Vasculopathic and thrombophilic risk factors for spontaneous preterm birth

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Background Mothers who give birth to preterm infants are at increased risk of mortality from coronary heart disease and stroke, but the biological pathways underlying these associations have not been explored.

Methods We carried out a case–control study nested in a large (n = 5337) prospective, multicentre cohort. All cohort women had an interview, examination and venipuncture at 24–26 weeks. Frozen plasma samples in spontaneous preterm births (n = 207) and 444 term controls were analysed for plasma homocysteine, folate, cholesterol (total, low-density lipoprotein and high-density lipoprotein) and thrombin–antithrombin (TAT) complexes. DNA was extracted and analysed for seven gene polymorphisms involved in thrombophilia or folate or homocysteine metabolism. Fresh placentas were fixed, stained and blindly assessed for histologic evidence of infarction and decidual vasculopathy.

Results High (above the median) plasma homocysteine and HDL cholesterol were significantly and independently associated with the risk of spontaneous preterm birth [adjusted odds ratios (ORs) = 1.9 (95% 1.1–3.3) and 0.5 (0.3–0.9), respectively]. A higher proportion of women with high homocysteine concentrations had decidual vasculopathy [(13.0 vs 6.8%; OR = 1.9 (1.1–3.5)], although the positive association between decidual vasculopathy and preterm birth did not achieve statistical significance [OR = 1.5 (0.9–2.7)]. No significant associations were observed with the DNA polymorphisms or with plasma TAT or folate levels.

Conclusions Similar vasculopathic risk factors may underlie preterm birth and adult coronary heart disease and stroke.

Keywords Preterm birth, coronary heart disease, stroke, biological pathways

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Introduction

The causal nature of the robust association between fetal growth restriction and adult chronic disease is a subject of lively debate. Several studies have strongly suggested a role for confounding by common genes or other maternal factors that may affect fetal growth, on the one hand, and type 2 diabetes, hypertension, coronary heart disease and stroke, on the other. Although most attention in this field has focussed on reduced fetal growth, mothers who give birth to preterm infants have also been reported to be at increased risk of mortality from coronary heart disease and stroke. This evidence suggests that common vascular risk factors may underlie preterm birth and adult vascular disease.

Several older studies have suggested that placental vascular lesions may cause some cases of preterm birth, but evidence of a vasculopathic causal pathway has not been convincingly demonstrated. Placental haemorrhage and thrombosis have also been reported to increase the risk of preterm birth in some, but not other studies. Moreover, several studies have reported associations between preterm birth and low maternal folate, high maternal homocysteine or high maternal cholesterol, as well as plasma levels of thrombin–antithrombin (TAT) complexes, folate, homocysteine and cholesterol.

Materials and methods

Our design, methods and procedures have been summarized previously. The study combines features of a prospective cohort and a nested case-control design. It also combines traditional interview methods with extensive biological measurements to explore the causal pathways and mediators that we hypothesized may explain socio-economic disparities in preterm birth. The nested case-control design improves efficiency by reducing the measurement costs of biological markers. Biological specimens are frozen and stored, with analysis limited to cases and approximately two controls per case (rather than all non-cases), thus providing statistical power nearly equivalent to analysis of the entire cohort. Only the psychosocial and other interview-derived variables, which require prospective data collection to avoid recall bias, are measured in the entire cohort.

The study is based in four large maternity hospitals affiliated with McGill University and l’Université de Montréal and Hôpital Maisonneuve-Rosemont. Most subjects were recruited at the time that women presented for routine dating ultrasound examinations (16–20 weeks), since obstetricians delivering women at the four study hospitals usually obtain their ultrasound examinations at the hospitals, rather than in private offices or free-standing radiology centres. A small number of subjects were also recruited at the time of prenatal blood drawing (usually 8–12 weeks) or in prenatal care clinics based at the study hospitals. The four study hospitals serve a wide socio-economic spectrum, including a large number of poor women and immigrant women, with good representation from both the majority French-speaking and minority English-speaking populations.

The Canadian health care system provides all women with free access to prenatal care, irrespective of their socio-economic status. Approval of this study was obtained from all obstetricians performing deliveries and by the ethics committees at the four study hospitals.

Eligibility criteria included age ≥18 years at the expected date of delivery, singleton gestation and fluency in French or English. Women with severe chronic illness (other than hypertension, asthma or diabetes) requiring ongoing treatment, placent previa, history of incompetent cervix diagnosed in a previous pregnancy, impending delivery at the time of initial contact or a fetus affected by a major anomaly were excluded.

Women who consented to participate in this study were asked to return to a special research clinic at the study hospital at 24–26 weeks of gestation, based on the ultrasound-based gestational age estimate obtained prior to the visit. At the clinic visit, a research nurse administered a questionnaire requesting data on age, parity, place of birth (no information was collected on race or ethnic origin), language spoken at home, maternal education, family income, marital and cohabitation status, height, pre-pregnancy weight and cigarette smoking. After the interview, the nurse performed a vaginal examination using a speculum, followed by a venipuncture (non-fasting).

The case room (delivery ward) of each of the four study hospitals was monitored daily (including weekends and holidays) for deliveries of study subjects. Each participant who delivered following spontaneous onset of labour before 37 completed weeks (based on the LMP if confirmed ±7 days by the dating ultrasound, otherwise by the dating ultrasound estimate) was classified as a case of spontaneous preterm birth. Spontaneous preterm birth cases were subdivided into those beginning with preterm pre-labour rupture of membranes (PPROM) vs those beginning with preterm labour, based on the mother’s history as recorded on the medical record. The mother’s report of leakage of fluid prior to onset of contractions was taken as evidence of PPROM, even if she was in...
labour at the time she was first examined by a physician. Women who delivered prior to 37 completed weeks after labour induction or pre-labour cesarean section were classified as having an ‘indicated’ preterm birth. For each case of spontaneous preterm, the next two study women who delivered at ≥37 completed weeks at the same study hospital were selected as term controls. Women whose menstrual and early ultrasound estimates of gestational age (GA) were within 7 days but resulted in a conflicting classification of case vs control status (i.e. one estimate classified the women as 36 weeks, while the other classified her as 37 weeks) fell into a ‘grey zone’ and were therefore classified as neither a case of preterm birth nor a control and excluded from the analysis. A total of 444 controls remained for biomarker analysis after final adjudication.

DNA was extracted from the thawed buffy coat and analysed for the following thrombophilic mutations: Factor V Leiden 1691G→A (rs6025, normal vs heterozygous or homozygous); prothrombin 20210G→A (rs1799963, normal or heterozygote vs homozygote); betaine–homocysteine methyltransferase (BHMT) 742G→A (rs1801131, normal or heterozygote vs homozygote); MTHFR 677C→T (rs1801133) and 1298A→C (rs1801131), (normal or heterozygote vs homozygote for each) and normal vs double heterozygote for both MTHFR polymorphisms simultaneously; methyltetrahydrofolate–homocysteine methyltransferase reductase (MTRR) 66A→G (rs1801394, normal or heterozygote vs homozygote); and reduced folate carrier (RFC1) 80A→G, (rs1051266, normal or heterozygote vs homozygote). These polymorphisms were chosen based on their known thrombophilic effects, their impact on folate or homocysteine metabolism, or their location in the coding sequence suggesting a functional effect on the protein produced.

Single nucleotide polymorphism (SNP) genotyping was carried out for the seven coding SNPs at the McGill University-Genome Quebec Innovation Center (Montreal) using the fluorescence polarization-single base extension (FP-SBE) method.24 FP-SBE reactions were performed in both orientations for each polymerase chain reaction (PCR) and were analysed using an Analyst HT reader (Molecular Devices, Sunnyvale, CA). For the minority of samples that did not yield consistent results for both DNA strands or for which there were no results using the FP-SBE method, manual genotyping was performed by PCR and restriction digestion using published procedures. Verification was also performed by PCR and restriction digestion on a 15% random selection of all samples.

TAT complexes were analysed using ELISA kits (Enzygmost TAT micro, Dade Behring Marburg, Germany). TAT levels were dichotomized as normal (≤8 µg/l) or high (>8 µg/l), based on the report by Elovitz et al.15 Maternal plasma homocysteine and folate were determined by IMx (Abbott Diagnostics, Abbott Park, IL, USA). IMx measures homocysteine by fluorescence polarization immunoassay, and folate by ion capture technology.

The lipoprotein fraction of maternal plasma of all cases and a random sample of 119 controls was analysed for total cholesterol, low-density lipoprotein cholesterol (LDLc) and high-density lipoprotein cholesterol (HDLc). HDLC was measured using a direct homogeneous two-reagent method with materials obtained from Equal Diagnostics (Exton, PA). Because blood samples were obtained in the non-fasting state, LDLc was measured directly using an automated spectrophotometric assay, LDL Direct Liquid Select (also from Equal Diagnostics). This method works well on non-fasting samples with triglyceride concentrations <14.7 mmol/l. Total cholesterol was determined using the enzymatic method of Allain et al.25

As previously described,26 placentas from cases and controls were placed immediately after delivery in a double plastic bag and refrigerated. Three transmural sections of 3 mm thickness (one each near the insertion of the umbilical cord, near a placental margin, and midway in between) were cut from the fresh placenta. All placental histopathologic features were evaluated by a single placental pathologist (MFC) blind to the case vs control status of the study subjects and to the results of all plasma and DNA analyses. We analysed both decidual vasculopathy and infarction in relation to spontaneous preterm birth and the vasculopathic and thrombotic factors under study. As previously reported,26 in a random sample of pathologic specimens re-examined by the same and a second placental pathologist, both decidual vasculopathy and infarction had high intra-observer agreement [(κ = 0.62 (0.36–0.88) and 0.78 (0.54–1.00), respectively]. Infarction also had good interobserver agreement [(κ = 0.78 (0.34–1.00)).

For each DNA polymorphism and plasma analyte, we compared spontaneous preterm births (n = 207) and controls (n = 444). The overall group of spontaneous preterm births was also subdivided by mode of onset [PPROM (n = 126) vs preterm labour (n = 81)] and by gestational age [<34 weeks (n = 34) and 34–36 weeks (n = 173)]. Because no subgroup differences were revealed by the latter analyses, however, results shown below are limited to the overall groups of cases vs controls. We also carried out multiple logistic regression analyses that simultaneously controlled for maternal age, parity, living arrangement (cohabitation), cigarette smoking, maternal birth place, language spoken at home, maternal education, family income, maternal height and pre-pregnancy body mass index (BMI). In the logistic analyses, biomarkers were analysed in two ways: above vs at or below the median, and by quartile, based on the distribution in the control group.

All statistical analyses were carried out using SAS version 9.1 (SAS Institute, Cary, NC).
Results

Table 1 compares the baseline characteristics of the 207 cases and 444 controls. As previously reported for the overall cohort, none of these characteristics differed significantly (based on $\chi^2$-tests) between cases and controls.

Based on goodness-of-fit $\chi^2$-tests of the observed allele frequencies, the control population was in Hardy–Weinberg equilibrium for all seven of the genetic polymorphisms under study, except for an unexpected single case of homozygosity for the prothrombin mutation. Table 2 compares the rates of genetic polymorphisms among the overall cases, and controls; no significant associations were observed. A similar lack of association was observed with high TAT levels (46.9 vs 50.1% in cases vs controls, respectively).

Mean plasma homocysteine concentration was significantly higher among cases than among controls (4.0 vs 3.7 μmol/l, $P = 0.001$) (see Table 3). In logistic regression analyses based on the median, the adjusted odds ratio (OR) for high homocysteine concentration was 1.8 (1.2–2.7). In the analyses based on quartiles, only Q4 (the highest quartile, range 4.2–15.4 μmol/l) was a significant risk factor [adjusted OR = 2.2 (95% CI 1.3–3.7) vs Q1]. No significant association was observed for plasma folate concentration.

As expected, mean homocysteine concentrations were higher among subjects with low folate levels (3.9 vs 3.6 μmol/l; $P < 0.001$), but the magnitude of the association between homocysteine and spontaneous preterm birth was not modified by plasma folate level ($P$ for interaction term = 0.28). Neither of the MTHFR polymorphisms (homozygote) or their double heterozygote was associated with plasma homocysteine concentration, nor did they significantly interact with low folate in relation to spontaneous preterm birth.

As also shown in Table 3, neither total cholesterol nor HDLC concentration was associated with preterm birth, but mean HDLC concentration was significantly lower among cases (1.6 vs 1.8 mmol/l, $P = 0.0001$) and was significantly associated with spontaneous preterm birth in both median- and quartile-based analyses. In logistic regression analyses controlling for the covariates shown in Table 1 and for homocysteine concentration, the adjusted OR was 0.5 (0.3–0.9) for high HDLC and 1.9 (1.1–3.3) for high homocysteine concentrations. No significant association was observed between HDLC and homocysteine concentrations.

We repeated all of the logistic regression analyses after excluding 49 women who either had pre-pregnancy diabetes or who were subsequently diagnosed with gestational diabetes. The results were unchanged after these exclusions.

Placental histopathologic evidence of spontaneous decidual vasculopathy was non-significantly associated with preterm birth [11.1 vs 7.5% among cases vs controls; OR = 1.5 (95% CI 0.9–2.7)].
Table 2  Thrombophilic and folate- or homocysteine-metabolizing DNA polymorphisms in cases and controls

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Percent with polymorphism</th>
<th>Crude OR (95% CI)</th>
<th>Adjusted(^a) OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Term controls ((n = 443))</td>
<td>Total cases ((n = 206))</td>
<td>(n = 443)</td>
</tr>
<tr>
<td>Factor V Leiden 1691G→A (heterozygote)</td>
<td>4.3</td>
<td>4.9</td>
<td>1.1 (0.5–2.5)</td>
</tr>
<tr>
<td>Prothrombin 20210G→A (heterozygote)</td>
<td>1.6</td>
<td>2.4</td>
<td>1.5 (0.5–4.9)</td>
</tr>
<tr>
<td>MTHFR 677C→T (homozygote)</td>
<td>13.8</td>
<td>15.1</td>
<td>1.1 (0.7–1.8)</td>
</tr>
<tr>
<td>MTHFR 1298A→C (homozygote)</td>
<td>9.0</td>
<td>7.3</td>
<td>0.8 (0.4–1.5)</td>
</tr>
<tr>
<td>MTHFR (double heterozygote)</td>
<td>21.4</td>
<td>20.9</td>
<td>1.0 (0.6–1.5)</td>
</tr>
<tr>
<td>BHMT 742G→A (homozygote)</td>
<td>8.6</td>
<td>9.7</td>
<td>1.1 (0.6–2.0)</td>
</tr>
<tr>
<td>MTRR 66A→G (homozygote)</td>
<td>25.1</td>
<td>22.3</td>
<td>0.9 (0.6–1.3)</td>
</tr>
<tr>
<td>RFC1 80A→G (homozygote)</td>
<td>28.2</td>
<td>32.0</td>
<td>1.2 (0.8–1.7)</td>
</tr>
</tbody>
</table>

\(^a\)Adjusted for all factors shown in Table 1.

Table 3  Homocysteine and folate concentrations in cases and controls

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Term controls Mean ± SD ((n = 441))</th>
<th>Total cases Mean ± SD ((n = 207))</th>
<th>Median-based OR (95% CI)</th>
<th>Quartile-based adjusted(^b) OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(\text{Mean} ± \text{SD}) (n = 441)</td>
<td>(\text{Mean} ± \text{SD}) (n = 207)</td>
<td>Crude</td>
<td>Adjusted(^b)</td>
</tr>
<tr>
<td>Homocysteine (μmol/l)</td>
<td>3.7 ± 0.9</td>
<td>4.0 ± 1.4(^b)</td>
<td>1.7 (1.2–2.4)</td>
<td>1.8 (1.2–2.7)</td>
</tr>
<tr>
<td>Folate (nmol/l)</td>
<td>40.4 ± 35.7</td>
<td>42.6 ± 45.0</td>
<td>1.3 (0.9–1.8)</td>
<td>1.6 (1.1–2.3)</td>
</tr>
<tr>
<td>Total cholesterol (mmol/l)</td>
<td>6.0 ± 1.1</td>
<td>6.1 ± 1.1</td>
<td>1.2 (0.8–1.9)</td>
<td>1.2 (0.7–2.0)</td>
</tr>
<tr>
<td>LDL cholesterol (mmol/l)</td>
<td>3.1 ± 0.8</td>
<td>3.0 ± 0.8</td>
<td>0.9 (0.6–1.5)</td>
<td>0.9 (0.5–1.5)</td>
</tr>
<tr>
<td>HDL cholesterol (mmol/l)</td>
<td>1.8 ± 0.4</td>
<td>1.6 ± 0.4(^b)</td>
<td>0.5 (0.3–0.8)</td>
<td>0.5 (0.3–0.9)</td>
</tr>
</tbody>
</table>

\(^a\)Adjusted for all factors shown in Table 1.

\(^b\)\(p \leq 0.001\) vs controls.
infarction was observed in 5.6 vs 6.6%, respectively, of cases and controls \( \text{OR} = 0.8 \) (95% CI 0.4–1.7)]. Women with high homocysteine concentrations were more likely to manifest both decidual vasculopathy [11.0 vs 6.0%; \( \text{OR} = 1.9 \) (1.1–3.5)] and infarction [8.0 vs 4.2%; \( \text{OR} = 2.0 \) (1.0–4.0)] than those with lower concentrations. High HDLC was not significantly associated with either decidual vasculopathy [10.7 vs 7.9%; \( \text{OR} = 1.4 \) (0.6–3.0)] or infarction [5.7 vs 6.8%; \( \text{OR} = 0.8 \) (0.3–2.1)]. None of the thrombophilic or folate or homocysteine-metabolizing polymorphisms was significantly associated with decidual vasculopathy or infarction.

**Discussion**

We were not able to replicate the findings of previous studies relating thrombophilic mutations to preterm birth. Erhardt et al. reported an increased risk of preterm birth among mothers with the Factor V Leiden mutation, and Göpel et al. reported similar increased risks among fetuses who themselves had either the Leiden or the prothrombin 20210G→A mutation. Gibson et al., however, reported reduced risks among fetuses who themselves had the Leiden mutation. Nor did we observe associations between thrombophilic mutations and histopathologic evidence of placental infarction or decidual vasculopathy. Although several studies have reported significant associations between low folate levels or intake and placental abruption (which can lead to preterm birth), few have found associations with preterm birth overall. Randomized trials of folate supplementation in pregnancy have not demonstrated a reduction in risk of preterm birth. And in contrast with temporal trends in neural tube defects, preterm birth rates have not diminished over time in countries (USA and Canada) that have fortified flour products with folic acid. Previous studies of polymorphisms involved in homocysteine or folate metabolism have reported mixed findings regarding associations with preterm birth. We observed no significant associations with these polymorphisms. Low statistical power due to our limited number of cases (\( n = 207 \)) may have prevented us from detecting modest associations. In addition, most previous studies have not excluded so-called ‘indicated’ preterm cases, and thus may have included pregnancies with severe pre-eclampsia, fetal growth restriction and other pathologies that may be associated with the studied polymorphisms.

Despite the weak evidence bearing on folic acid, one large Norwegian study reported a significant association between preterm birth and maternal plasma homocysteine obtained often after many years after the pregnancy, whereas a study of Chinese women based on only 29 cases of preterm birth observed a significantly increased risk with high preconceptional homocysteine levels. Homocysteine has primarily been investigated as a risk factor for coronary heart disease and has not been frequently investigated in biomarker studies of adverse pregnancy outcomes other than pre-eclampsia. Attention has focused on its role in endothelial function and vascular occlusion, which may also underlie its possible etiologic role in preterm birth. We observed a nearly 2-fold higher prevalence of decidual vasculopathy among women with a high plasma homocysteine concentration. These results suggest that, as with the coronary vasculopathy, high homocysteine concentrations may cause, or be a marker for, placental vascular changes that stimulate hormonal, inflammatory or cellular (e.g. increase in gap junctions) changes that initiate or accelerate the cascade of events leading to preterm labour or PPROM. High plasma homocysteine may act through a direct mechanism, however; one in vitro study reported that homocysteine increased the frequency of spontaneous contractions of human pregnant myometrium suspended in an organ bath.

Low HDLC is considered a strong cardiovascular risk factor and is the most frequent lipoprotein disorder, yet controversy still surrounds its causal role in atherosclerosis. Experimental evidence demonstrates that the atheroprotective effects of HDL extend beyond removing cholesterol from lipid-laden macrophages in the atherosclerotic plaque, an effect known as reverse cholesterol transport. HDL also has anti-inflammatory effects, prevents oxidation of low-density lipoproteins, shows anti-thrombotic properties, modulates vasomotor tone, and may improve endothelial cell survival (by preventing apoptosis), migration and proliferation.

The protective effect of high HDLC observed in our study could be a chance finding; such an effect was not hypothesized a priori and requires confirmation in other studies. Second-trimester serum total cholesterol was recently reported to be positively associated with spontaneous preterm birth in one study but to have a U-shaped relation with overall (spontaneous plus indicated) preterm birth in another. Neither study separately examined LDLC and HDLC. A recent randomized trial of a cholesterol-lowering diet reported a markedly reduced risk of preterm birth in Norwegian women randomized to the diet. We found no evidence that HDLC was associated with placental infarction or maternal vasculopathy. And unlike a recent study from some members of our group in mice and in adult men with coronary heart disease, as well as a large cross-sectional study of middle-aged Norwegian men and women, we found no association between high homocysteine concentration and low HDLC levels. HDLC concentration has been reported to rise over the course of normal pregnancy, and HDLC is actively taken up from maternal blood by fetal trophoblasts. Thus low maternal HDLC may lead to poor placental function, rather than directly causing decidual vasculopathy and (secondarily) causing preterm birth.
Our study has both strengths and limitations. The strengths include a multicentre sampling frame, rigorous assessment of gestational age, restriction to spontaneous preterm birth, separate characterization of PPROM and preterm labour, blood sampling within a narrow gestational window (24–26 weeks), and control for an extensive list of potentially confounding demographic, socio-economic and clinical variables. Despite the large size of our study cohort, the low number of spontaneous preterm cases \( n = 207 \) provides low power to detect associations of modest magnitude, especially for the studied genetic polymorphisms. Blood samples were obtained in the non-fasting state, which may have affected cholesterol and/or homocysteine levels. We attempted to minimize the effect of non-fasting by measuring LDLC directly. Moreover, the additional measurement variation caused by non-fasting should be non-differential (i.e. similar in women who subsequently delivered preterm vs at term) and thus should bias the associations under study toward the null. Finally, the fact that our cohort was recruited after fortification of flour products was instituted in Canada, and that the majority of our study women were taking folic acid and/or multivitamin supplements at the time of the study interview and blood sampling, may have prevented us from observing an association that might be evident in unsupplemented women consuming non-fortified foods.

Given the large number of analytes examined, some of the associations we observed may have arisen by chance (type 1 errors) and thus require confirmation in other studies. Even if confirmed, it remains unclear whether high maternal plasma homocysteine or low HDLC concentrations may either cause or be causal markers of this increased risk. Our placental pathologic findings suggest that the biological pathway may involve decidual vasculopathy. We found no evidence that genetic polymorphisms involved in thrombophilia or folate or homocysteine metabolism play a causal role.

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**Conflict of interest:** None declared.

**KEY MESSAGES**

- Mothers who deliver preterm are known to be at increased risk of mortality from coronary heart disease and stroke.
- The biological pathways underlying this increased risk are unknown.
- Our case–control study nested in a large, prospective, multicentre cohort suggests that elevated maternal homocysteine and low HDLC concentrations may either cause or be causal markers of this increased risk.
- Our placental pathologic findings suggest that the biological pathway may involve decidual vasculopathy.
- We found no evidence that genetic polymorphisms involved in thrombophilia or folate or homocysteine metabolism play a causal role.
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