean ± SD homocysteine µmol/L		Total score (9 ✵ max)	Comparability score: GA (1 ✵) and other factors (1 ✵) †
Chaudhry, 2019	Canada,	2002-2009	Prospective cohort	Antepartum bleeding and retroplacental thrombus	65	4.82 ± 1.20	7435	4.83 ± 1.28	0.9238	7 ✵	

Abbreviations: GA, gestational age

* Newcastle-Ottawa Scale, Score components: Selection (4 ✵), Comparability (2 ✵), Exposure (case-control study) or Outcome (cohort study) (✵ *)

† Newcastle-Ottawa Scale, Comparability component: Comparability of most important factor gestational age (GA), 1✵; Comparability of other factors: BMI, serum folate/folic acid supplementation, smoking, or maternal age, 1✵

Table D6: Studies reporting effect estimates for the association of homocysteine with Placental abruption

Study (Author, year)	Country	Enrollment	Design	Definition of placental abruption	Cases	Controls	Threshold homocysteine	OR (95% CI)	Newcastle-Ottawa Scale *	
									Total score (9 ✵ max)	Comparability score: GA (1 ✵) and other factors (1 ✵)†
Chaudhry, 2019	Canada	2002-2009	Prospective cohort	Antepartum bleeding and retroplacental thrombus	65	7435	5 µmol/L increase	1.005 (0.59, 1.71)	9 ✵	GA ✵ Other ✵

Abbreviations: GA, gestational age

* Newcastle-Ottawa Scale, Score components: Selection (4 ✵), Comparability (2 ✵), Exposure (case-control study) or Outcome (cohort study) (3 ✵)

† Newcastle-Ottawa Scale, Comparability component: Comparability of most important factor gestational age (GA), 1✵; Comparability of other factors: BMI, serum folate/folic acid supplementation, smoking, or maternal age, 1✵

Studies that investigated the outcome pregnancy loss

Table D7: Studies reporting homocysteine concentration in Pregnancy loss cases versus comparison

Study (Author, year)	Country	Enrollment	Design	Definition of pregnancy loss	Cases		Controls		p- value	Newcastle-Ottawa Scale *	
					n	Mean ± SD homocysteine µmol/L	n	Mean ± SD homocysteine µmol/L		Total score (9 ✵ max)	Comparability score: GA (1 ✵) and other factors (1 ✵) †
Ronnenberg, 2002 (OG)	China	1996-1998	Nested case-control	Fetal death (via hCG) of a clinically recognized pregnancy by 100 days' gestation	49	7.7 ± 3.3074	409	8.0 ± 3.086	0.59	7 ✵	GA ✵
Rodriguez, 2009	Mexico	2001-NR	Nested case-control	Spontaneous abortion <20 weeks	23	Median: 10.2 (P10-P90: 5.7-14.8)	74	Median: 9 (P10-P90: 6.8-13.4)	0.53	7 ✵	GA ✵
Chaudhry, 2019	Canada	2002-2009	Prospective cohort	Intrauterine demise <20 weeks or stillbirth	85	5.19 ± 1.54	7415	4.83 ± 1.27	0.0332	7 ✵	
Mascarenhas, 2014	India	2009-2011	Prospective cohort	Loss and stillbirth	8	24.65 ± 4.51	82	13.51 ± 7.47	0.0002	5 ✵	

Abbreviations: GA, gestational age; OG, Obstetrics & Gynecology (Journal); P10-P90, 10th to 90th percentile

* Newcastle-Ottawa Scale, Score components: Selection (4 ✵), Comparability (2 ✵), Exposure (case-control study) or Outcome (cohort study) (3 ✵)

† Newcastle-Ottawa Scale, Comparability component: Comparability of most important factor gestational age (GA), 1✵; Comparability of other factors: BMI, serum folate/folic acid supplementation, smoking, or maternal age, 1✵

Table D8: Studies reporting effect estimates for the association of homocysteine with Pregnancy loss

Study (Author, year)	Country	Enrollment	Design	Definition of pregnancy loss	Cases	Controls	Threshold homocysteine	OR (95% CI)	Newcastle-Ottawa Scale *	
									Total score (9 ✵ max)	Comparability score: GA (1 ✵) and other factors (1 ✵) †
Murakami, 2001	Japan	1996-1998	Prospective cohort	Stillbirth	12	737	≥ mean + 1.96 standard deviation	4.3 (0.90, 20)	7 ✵	
Ronnenberg, 2002 (OG)	China	1996-1998	Nested case-control	Fetal death (confirmed via hCG) of clinically recognized pregnancy up to 100 days gestation	49	409	≥95th percentile (15.5 µmol/L)	2.0 (0.6, 6.8)	7 ✵	GA ✵
Dodds, 2008	Canada	2002-2005	Prospective cohort	Spontaneous fetal death	103	2016	≥90th percentile in normotensive controls with live-born infants not SGA (cut-off determined per 2-week GA interval)	2.1 (1.2, 3.6)	9 ✵	GA ✵ Other ✵
Chaudhry, 2019	Canada	2002-2009	Prospective cohort	Intrauterine demise <20 weeks or stillbirth	85	7415	5 µmol/L increase	1.392 (0.88, 2.21)	9 ✵	GA ✵ Other ✵

Abbreviations: GA, gestational age; OG, Obstetrics & Gynecology (Journal)

* Newcastle-Ottawa Scale, Score components: Selection (4 ✵), Comparability (2 ✵), Exposure (case-control study) or Outcome (cohort study) (3 ✵)

† Newcastle-Ottawa Scale, Comparability component: Comparability of most important factor gestational age (GA), 1✵; Comparability of other factors: BMI, serum folate/folic acid supplementation, smoking, or maternal age, 1✵

Studies that investigated a composite outcome

Table D9: Studies reporting homocysteine concentration in composite outcome cases versus comparison

Study (Author, year)	Country	Enrollment	Design	Definition of composite outcome	Cases		Controls		p- value	Newcastle-Ottawa Scale *	
					n	Mean ± SD homocysteine µmol/L	n	Mean ± SD homocysteine µmol/L		Total score (9 ✵ max)	Comparability score: GA (1 ✵) and other factors (1 ✵) †
Onalan, 2006	Turkey	2004-2005	Nested case-control	Any adverse outcome (preeclampsia, isolated SGA <5th centile, placental abruption, or stillbirth)	65	6.4 ± 3.425	324	5 ± 3.917	<0.001	7 ✵	
Chaudhry, 2019	Canada	2002-2009	Prospective cohort	Any placenta-mediated complication (SGA >10th percentile, preeclampsia, Placental abruption, pregnancy loss)	745	5.03 ± 1.51	6676	4.81 ± 1.25	0.0001	7 ✵	
Maged, 2017	Egypt	2015-2016	Prospective cohort	Other complications (pregnancy loss and placental abruption: separation of normally implanted placenta	59	6.602 ± 2.469	297	4.70 ± 2.082	<0.05	7 ✵	

				resulting in concealed or revealed antepartum hemorrhage)							

Abbreviations: GA, gestational age

* Newcastle-Ottawa Scale, Score components: Selection (4 ✵), Comparability (2 ✵), Exposure (case-control study) or Outcome (cohort study) (3 ✵)

† Newcastle-Ottawa Scale, Comparability component: Comparability of most important factor gestational age (GA), 1✵; Comparability of other factors: BMI, serum folate/folic acid supplementation, smoking, or maternal age, 1✵

Table D10: Studies reporting effect estimates for the association of homocysteine with a composite outcome

Study (Author, year)	Country	Enrollment	Design	Definition of composite outcome	Cases	Controls	Threshold homocysteine	OR (95% CI)	Newcastle-Ottawa Scale *	
									Total score (9 ✵ max)	Comparability score: GA (1 ✵) and other factors (1 ✵) †
Onalan, 2006	Turkey	2004-2005	Nested case-control	Any adverse outcome (preeclampsia, isolated SGA <5th centile, placental abruption, or stillbirth)	65	324	≥95th percentile in controls (6.3 µmol/L)	22.2 (10, 49.3)	8 ✵	Other ✵
Chaudhry, 2019	Canada	2002-2009	Prospective cohort	Any placenta-mediated complication (SGA >10th percentile, preeclampsia, placental abruption, pregnancy loss)	745	6676	5 µmol/L increase	1.63 (1.23, 2.16)	9 ✵	GA ✵ Other ✵

Abbreviations: GA, gestational age

* Newcastle-Ottawa Scale Score components: Selection (4 ✵), Comparability (2 ✵), Exposure (case-control study) or Outcome (cohort study) (3 ✵)

† Newcastle-Ottawa Scale Score Comparability component: Comparability of most important factor gestational age (GA), 1✵; Comparability of other factors: BMI, serum folate/folic acid supplementation, smoking, or maternal age, 1✵

APPENDIX D. SUPPLEMENTARY DATA FOR CHAPTER 6

Table S1: Multivariable linear regression analysis of the determinants of plasma homocysteine, with plasma homocysteine as a continuous dependent variable, examining the effect of MTHFR 677C>T genotype (n=4006)

Variable	Effect (95% CI)		p-value [1]
Gestational age at blood work (restricted cubic spline (rcs), five knots)			<0.0001
13.7 versus 12.4 weeks	0.018	(-0.094, 0.130)	
Age (rcs, three knots)			0.0024
34 versus 27 years	-0.034	(-0.095, 0.026)	
Race			<0.0001
African	-0.087	(-0.336, 0.163)	
Middle eastern	-0.397	(-0.594, -0.200)	
Asian	-0.421	(-0.574, -0.267)	
Caucasian	Reference		
Other	-0.407	(-0.707, -0.107)	
Education			0.5001
College/University completed versus less than completed	0.036	(-0.068, 0.140)	
Nulliparous			0.0468
Yes versus no	0.099	(0.001, 0.196)	
Smoking			<0.0001
No	Reference		
Second-hand	0.204	(-0.190, 0.599)	
Med/light smoker (<10 cigarettes per day)	0.406	(0.226, 0.587)	
Heavy smoker (≥10 cigarettes per day)	0.991	(0.765, 1.216)	
Diabetes			0.3769
Yes versus no	-0.142	(-0.456, 0.173)	
BMI (rcs, three knots)			0.0078
27.3 versus 21.1 kg/m^2	-0.098	(-0.167, -0.028)	
Hormonal birth control prior to conception			0.1184
No	Reference		
Oral	-0.084	(-0.172, 0.003)	
Injection or IUD	0.115	(-0.199, 0.429)	
Chronic hypertension			0.0029
Yes versus no	0.524	(0.180, 0.869)	
History of PMC (Preeclampsia, placental abruption, SGA, stillbirth/loss)			0.0136
Yes versus no	-0.158	(-0.283, -0.032)	
Folic acid supplementation			0.3292
None	Reference		
>0 and ≤1 mg	-0.043	(-0.232, 0.146)	
>1 mg	-0.115	(-0.323, 0.093)	
Serum folate*MTHFR genotype [2]			<0.0001
Serum folate (rcs, five knots)			<0.0001
TT: 45.1 versus 30.6 nmol/L	0.304	(0.011, 0.597)	
CC/CT: 45.1 versus 30.6 nmol/L	0.051	(-0.055, 0.158)	
MTHFR genotype			<0.0001
CC/ CT (wild type/ heterozygous)	Reference		
TT (homozygous)	0.106	(-0.125, 0.337)	

[1] Wald test of most meaningful hypotheses, pooled across multiple imputation data sets

[2] Interaction effect (Factor + higher order factors)

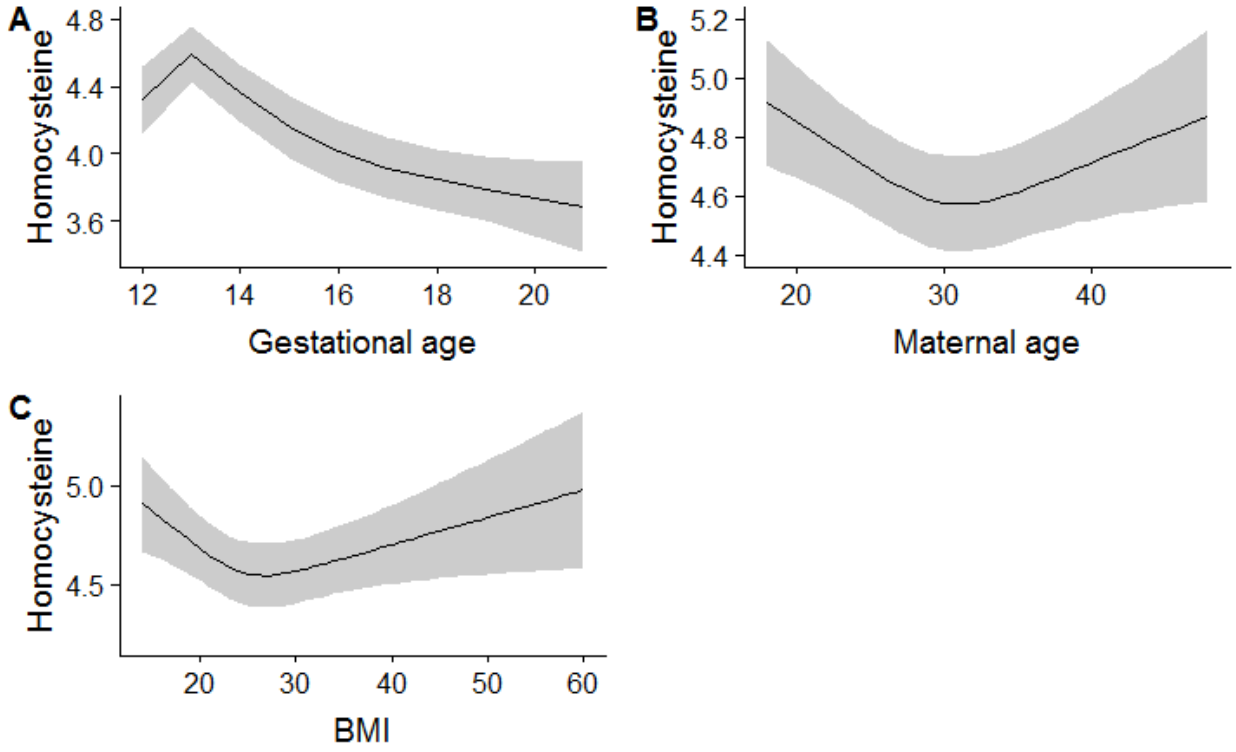

Figure S1: For the multivariable model reported in Table S1, associations between homocysteine and continuous variables modeled as a restricted cubic spline: A) gestational age at blood work with five knots at 12.1, 12.4, 12.8, 13.4, and 19; B) maternal age with three knots at 24, 30, and 37; and C) BMI with three knots at 19.6, 23.5, and 32.2. Shaded area represents 95% CI.

Table S2: Multivariable linear regression analysis of the determinants of plasma homocysteine, with the dependent variable as a continuous z-score calculated from homocysteine mean and SD for gestational week at recruitment (n=7587)

Variable	Effect (95% CI)		p-value [1]
Gestational age at blood work (linear)			0.5637
13.7 versus 12.4 weeks	-0.004	(-0.017, 0.009)	
Age (rcs, three knots)			0.0014
34 versus 27 years	-0.014	(-0.048, 0.021)	
Race			<0.0001
African	-0.285	(-0.438, -0.133)	
Middle eastern	-0.348	(-0.472, -0.225)	
Asian	-0.365	(-0.460, -0.270)	
Caucasian	Reference		
Other	-0.309	(-0.475, -0.144)	
Education			0.4297
College/University completed versus less than completed	-0.024	(-0.082, 0.035)	
Nulliparous			<0.0001
Yes versus no	0.121	(0.068, 0.173)	
Smoking			<0.0001
No	Reference		
Second-hand	0.265	(0.105, 0.425)	
Med/light smoker (<10 cigarettes per day)	0.324	(0.228, 0.420)	
Heavy smoker (≥10 cigarettes per day)	0.682	(0.556, 0.808)	
Diabetes			0.6017
Yes versus no	-0.047	(-0.225, 0.130)	
BMI (rcs, 3 knots)			0.0082
27.3 versus 21.1 kg/m^2	-0.059	(-0.098, -0.021)	
Hormonal birth control prior to conception			0.2208
No	Reference		
Oral	-0.033	(-0.082, 0.016)	
Injection or IUD	0.072	(-0.070, 0.214)	
Chronic hypertension			0.0001
Yes versus no	0.408	(0.208, 0.609)	
History of PMC (Preeclampsia, placental abruption, SGA, stillbirth/loss)			0.0170
Yes versus no	-0.089	(-0.161, -0.016)	
Folic acid supplementation			<0.0001
No	Reference		
>0 and ≤ 1 mg	-0.251	(-0.356, -0.147)	
>1 mg	-0.268	(-0.386, -0.151)	
Serum folate (rcs, five knots)			<0.0001
45.1 versus 30.6 nmol/L	0.106	(0.048, 0.164)	

[1] Wald test of most meaningful hypotheses, pooled across multiple imputation datasets

Table S3: Multivariable logistic regression analysis of the determinants of plasma homocysteine, with the binary dependent variable as plasma homocysteine greater than the 90th percentile for gestational week at recruitment (n=7587)

Variable	Odds ratio (95% CI)		p-value [1]
Gestational age at blood work (restricted cubic spline (rcs), four knots)			<0.0001
13.7 versus 12.4 weeks	0.702	(0.612, 0.804)	
Age (linear)			0.4675
34 versus 27 years	0.966	(0.881, 1.060)	
Race			<0.0001
African	0.511	(0.318, 0.822)	
Middle eastern	0.416	(0.265, 0.653)	
Asian	0.491	(0.357, 0.676)	
Caucasian	Reference		
Other	0.565	(0.335, 0.953)	
Education			0.4195
College/University completed versus less than completed	0.939	(0.806, 1.094)	
Nulliparous			0.0012
Yes versus no	1.268	(1.098, 1.465)	
Smoking			<0.0001
No	Reference		
Second-hand	1.409	(0.953, 2.082)	
Med/light smoker (<10 cigarettes per day)	1.666	(1.323, 2.098)	
Heavy smoker (≥10 cigarettes per day)	2.346	(1.761, 3.125)	
Diabetes			0.1742
Yes versus no	1.371	(0.870, 2.162)	
BMI (rcs, three knots)			0.0356
27.3 versus 21.1 kg/m^2	0.880	(0.794, 0.976)	
Hormonal birth control prior to conception			0.0563
No	Reference		
Oral	0.858	(0.749, 0.982)	
Injection or IUD	1.118	(0.787, 1.590)	
Chronic hypertension			0.0010
Yes versus no	2.215	(1.377, 3.565)	
History of PMC (Preeclampsia, placental abruption, IUGR, stillbirth, loss)			0.1605
Yes versus no	0.862	(0.700, 1.061)	
Folic acid supplementation			0.0003
Yes versus no	0.626	(0.486, 0.807)	
Serum folate (rcs, 5 knots)			<0.0001
45.3 versus 30.7 nmol/L	1.038	(0.883, 1.220)	

[1] Wald test of most meaningful hypotheses, pooled across multiple imputation datasets