MARKERS OF CARDIOVASCULAR DYSFUNCTION AFTER PREGNANCIES COMPLICATED BY PRE-ECLAMPSIA

by

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Abstract

Pre-eclampsia (PE) is a hypertensive disorder of pregnancy characterized by maternal systemic endothelial dysfunction. While the clinical manifestations resolve soon after delivery, a large body of epidemiological evidence indicates significant long-term maternal risk for cardiovascular disease (CVD) after PE. The mechanisms by which PE and future CVD are associated are unclear, although shared constitutional risk factors likely contribute to the features of endothelial dysfunction characteristic to both. We postulate that PE offers a window of opportunity for the identification of unique markers of dysfunction in the earliest stages of disease that may be used to validate cardiovascular risk screening in the early postpartum period.

The studies presented in this thesis provide evidence of changes in circulating factors in women with a recent history of PE. Using blood samples collected within the first year of pregnancy, unique patterns of microRNA expression, enrichment of coagulation system proteins and endothelial progenitor cell dysfunction were described. Many of the described changes appear to be independent of cardiovascular risk. In addition to alterations in circulating factors however, longitudinal postpartum assessments demonstrated that microvascular and cardiac abnormalities were evident in the early periods postpartum after a pre-eclamptic pregnancy.

Collectively, the data presented in this thesis reveal that physiological alterations in women with a recent history of PE are not necessarily dependent on clinical parameters of cardiovascular risk, and that resulting dysfunction may be demonstrated within the first year postpartum. Importantly, the biomarkers presented herein are all demonstrated elsewhere in the literature to benefit from lifestyle modification and risk reduction. In closing, the findings of this thesis support a need for cardiovascular risk screening based on obstetrical history, namely after pregnancies complicated by PE.
Co-Authorship

All experiments presented in this thesis are original. Malia SQ Murphy and Graeme N Smith are responsible for the experimental design, interpretation of data and manuscript preparation. Malia SQ Murphy completed all experimental work and analysis of data with contributions from the co-authors listed below. All co-authors contributed revisions to the published papers and consented to their publication. Graeme N Smith is the primary principal investigator for these studies.

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Contributions to study: MSQM and GNS created the experimental design. MSQM completed all experimental work. RCC contributed methodological guidance and technical assistance. CT provided critical guidance on the project and aided in interpretation of findings. The manuscript was drafted by MSQM and edited by CT and GNS.


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Contribution to study: MSQM completed and directed all experimental work with technical assistance from MV. The manuscript was drafted by MSQM and edited by GNS.


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List of Abbreviations

ACh  Acetylcholine
Ac-LDL  Acetylated-low density lipoprotein
ANOVA  Analysis of variance
BMI  Body mass index
CAC  Circulating angiogenic cell
cDNA  Complementary deoxyribonucleic acid
CFU-EPC  Colony forming unit endothelial progenitor cells
CHD  Coronary heart disease
CI  Confidence interval
cm  Centimeter
COX  Cyclooxygenase
Ct  Cycle threshold
CTRL  Control
CV  Coefficient of variance
CVD  Cardiovascular Disease
Da  Dalton
dL  Deciliter
ECFC  Endothelial colony forming cell
ECG  Electrocardiograph
EDHF  Endothelial derived hyperpolarizing factor
EDTA  Ethylenediaminetetraacetic Acid
emPAI  Exponentially modified protein abundance index
eNOS  Endothelial nitric oxide synthase
EPC  Endothelial progenitor cells
g  Gram
gh  Gravitational force
HCl  Hydrochloric acid
HDL-C  High density lipoprotein cholesterol
HELLP  Hemolysis elevated liver enzymes and low platelets
HF  High frequency
HIP  Health improvement after pregnancy
HRV  Heart rate variability
hs-CRP  High sensitivity C-reactive protein
HUVEC  Human umbilical vein endothelial cells
Hz  Hertz
IPA  Ingenuity pathways analysis
kDa  Kilodalton
kg  Kilogram
KGH  Kingston General Hospital
kHz  Kilohertz
LC/MS/MS  Liquid chromatography – mass spectrometry
LDL-C  Low density lipoprotein cholesterol
LF  Low frequency
LF:HF  Low frequency to high frequency ratio
m  Meter
mC  Millicoulomb
MetS  Metabolic syndrome
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>mg</td>
<td>Milligram</td>
</tr>
<tr>
<td>miR</td>
<td>Micro ribonucleic acid</td>
</tr>
<tr>
<td>miRNA</td>
<td>Micro ribonucleic acid</td>
</tr>
<tr>
<td>mM</td>
<td>Millimolar</td>
</tr>
<tr>
<td>mmHg</td>
<td>Millimeters of mercury</td>
</tr>
<tr>
<td>mmol</td>
<td>Millimoles</td>
</tr>
<tr>
<td>MMPs</td>
<td>Matrix metalloproteinase</td>
</tr>
<tr>
<td>mL</td>
<td>Millilitre</td>
</tr>
<tr>
<td>MotHERS</td>
<td>Mothers’ Health Education Research and Screening</td>
</tr>
<tr>
<td>ms</td>
<td>Millisecond</td>
</tr>
<tr>
<td>MSC</td>
<td>Mesenchymal stem cell</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>NP</td>
<td>Never-pregnant</td>
</tr>
<tr>
<td>n.u.</td>
<td>Normalized units</td>
</tr>
<tr>
<td>OR</td>
<td>Odds ratio</td>
</tr>
<tr>
<td>PAR</td>
<td>Population attributable risk</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PE</td>
<td>Pre-eclampsia</td>
</tr>
<tr>
<td>PE-NET</td>
<td>Pre-eclampsia New Emerging Team</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>PGF</td>
<td>Placental growth factor</td>
</tr>
<tr>
<td>pNN50</td>
<td>Proportion of R-R intervals differing from their directly adjacent R-R intervals &gt;50ms</td>
</tr>
<tr>
<td>PU</td>
<td>Perfusion units</td>
</tr>
<tr>
<td>r</td>
<td>Correlation coefficient</td>
</tr>
<tr>
<td>RMSSD</td>
<td>Root mean squared successive difference</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RR</td>
<td>Relative risk</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcription polymerase chain reaction</td>
</tr>
<tr>
<td>SAPW</td>
<td>Signal averaged P-wave</td>
</tr>
<tr>
<td>SAQRS</td>
<td>Signal averaged QRS</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SDNN</td>
<td>Standard Deviation of Normal-Normal RR Intervals</td>
</tr>
<tr>
<td>sFlt-1</td>
<td>Soluble fms-like tyrosine kinase-1</td>
</tr>
<tr>
<td>SNP</td>
<td>Sodium nitroprusside</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumour necrosis factor alpha</td>
</tr>
<tr>
<td>μA</td>
<td>Microampules</td>
</tr>
<tr>
<td>μL</td>
<td>Microliter</td>
</tr>
<tr>
<td>UTR</td>
<td>Untranslated region</td>
</tr>
<tr>
<td>v/v</td>
<td>Volume to volume</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>VEGFRT-2</td>
<td>Vascular endothelial growth factor receptor 2</td>
</tr>
<tr>
<td>$\chi^2$</td>
<td>Chi-squared</td>
</tr>
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</table>
Chapter 1

General Introduction

and Literature Review
1.1 Introduction

Cardiovascular disease (CVD) accounts for approximately 30% of deaths in American and Canadian women, making it the leading cause of death in women in each of these North American countries (1, 2). CVD is becoming increasingly prevalent amongst younger women who are now exhibiting higher rates of heart disease, hypertension, diabetes and obesity (3). While CVD risk factors in women have been well documented (4), CVD prevention guidelines have only recently targeted the need for gender-based management strategies (5).

The underlying contributors of many cardiovascular events (i.e., hypertension, diabetes and dyslipidemia) are often present decades before the onset of clinical symptoms, and the presence of risk factors in early life significantly influences risk of premature CVD. With over 88% of Canadian women over the age of 20 exhibiting at least one risk factor for CVD (6), early-onset heart disease, hypertension and diabetes are likely to pose substantial burdens on health care resources as individuals require longer term and more intensive treatment. The American Heart Association projects that total direct costs of all CVD in the United States will increase by 200% in the next 20 years (7). Thus the considerable burden of CVD in women and on health care resources necessitates an emphasis on prevention and early risk screening before the development of the disease itself. The aims of this review are to examine cardiovascular risk factors in young women and their role in the development of premature CVD, with particular attention paid to pre-eclampsia as a marker of cardiovascular susceptibility. Current screening practices will be discussed, as will their influences on identifying and reducing cardiovascular risk and subsequently CVD in younger women.

1.2 Risk factors for Cardiovascular Disease in Women

Atherosclerotic CVD encompasses coronary heart disease (CHD), cerebrovascular disease, and peripheral arterial disease. Women generally present with more atypical symptoms of acute
coronary syndromes than do men (8, 9), and are more likely to be misdiagnosed upon presentation (10, 11). As a result, women are less likely to receive optimal evidence-based treatment and are more likely to experience poorer outcomes including death following cardiac episodes (10, 12, 13).

Importantly, a large proportion of CVD is avoidable through risk factor modification. Although traditional risk factors for CVD are similar to both sexes, their individual and cumulative impacts vary greatly. The INTERHEART study, an international population-based study that assessed the risk for myocardial infarction found that among biophysical parameters, high blood pressure and diabetes mellitus were greater contributors to the population-attributed risk (PAR) of CVD in women than in men (14). The PAR (the reduction in mortality if all disease was eliminated) for hypertension in women is estimated at 35.8% in individuals at risk for myocardial infarction (14) – 31.9% in women less than 65 years old, and 25.4% in women older than 65 years. The PAR for select risk factors for myocardial infarction in men and women as determined in the INTERHEART study are summarized in Table 1.1.

Recent improvements in primary and secondary preventative strategies have promoted better risk factor reduction in women (5, 15), although an understanding of the role traditional cardiovascular risk factors play in the development of CVD in women remains important when considering the potential for interventions.

1.2.1 Hypertension

Hypertension is one of the strongest risk factors for vascular disease. Globally, 51% of deaths from stroke and 45% of deaths from ischemic heart disease are attributable to the effects of hypertension. The long-term effects of chronic hypertension are reflected in structural and functional changes in target organs. Central to the regulation of blood pressure is the renin-angiotensin system, and specifically its end effector molecule, angiotensin II; activation of angiotensin II type I receptors contribute to acute vasoconstriction, pathogenic vascular growth
Table 1.1. Population Attributable Risks for select risk factors of acute myocardial infarction in men and women. (14)

<table>
<thead>
<tr>
<th>Risk Factor</th>
<th>Male</th>
<th>Female</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Odds Ratio (99% CI)</td>
<td>PAR (99% CI)</td>
</tr>
<tr>
<td>Current Smoking</td>
<td>3.05 (2.78-3.33)</td>
<td>44.0 (40.9-47.2)</td>
</tr>
<tr>
<td>Diabetes</td>
<td>2.67 (2.36-3.02)</td>
<td>10.1 (8.9-11.4)</td>
</tr>
<tr>
<td>Hypertension</td>
<td>2.32 (2.12-2.53)</td>
<td>19.5 (17.7-21.5)</td>
</tr>
<tr>
<td>Abdominal Obesity</td>
<td>2.24 (2.03-2.47)</td>
<td>32.1 (28.0-36.5)</td>
</tr>
<tr>
<td>Physical activity</td>
<td>0.77 (0.69-0.85)</td>
<td>22.9 (16.9-30.2)</td>
</tr>
<tr>
<td>Daily consumption of fruits and vegetables</td>
<td>0.74 (0.66-0.83)</td>
<td>10.3 (6.9-15.2)</td>
</tr>
</tbody>
</table>

Data are adjusted for age, sex and geographic region. PAR, population attributable risk; CI, confidence interval.
and endothelial dysfunction(16). Disruption of vascular endothelial integrity as a result of shear forces generated by chronically elevated blood pressure accelerate the process of atherosclerosis (17).

Increases in normal blood pressure greater than 20 mmHg of systolic blood pressure or 10 mmHg of diastolic blood pressure roughly double mortality due to cardiovascular-related disease (18). Hypertensive women are twice as likely to develop CVD (19) compared to their normotensive counterparts although incremental increases in blood pressure even within the normal non-hypertensive range have also been found to increase CVD-related risks. Indeed, compared to optimal arterial pressures, high-normal blood pressures in women are associated with CVD (adjusted hazard ratio 2.5[95%CI 1.6-4.1]) (20). Of particular concern, a recent public health report found that nearly 15% of women were unaware that they have hypertension, and that even with a diagnosis, hypertension remained uncontrolled in 18% of females using antihypertensive medication (21).

Elevated arterial pressures often cluster with other major risk factors for CVD including obesity, dyslipidemia and glucose intolerance. The degree of risk conferred by hypertension alone therefore, is difficult to ascertain, although it appears that the degree of risk for CVD increases more steeply in women than in men with the addition of each individual risk factor (22).

1.2.2 Obesity and adipose distribution

36.2% of American and 23.9% of Canadian females between the ages of 20-79 have body mass indices (BMI) greater than 30 kg/m² (23) – a nearly 10% increase in the prevalence of obesity in North America over the past 20 years. These weight increases have been the greatest amongst North American women of child-bearing years (23). Relative body weight in women is a particularly strong independent risk factor for the future development of CVD and death (24, 25), and each unit increase of BMI accounts for nearly a 10% increase in individual risk for CHD (26). The relationship between subcutaneous and visceral adiposity to coronary artery and
abdominal aortic calcification has also been established, suggesting that distribution of body fat, coupled with altered blood and lipid profiles may well play an important role in CVD development (27).

The prevalence of co-morbidities associated with obesity including insulin resistance, type 2 diabetes mellitus, high cholesterol and hypertension increases steeply with advancing weight class and are even greater amongst younger women (28). Obesity is a particularly strong predictor of hypertension, and in women, a BMI ≥ 25 kg/m² accounts for approximately 62% of hypertension (29). The incidence of metabolic abnormalities also increases with adiposity. Indeed, obesity appears to be the driving force behind the development of the metabolic syndrome (MetS); the presence of a constellation of risk factors that predict the development of CVD and Type 2 diabetes. Indeed, the Third National Health and Nutrition Examination Survey reports that 28.1% of overweight and 50% of obese women also had MetS (30).

1.2.3 Lipid abnormalities

High levels of total cholesterol, low density lipoprotein cholesterol (LDL-C) and triglycerides as well as low levels of high density lipoprotein cholesterol (HDL-C) are important risk factors for all types of CVD. The athero-protective properties of HDL-C appear to be more pronounced in women than in men. Strong independent inverse relationships between HDL-C concentrations and CVD mortality have been reported in women (31), and Framingham studies have established that 10mg/dL (0.3 μmol/L) increases in HDL-C concentrations result in 40-50% reductions in CHD risk (32). Women are particularly sensitive to decreases in HDL-C (33, 34) with low levels being stronger predictors of CHD mortality in women than in men (35, 36). Even low-normal ranges of HDL-C concentrations increase risk for CHD morbidity and mortality. As a result of these discrepancies, evidence-based guidelines from the American College of Cardiology now recommend higher HDL-C target concentrations in women (37). Triglyceride levels are reliable predictors of risk in women, especially when considered with by HDL-C. Elevated triglyceride
and low HDL-C levels independently predict CHD (38, 39) while the relative risks of high total cholesterol and LDL-C levels in women under 65 years are more than double that of older women (35).

Increased duration of dyslipidemia was associated with elevated atherosclerotic plaque burden in the INTERHEART study. Here, abnormal lipids had the highest PAR in women (47.1%) of all nine major risk factors for myocardial infarction, with significant contributions from abdominal obesity (14). Lipid abnormalities are well documented up to 10 years in advance of the clinical onset of diabetes (40). Importantly total cholesterol, HDL-C and triglycerides are determinants in cardiovascular risk calculations and for the diagnosis of MetS.

1.2.4 Type 2 diabetes mellitus
Cardiovascular events are the leading cause of death in women with type 2 diabetes mellitus. Diabetes accelerates atherosclerosis through a variety of mechanisms, with the overall effect of increasing an individual’s physiologic vascular age by roughly 15 years (41). A large body of epidemiological evidence implicates diabetes as an independent risk factor for CVD (42, 43). While the impact of diabetes in women appears to diminish with age, its contribution to the risk of CVD death remains more dramatic in women than in men (40). Whether this represents a difference between length of exposure and relative metabolic and vascular damage incurred as a result of diabetes remains unclear, although increases in levels of glucose in non-diabetic women are also independently associated with an increased risk of CVD mortality, however (44).

Insulin resistance is a component of many traditional risk factors, although it is a particularly important metabolic disturbance in the pathogenesis of type 2 diabetes mellitus. Both insulin resistance and type 2 diabetes mellitus are associated with risk for CVD potentially through the development of hyperglycaemia as a causative mechanism in the vascular complications associated with these conditions (45, 46). Generation of reactive oxygen species (ROS) and impaired anti-oxidant defense mechanisms contribute to the cumulative states of oxidative stress.
and endothelial dysfunction. Increased non-enzymatic glycation reactions affect diabetic patients at many levels. Glycated LDL-C enhances susceptibility for oxidation and foam cell accumulation (47) while glycation of fibrin and platelet proteins have anti-fibrinolytic and pro-thrombotic repercussions (48).

Type 2 diabetes also has a tendency to cluster with traditional cardiovascular risk factors. Up to 75% of CVD in diabetes is attributable to hypertension (49) and 44% of global diabetes burden is attributable to overweight and obesity (50). Strong Heart Study data suggest that diabetic women display more adverse changes in cardiovascular risk factors including HDL-C, apoB, apoA1 and LDL-C (51). Moreover, the risk of CVD among women with diabetes is over three times greater (14, 40, 52) and mortality is higher than in non-diabetic women and in men (53-56). Thus, it would appear that the anti-atherogenic effects of female sex hormones appear to be lost amongst pre-menopausal diabetic women (57).

1.2.5 Metabolic syndrome

The metabolic syndrome describes a composite of modifiable cardiovascular risk factors that, when present, increase the risk for not only CVD but also type 2 diabetes. The Third Report of the National Cholesterol Education Program Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (ATP-III) (58) stresses the importance of establishing prevention strategies amongst this high-risk population. The current working definition for MetS in women is summarized in Table 1.2 (58, 59).

Recent reports indicate that 20.5-22.9% of Canadian women meet the criteria for MetS (60) while data from the U.S. indicate that rates of MetS in women have increased from 22.6% to 32.6% between 2003 and 2006. These numbers are expected to continue to rise with increasing rates of obesity (30, 61). While there does not appear to be discordance by sex in regards to the prevalence of the syndrome, the associated risk factors do appear to cluster differently in women than in men yet remain closely associated with BMI. In comparison to men, of the five MetS
Table 1.2. Criteria for the clinical diagnosis of metabolic syndrome in women. (58, 59)

<table>
<thead>
<tr>
<th>Clinical Measure (any 3)</th>
<th>Categorical thresholds</th>
</tr>
</thead>
<tbody>
<tr>
<td>Waist circumference</td>
<td>≥88 cm; ≥80 cm for women of Asian descent</td>
</tr>
<tr>
<td>Serum triglycerides</td>
<td>≥1.7 mmol/L; or on drug treatment for elevated triglycerides</td>
</tr>
<tr>
<td>HDL-C</td>
<td>≤1.3 mmoL/L; or on drug treatment for reduce HDL-C</td>
</tr>
<tr>
<td>Systolic blood pressure</td>
<td>≥130 mmHg; or on drug treatment for elevated blood pressure</td>
</tr>
<tr>
<td>Diastolic blood pressure</td>
<td>≥85 mmHg; or on drug treatment for elevated blood pressure</td>
</tr>
<tr>
<td>Fasting blood glucose</td>
<td>≥5.6 mmol/L; or on drug treatment for elevated fasting glucose.</td>
</tr>
</tbody>
</table>

HDL-C; High density lipoprotein cholesterol
criteria, current data indicate that women have higher rates of abdominal obesity at all ages, and lower HDL-C levels, particularly in the younger age brackets (30, 60). In women, overweight and obesity dramatically increase the risk of MetS (OR 5.48; 95%CI 3.75, 8.02 and OR 17.14; 95%CI 12.54, 23.44) (61). It is estimated that roughly 16% of women under 40 years meet the criteria for MetS, after which rates increase sharply to 37% in peri-menopausal women, and over 54% in women 60 and older (61). Frequent updates to the diagnostic standards used to assess the presence of MetS have affected adequate comparison of the literature regarding changes in its epidemiology. Moreover, as the components of MetS aren’t yet weighted by age, sex or race it is difficult to surmise whether some of the criteria may be more potent CVD risk factors in specific populations.

1.2.6 Lifestyle

Smoking increases the risk of all cardiovascular events. As many as 15.8% (95%CI 12.9, 19.3) of acute myocardial infarctions in women are attributable to current smoking, with greater effect on risk in adults under 65 years (PAR 20.8%; 95%CI 15.7, 26.9 vs. PAR 8.2%; 95%CI 4.1, 15.7 in women >65 years) (14). The relationship between myocardial infarction and smoking is dose-dependent in women, dramatically increasing from those who smoke 1-5 cigarettes a day (OR 2.48; 95%CI 1.13, 5.52) to those who smoke over 40 cigarettes daily (OR 74.64; 95%CI 32.97, 168.9) in comparison to non-smokers (62). Smoking also increases the risk of cerebrovascular disease, peripheral artery disease and congestive heart failure. Free-radical mediated oxidative stress appears to be the defining mechanism through which cigarette smoke mediates the progression of CVD. Indeed, inflammatory and thrombotic states accompanied by endothelial dysfunction and lipid peroxidation are characteristic of smoking profiles (63). Antioxidant agents and increased nitric oxide (NO) availability have been shown to attenuate the pro-atherogenic attributes of cigarette smoke (64, 65).
Dietary patterns are known to modulate baseline risk for CHD (66), and poor dietary intake accounts for nearly 20% of the risk for myocardial infarction in North American women (14). The Nurses’ Health Study reported a doubling of the relative risk of coronary events for women with the poorest dietary scores compared to those with optimal dietary intake (RR 1.9; 95% CI 1.55, 2.34) (67). Using PAR estimates, this study found that 82% (95% CI 58, 93) of all documented coronary events might have been prevented had all participants adhered to low-risk lifestyle patterns based on diet, exercise and abstinence from smoking in conjunction with weight management. Epidemiologic studies have consistently demonstrated that women who are more sedentary are more likely to be overweight or obese. In addition to weight loss benefits, modest increases in physical activity have demonstrated improvements in blood pressure, lipid profiles and inflammatory and hemostatic regulators (68). Physical activity as minimal as brisk walking has been associated with lower risk for ischemic stroke compared with casual paces. Questionnaires completed in the Nurses’ Health Study from 1986-1992 found that increasing quintiles of walking hours per week were inversely associated with risk of ischemic stroke (69). The risk benefits of walking were similar to those of vigorous non-walking physical activity, and those who became active later in life appear to be conferred the same benefits as those who remain active throughout.

1.2.7 Family history

Family history is a critical component in the assessment of any individual’s cardiovascular risk. A woman with a first degree relative with premature CVD (CVD at <55 years in a male relative, <65 years in a female relative) is considered to be at risk (37). A woman’s risk doubles with positive parental history of premature CVD (OR 2.3; 95% CI 1.3, 4.3) (70) compared to women with no documented history, and similarly, having a sibling with documented CVD substantially increases an individual’s risk (71). Family history of CVD likely augments personal risk through a combination of inherited factors and shared lifestyle characteristics. In addition, the effects of
cigarette smoke and poor nutritional intake are exposures that are more likely to be shared within the household environment (72, 73).

1.3 Pregnancy and Cardiovascular Risk

Pregnancy has been likened to a maternal stress test that has the potential to reveal a woman’s vascular or metabolic susceptibility for disease (74). The 2011 update to the *American Heart Association’s Effectiveness-Based Guidelines* for the prevention of CVD (15) acknowledges the contribution of the common pregnancy-related medical complications to a woman’s cardiovascular risk, identifying pre-eclampsia, gestational hypertension, and gestational diabetes mellitus as risk factors for heart disease and stroke.

1.3.1 Pre-eclampsia

Hypertensive disorders are the most common complications arising in pregnancy, affecting 6-8% of pregnancies worldwide. The *American College of Obstetricians and Gynaecologists Task Force on Hypertension in Pregnancy* consider four classifications of hypertension-complicated pregnancies (75); 1) pre-eclampsia-eclampsia, 2) chronic hypertension, 3) chronic hypertension with superimposed pre-eclampsia and 4) gestational hypertension. Of these, pre-eclampsia-eclampsia, or simply pre-eclampsia (PE) in the absence of development of convulsions is the most common form of high blood pressure complicating pregnancy.

Affecting about 3-5% of pregnancies, PE is a common cause of maternal and neonatal morbidity and mortality. Normal pregnancy is accompanied by myometrial vascular remodelling, such that the maternal spiral arterioles are transformed into high-capacitance, low-resistance vessels capable of sufficiently supporting the placental-fetal unit. In PE, deficient arteriole remodelling results in impaired utero-placental circulation. As a consequence, the myometrial layer of maternal spiral arterioles remains unmodified, leaving high resistance vessels that are detrimentally reactive to circulating vasopressors (76). As fetal metabolic demands increase over
the course of gestation the utero-placental environment becomes increasingly hypoxic with reduced utero-placental blood flow. Vascular lesions found in PE placental beds are characterized by the accumulation of foam cells and invading lymphocytes, and so have been termed “acute atherosis” due to their similarity to atherosclerotic plaques (77).

A two-stage model of PE suggests that in response to relative or intermittent ischemia, placental tissue releases soluble factors including placental microthrombemae, microvesicles and angiogenic factors into the maternal circulation. As a consequence, a second maternal stage of the disorder arises, characterized by exacerbated inflammation, coagulation and metabolic abnormalities that result in widespread maternal endothelial dysfunction and eventually the clinical manifestations of maternal hypertension and proteinuria (50). Many women with PE have evidence of endothelial dysfunction early in the development of pregnancy, before the onset of clinical symptoms, which has led many researchers to suggest that underlying this endothelial dysfunction (and potentially premature atherosclerosis) may, in fact, be a cause of the disorder rather than a result of the maternal syndrome of PE (Figure 1.1).

1.3.2 Pre-pregnancy risk factors for pre-eclampsia

Women who develop PE likely have underlying risk factors that manifest as PE in pregnancy, which then subside shortly after delivery. In 2002 Sattar and Greer proposed a model whereby pregnancy acts as an acute stress test predictive of long-term cardiovascular outcomes (78). This model suggests that subclinical metabolic and vascular susceptibility present in early life is exacerbated by the physiologic demands of pregnancy and thus primes the maternal endothelium for later vascular dysfunction and disease.

Women who develop PE often exhibit pre-pregnancy cardiovascular risk factors including elevated blood pressure, high BMI and abnormal lipid profiles. Linkage-based studies performed by Magnussen et al. (79) indicate strong associations of high pre-pregnancy systolic (>130 mmHg) and diastolic (>78 mmHg) blood pressures with risk of PE. Indeed, American and
Figure 1.1. Two-stage model of pre-eclampsia.

The two-stage model of PE whereby poor placental perfusion (Stage 1) releases factors into the maternal circulation. The maternal response to the unfavourable placenta environment and released placental factors contribute to the clinical manifestations of PE (Stage 2). This model is dependent on maternal constitutional factors, which provide the background upon which pregnancy progresses.
Canadian data indicate that prevalence of PE in women with pre-pregnancy hypertension far exceed the rates in the general obstetric population (20-25% vs. 3-5%) (80, 81). Risk of superimposed PE (i.e., chronic hypertension with the development of PE) appears to be strongly correlated with longer durations of hypertension (80) and even in the absence of superimposed PE, chronic hypertension independently increases the risks of adverse outcomes including fetal growth restriction (FGR), preterm birth (81, 82) and placental abruption (83).

Obesity strongly impacts maternal and obstetrical complications during pregnancy (84) by increasing risk of hypertensive disorders of pregnancy, gestational diabetes mellitus, and obesity-attributable indications for caesarian section (85-88). The highest indices of both BMI and waist circumference are associated with a nearly 2-fold increase in risk for PE (OR 2.2, 95%CI 1.2, 3.8) (79). In a large retrospective study of 96 801 women, obese (BMI ≥30 kg/m²) and overweight (BMI 25-29.9 kg/m²) women were at higher risk of gestational diabetes (OR 5.2; 95%CI 4.3, 6.2 and OR 2.4; 95%CI 2.0, 2.9 respectively), PE (OR 3.3; 95%CI 3.0, 3.7 and OR 2.0; 95%CI 1.8, 2.2 respectively), and eclampsia (OR 3.0; 95%CI 2.1, 4.4 and OR 2.0; 95%CI 1.4, 2.9 respectively), compared to women in the lowest BMI category (<20.0 kg/m²) (87). Interestingly, a relation between obesity and the PE-related syndrome of hemolysis, elevated liver enzymes, and low platelet count (HELLP) has not been validated (89).

Pre-pregnancy serum triglycerides, total cholesterol and LDL-C increase risk of PE even within clinically normal ranges, while levels of high-density lipoprotein cholesterol and fasting glucose are less indicative of PE risk (79). The significant contribution of the pre-pregnant state to PE risk and future CVD was recently examined in a longitudinal analysis of Norwegian population-based data sets (the Nord-Trøndelag Health Study [HUNT]) (90). Here, differences in post-pregnancy cardiovascular risk factors in women who had and had not developed hypertensive disorders of pregnancy (i.e., gestational hypertension or PE) were attenuated by more than 40%
after adjustment for pre-pregnancy measurements including BMI, systolic and diastolic blood pressure as well as lipoproteins and serum triglycerides.

Alternative to the hypothesis suggesting that PE exacerbates and unmasks underlying risk factors is one in which endothelial dysfunction and metabolic abnormalities are incurred with the onset of PE, resulting in a permanent disruption of vascular integrity. In this alternative scenario, PE directly affects the development of future disease. While published evidence supports the influence of familial inheritance on the risk of PE, an early study by Epstein et al. (91) found that both male and female siblings of women who had developed PE exhibited lower blood pressures and rates of hypertension than their pre-eclamptic siblings 15 years postpartum. The authors of this study suggested that PE might independently predispose to the development of later disease.

Postpartum changes documented in animal models of PE also support this hypothesis. Long-term changes in the mouse proteome after the induction of experimental PE are found to be consistent with changes observed in CVD (92). Furthermore, there appears to be a dose-dependent relationship between timing of onset or severity with risk of disease; women who develop early-onset or severe PE are at greatest risk of experiencing recurrent PE in subsequent pregnancies (15.0; 95%CI 6.3, 35.4) (93), and also for ischemic heart disease (16.0; 95%CI 1.86, 2.52) (94).

It remains unclear therefore whether vascular dysfunction is present before or incurred as a result of pregnancy and PE development. It may not be relevant however whether the link between PE and CVD is a manifestation of either of these hypotheses in isolation, or a combination of the two. Given that many risk factors for PE are modifiable and are also traditional risk factors for CVD it seems reasonable that high-risk women identified through both pre-pregnancy cardiovascular risk factors and the development of PE in pregnancy ought to be targeted for CVD screening and life-style intervention early within the postpartum period (97) (Figure 1.2).
Figure 1.2. Pregnancy as a stress test for life.

Pregnancy as a vascular and metabolic stress test for susceptibility to cardiovascular disease, and the effect of targeted screening and intervention in high-risk women following pregnancies complicated by pre-eclampsia. Three groups of women are hypothesized to enter pregnancy – 1) women with known cardiovascular risk factors (CVR) who are at high risk of obstetrical complications and later cardiovascular disease, 2) women with unknown or unrecognized CVR who develop an obstetrical complication and 3) women with no CVR who are at low risk of obstetrical complications and low risk for cardiovascular disease. Women with unknown or unrecognized CVR may be identified in the early postpartum period based on the development of unfavorable obstetrical complications and sent for targeted postpartum cardiovascular counselling and risk reduction. Adapted from Gn Smith, MSQ Murphy, K Nerenberg. Pregnancy as a Window of Future Health. In Press 2015 (In Press).
1.3.4 Pre-eclampsia as a risk factor for cardiovascular disease

Large retrospective population-based studies have consistently identified the risk for CVD associated with the development of PE. A recent meta-analysis examining nearly 50 individual case-control and cohort studies highlights the risk of early cardiac and cerebrovascular sequelae in this group of women (95). The analysis demonstrated a relative risk of 3.13 (95%CI 2.51, 3.89) for hypertension, an odds ratio of 2.28 (95%CI 1.87, 2.77) for CVD. Additionally the odds of cerebrovascular disease was 1.77 (95%CI 1.43, 2.21) (95). Early-onset (96), severity (97, 98) and recurrence (99) of PE further increase the risk of CVD, particularly when associated with pre-term delivery (<34 weeks of gestation) (100).

Early studies investigating the influence of pregnancy on future cardiovascular sequelae first noted the effect of PE on future blood pressure. In 1961 Adams and MacGillivray reported on the effect of previous mild/severe PE, normotensive pregnancy and nulliparity on blood pressure in middle aged women (101). In contrast to common opinion however, the hypertensive effect of mild PE appeared to be stronger than that observed for severe PE, and nulliparity was similarly associated with higher blood pressures compared to women who remained normotensive in pregnancy. While delivery of the placenta remains the only definitive ‘cure’ of maternal symptoms, maternal hypertension often persists into the postpartum period. Arterial blood pressures typically normalize by six weeks postpartum, although women may require anti-hypertensive management until this is achieved. International guidelines indicate that hypertension as a result of PE should resolve within three months of pregnancy although persistence or development of hypertension months to years postpartum is not uncommon following hypertensive obstetrical disorders (102, 103). Prolonged diagnosis-delivery intervals and maximal arterial pressures are correlated with the time to postpartum resolution of blood pressure, suggesting a relationship between degree and length of exposure to endothelial damage and remote cardiovascular health (104). Despite eventual return to pre-pregnancy pressures, future risk of chronic hypertension in women with previous PE is roughly triple that of
individuals with uncomplicated obstetrical histories (RR 3.13; 95% CI 2.51, 3.89) (94, 105, 106), and is nearly 7-fold higher in women with recurrent PE compared to a single episode (99).

In addition to persistent hypertension, women with a history of PE may exhibit other cardiovascular risk factors both early in the postpartum period, and decades from delivery. Data from the prospective cohort study of Pre-Eclampsia New Emerging Team (PE-NET) have shown that women with recent PE display greater propensity for weight retention, hypertension, higher levels of total and LDL cholesterol, elevated microalbumin/creatinine levels, and increased HOMA$_{IR}$ indices at one year postpartum (107). The PE-NET cohort, comprised of normotensive and PE women followed from delivery to 1 year, 3 years, and 5 years postpartum, report elevated risk of cardiovascular events amongst PE women using 10-year, 30-year and lifetime CVD models (108). PE-NET data have further demonstrated that risk of MetS amongst formerly PE women is greater than in control participants, at both 1 year (RR 2.68; 95% CI 1.22, 5.90) and 3 years postpartum (RR 3.43; 95% CI 1.06, 11.04) (109). Similar findings have been corroborated by others (110-112). Furthermore, data from a large population-based retrospective cohort, found that while a woman’s risk of CVD was elevated in the presence of hypertension, diabetes, obesity, dyslipidemia or MetS, the addition of a placental syndrome of pregnancy, including PE, to any one of these risk factors markedly increased a woman’s risk of CVD (113).

1.4 Hemodynamic Function in Pre-eclampsia

1.4.1 Endothelial dysfunction

Endothelial dysfunction refers to a systemic pathological condition that arises as a result of reduced production and bioavailability of vasodilators. In particular, impaired endothelial nitric oxide synthase (eNOS) activity (114, 115) and accelerated degradation of nitric oxide (NO) (115) are characteristic of endothelial dysfunction, the functional consequences of which are observed as impaired vascular relaxation. Vascular function is largely dependent on the stimulation of NO
production within endothelial cells to stimulate vascular smooth muscle cell relaxation. Increases in intracellular calcium concentrations promote the conversion of L-arginine to NO and L-citrulline by eNOS within the endothelium. NO stimulation of guanylyl cyclase activity within the smooth muscle layer of the vasculature increases cyclic guanylyl monophosphate levels, and directly induces vasodilation. Other mediating pathways of vascular relaxation include cyclooxygenase (COX) driven production of prostacyclin, and activation of K+ channels by endothelial derived hyperpolarizing factors.

Vascular function or dysfunction is typically taken as a measure of relative degrees of endothelial-dependent and -independent vasodilation. Assessment of endothelial-dependent processes of vasodilation typically involves the delivery of pharmacological agonists of eNOS, COX or K+ channels, or the induction of shear stress to elicit vasodilation by reactive hyperemia. NO donors including glyceryl trinitrate and sodium nitroprusside directly stimulate guanylyl cyclase activity within smooth muscle and are used to measure vasodilation independent of endothelial pathways (Figure 1.3). Endothelial vasomotor function may be measured by minimally invasive or non-invasive techniques in the coronary arteries, conduit arteries, and peripheral resistance arteries as well as within the peripheral microvasculature, thereby demonstrating that endothelial dysfunction is a systemic disorder, affecting a variety of vascular beds.

Also characteristic of endothelial dysfunction are states of endothelial activation in which an inflammatory and coagulatory milieu promotes atherogenic progression. This relationship between endothelial dysfunction and atherogenesis favours the use of measurements of vascular reactivity to vasodilators and circulating markers of activation in conjunction with the presence of risk factors to examine populations at risk. Persistence or development of cardiovascular risk factors postpartum likely contributes to the relationship between maternal endothelial dysfunction of PE and future CVD. Indeed, as discussed below, postpartum endothelial dysfunction has been
Figure 1.3. Mechanisms of microvascular endothelial-dependent and -independent vasodilation.

Receptor and shear stress mediated production of nitric oxide through activation of COX, eNOS and EDHF pathways represent endothelial-dependent pathways of vascular dilation. Alternatively, direct delivery of nitric oxide to the smooth muscle compartment directly stimulates guanylyl cyclase activity to induce vasodilation; an endothelial independent process. COX-1, cyclooxygenase 1; eNOS, endothelial nitric oxide synthase; EDHF, endothelial derived hyperpolarizing factor; PGI2, prostacyclin; cAMP, cyclic adenosine monophosphate; cGMP, cyclic guanylyl monophosphate.
documented in women many years after index pregnancies. Persistent disruption of endothelial integrity can exhaust endogenous antioxidant defense mechanisms, homeostatic regulatory systems for inflammation and coagulation that may confer greater risk of CVD.

1.4.2 Angiogenic and anti-angiogenic factors

Circulating factors within biofluids contain a multitude of secreted, shed and released regulatory molecules that can impact endothelial and vascular homeostasis. Of these, angiogenic factors and in particular disturbances in angiogenic factor levels may also confer cardiovascular risk to women who develop PE. In normal pregnancy, circulating angiogenic molecules including vascular endothelial growth factor (VEGF) and placental growth factor (PGF) promote placental growth and adequate sustenance of the maternal vasculature (116). Proteins of the VEGF family are important in the stabilization of vascular endothelial cells. The downstream signaling effects of VEGF promote the release of endothelium derived vasodilatory substances and stimulate eNOS activity in the vasculature (117, 118). A splice variant of VEGF receptor-1, soluble fms-like tyrosine kinase 1 (sFlt-1) serves as a VEGF/PGF protein antagonist. Levels of this anti-angiogenic factor normally remain low until the third trimester at which point levels increase to attenuate placental angiogenesis (119). Elevated levels of maternal sFlt-1 and other anti-angiogenic molecules often precede the clinical manifestations of PE and are correlated with severity of the disorder (120, 121). Lasting persistence of sFlt-1 has been described from months to years after delivery (112, 122). In animal experiments, administration of sFlt-1 induces hypertension, proteinuria and glomerular endotheliosis in pregnant rodents (123, 124). While these animals demonstrate normal circulating levels of sFlt-1, absence of proteinuria and vascular dysfunction at six months postpartum (125), long-term changes in maternal proteome have been measured (92). Thus, at present the roles of sFlt-1 and other angiogenic mediators of PE in perpetuating CVD risk postpartum remains unclear, and ultimately exploration of other markers of dysfunction is needed.
1.4.3 Vasomotor function and pre-eclampsia
The hemodynamic adaptations required of normal pregnancy are dysregulated in PE, with a relative impairment of vasodilation and arterial stiffness that have been shown to persist beyond pregnancy. Evaluation of local vasodilator responses in large resistance arteries have demonstrated impaired postpartum endothelial function in PE patients. Studies of brachial artery flow-mediated dilation using high-resolution external vascular ultrasound report reduced endothelial-dependent vasodilation in PE women, both during and after pregnancy (126-128), independent of smoking status, maternal obesity, blood pressure and metabolic disturbances. Examination of the microvasculature using laser Doppler imaging and flowmetry techniques to measure cutaneous perfusion has found evidence of altered endothelial-dependent and -independent dilation, suggestive of impairments not only at the endothelial level (129, 130) but also within the vascular smooth muscle layer (131, 132). While the consequences of this dysfunction can vary depending on the vascular bed, there is evidence to suggest that findings within the microvasculature can be extrapolated to function in larger resistance vessels (133, 134). As the microvasculature is likely the initial site of disruption (135), microvascular damage may well be one of the earliest recognizable signs of clinically measurable dysfunction within groups at high risk for vascular disease.

1.5 Postpartum Risk Screening
Risk estimation for CVD has become an important determinant for treatment and eligibility for vascular risk reduction programs. The 10-year risk estimations derived from the Framingham cohort data are the most widely used models of risk estimation. In this risk model, levels of traditional cardiovascular risk factors are assigned weighted values, and the clustering of individual risk factors including age, lipid levels, systolic blood pressure and smoking status contribute to a total score used to predict 10-year risk of CVD.
Age is a heavily weighted variable in most short-term prediction models such that in young individuals, in particular women under the age of 65, modest increases in other risk factors have relatively little effect on CVD risk estimates (136, 137). As a result, the effect that a given risk factor may have in a young woman is obscured. Indeed, many young women do not reach the threshold scores required for preventative interventions based on current CVD recommendations (138, 139), even though data from non-invasive imaging studies suggest that the presence of risk factors contribute substantially to the burden of asymptomatic atherosclerosis in young adulthood (140, 141). In an attempt to overcome these shortcomings, risk factor management guidelines for young women with elevated risk factors recommend calculating 10-year risk estimates as though young women were 60 years of age (142).

The introduction of 30-year and lifetime risk prediction models has served to re-examine an individual woman’s risk for cardiovascular outcomes. The use of lifetime risk prediction models removes age as a component of these risk calculations, and are likely more useful in assessing young women at risk of CVD. Recent data from our group has demonstrated that the proportion of women with a history of PE whose risk is “hidden” by the factor of age is substantial (108). Until 2011, cardiovascular risk assessment and counselling did not acknowledge the importance of a woman’s obstetrical history in individual CVD risk prediction. The American Heart Associations’ 2011 update on Effectiveness-Based Guidelines for the Prevention of Cardiovascular Disease in Women were the first guidelines to recognize that pregnancy offered a unique opportunity to identify women at risk of CVD (15). A comparison of the variables included in 10-year, 30-year and lifetime cardiovascular risk calculations is provided in Table 1.3.

Whether subclinical risk factors drive the development of PE or whether PE exacerbates pre-existing risk factors is uncertain. Regardless of whether PE, among other complications of pregnancy, is indeed an independent risk factor for the development of atherosclerosis, or if it simply unmask a subclinical vulnerability to endothelial dysfunction, a diagnosis of PE should
Table 1.3. A comparison of 10-year, 30-year and lifetime cardiovascular risk scoring variables.

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</tbody>
</table>

be considered an essential tool for the identification of women at risk of CVD. Risk factors including obesity, dyslipidemia and diabetes are shared by PE and CVD necessitating the consideration of their relationship in current cardiovascular risk evaluations (Figure 1.4). Recent examination of the effectiveness of postpartum lifestyle interventions in high-risk populations has demonstrated the value of risk reduction in recently PE women (146). Furthermore, survey-based analysis identifies women with PE to be highly motivated towards lowering their cardiovascular risk, although also highlights a need for professional counselling and involvement (147).

Therefore, in the struggle to identify young women at high risk of CVD, obstetrical history may prove to be a promising first step. The next step requires action through implementation of interventions that effectively reduce an individual’s risk of CVD. Data suggests that general awareness of CVD risk amongst women is associated with initiation of preventative actions (148) thereby highlighting the need for targeted CVD educational programs. Combined educational, nutritional and physical activity programs targeting an individual’s specific needs based on health history are likely to be the most effective in the reduction of cardiovascular risk. Most CVD risk in women is modifiable. Even modest modifications of various lifestyle parameters have been shown to reduce risk factors for CVD. Physical activity of moderate intensity in particular may ameliorate the adverse systemic effects of obesity. A 5-10% weight loss when accompanied with physical activity is shown to beneficially affect lipid and cholesterol levels as well as various hemostatic and inflammatory factors (149, 150). Primary prevention is a more effective approach to minimizing the impacts of disease at a population-level than secondary prevention from both a patient health and health care cost perspective. As such, promotion of a healthy lifestyle in conjunction with close monitoring and treatment of individual CVD risk factors is likely to be the most promising approach to CVD prevention in young at-risk women.
Figure 1.4. Endothelial dysfunction is characteristic of both pre-eclampsia and cardiovascular disease.

Although the clinical manifestations of pre-eclampsia resolve following delivery of the placenta, unaddressed cardiovascular risk factors may persist postpartum and when coupled with underlying endothelial dysfunction exacerbated as a result of PE likely contribute to future risk of re-current pregnancy complications and CVD.
A recent review examining postpartum cardiovascular risk management has called for the
development of structured postpartum cardiovascular screening programs for women after
hypertensive disorders of pregnancy (151). Such a clinic, The Maternal Health Clinic, has already
been established by our group at Kingston General Hospital in association with Queen’s
University, in Kingston, Ontario, Canada. The Maternal Health Clinic targets women at risk of
CVD based on obstetrical complications (e.g., hypertensive disorders of pregnancy, gestational
diabetes mellitus, pre-term delivery, intrauterine growth restriction, etc.). Here, postpartum
women undergo expanded cardiovascular risk screening including an assessment of traditional
cardiovascular risk factors and pregnancy-related risk indicators (152). Data from the first 17
months of operation of this clinic indicate that by six months postpartum a significant proportion
of women who develop pregnancy related cardiovascular risk indicators exhibit unfavourable
blood pressure, lipid values, hs-CRP levels and urinary microalbumin/creatinine ratios (153). Few
other such clinics have been established since the association between PE and CVD risk was first
recognized. Due to the fact that these programs are still in their infancy, the direct long-term
impact that they may have on risk reduction in the postpartum PE population remains to be seen.
In this light, the provision of evidence towards subclinical cardiovascular dysfunction soon after
PE is necessary to provide the impetus for the development of postpartum screening clinics
targeting this population.

1.6 Hypotheses and Objectives

Identification of women with a history of PE in the early postpartum period may provide an
opportunity to address the gender-based disparity in the diagnosis and management of CVD in
women. In order to prevent CVD in women, a necessary first step is identification of individuals
at increased risk of CVD through the use of age-appropriate risk models and obstetrical history,
followed by effective implementation of targeted risk reduction programs. Scattered evidence of
cellular and vascular dysfunction postpartum of PE underscores the need for a thorough exploration of non-traditional markers of cardiovascular risk and dysfunction in this young at-risk population to provide the basis for the development of such programs.

Under the proposal that pregnancy and the postpartum provide windows of opportunity to improve a woman’s long-term cardiovascular health, the OVERALL HYPOTHESIS of this thesis is that minimally invasive biomarkers can be used to identify persistent abnormalities in cardiovascular health in women with a recent history of PE. Herein we present a series of observational studies designed to establish a better understanding of the pathophysiologic link between PE and underlying or future cardiovascular disease. The specific hypotheses and objectives of the enclosed five studies are listed below:

1.6.1 Chapter 2: Differential expression of plasma miRNA in pre-eclamptic patients at delivery and one year postpartum.

MicroRNAs (miRNAs) are a highly conserved group of 18-25 nucleotide regulatory RNAs which play an important role in gene expression by binding to the 3’-untranslated region of mRNAs to orchestrate growth, development, function and stress responses of various organs (154). Therefore, in addition to playing an important role in the pathogenesis of a disease, miRNAs may also be used as potential biomarkers of disease or disease progression. Indeed, evidence of miRNAs circulating in human biofluids provides promise for their development as predictive or prognostic tools (155). Involvement of miRNAs in the pathogenesis of PE has been recently demonstrated and is an emerging field of investigation (156-158). No studies have examined miRNAs beyond the context of pregnancy, and this leaves a substantial gap in knowledge regarding their role in mediating future risk for CVD after PE. As such, we HYPOTHESIZE that unique patterns of miRNA expression in the maternal circulation will be evident in women with a recent history of PE.
Main Objectives:

1. To determine if the expression profiles of select non-placenta and non-pregnancy specific miRNA differ in the plasma of pre-eclamptic women as compared with that of normotensive controls.

2. To determine if the expression profiles of selected miRNA differ in the plasma of the same PE and control women at one year postpartum.

3. To determine if plasma expression profiles of select miRNA differ by PE severity (mild PE, severe PE) compared to normotensive controls.

4. To determine the biological impact of differential miRNA expression by global network analysis of miRNA function.

1.6.2 Chapter 3: Long-term alterations to the maternal circulating proteome after PE in women with and without cardiovascular risk factors.

Biofluids provide an important source of biomarkers with predictive potential for predisposition towards, or as early indicators of disease. Specifically, the plasma proteome, as a circulating biofluid, contains within it secreted, shed and released proteins reflecting the physiological state of all organ systems in the body (159). Recent investigations have identified peptides enriched for coagulation and complement activation in the plasma proteome of women with PE (160, 161), although no literature on the long-term alterations to maternal circulating peptide profiles are available. In light of this, we HYPOTHESIZE that examination of the maternal circulating proteome long after PE will provide evidence of differentially expressed and unique circulating peptides enriched for coagulation and inflammatory processes.

Main Objectives:

1. To determine if women with a recent history of PE have detectable alterations in the circulating maternal proteome.

2. To determine if the presence of cardiovascular risk factors mediates changes to circulating peptide profiles in PE subjects.

3. To determine the biological impact of differential peptide expression by global network analysis of peptide function.
1.6.3 Chapter 4: Postpartum alterations in circulating endothelial progenitor cells in women with a history of pre-eclampsia.

Endothelial damage or dysfunction is recognized as a hallmark of both PE and atherosclerosis. In addition to damages sustained as a result of complications in pregnancy, mechanisms preventing recovery of endothelial homeostasis likely contribute to the association of PE with future CVD. Bone-marrow derived endothelial progenitor cells (EPCs) are mobilized into the circulation to support angiogenesis and to promote healthy maintenance of the endothelium (162). Emerging data suggests that numbers of EPCs increase in maternal circulation over the course of normal pregnancy (163). Moreover, recent literature suggests impaired mobilization and function of EPCs in women with PE (163-165), which may account for or contribute to the endothelial dysfunction characteristic of the disorder. It remains unclear however, how pregnancy may affect postpartum maternal EPC physiology, and in particular the capacity for recovery of EPC physiology after pregnancies complicated by PE. In light of this we HYPOTHESIZE that persistent postpartum differences in endothelial progenitor cell number and function represent a pathophysiologic link between PE and CVD risk.

Main Objectives:

1. To determine if levels of circulating EPCs are reduced in the blood of women with a recent history of PE compared to postpartum time-matched controls.

2. To determine if the colony forming capacity of circulating angiogenic cells obtained from women with a recent history of PE is impaired compared to postpartum time-matched controls.

3. To determine the degree of cardiovascular risk and incidence of MetS in PE women at 6 months postpartum.

1.6.4 Chapter 5: Increased microvascular vasodilation and cardiovascular risk following a pre-eclamptic pregnancy.

Central to the paradigm of endothelial function is the homeostasis of vasoreactive factors mediating vascular responses based on physiological demand. It is well established that women
with PE exhibit distinct endothelial-dependent and -independent vascular impairments vessels (128, 131). Over what time course that these findings return to “normal” postpartum, if at all, is unknown however. As the microcirculation is likely the initial site for endothelial damage and dysfunction in subjects at risk of CVD, we HYPOTHESIZE that examination of microvascular function in women with a recent history of PE will reveal postpartum differences in microvascular reactivity. In addition, we expect that unique changes in vascular function will correlate with the presence of other physical and biochemical cardiovascular risk factors in women who developed PE.

**Main Objectives:**

1. To characterize the effect of normotensive pregnancy on endothelial dependent and -independent microvascular vasodilation and recovery of microvascular reactivity postpartum.

2. To characterize the effect of a recent history of PE on endothelial-dependent and -independent microvascular vasodilation compared to postpartum time-matched and never-pregnant controls.

3. To determine if differences in microvascular reactivity between PE and control groups were augmented by PE severity and cardiovascular risk.

**1.6.5 Chapter 6: Reduced heart rate variability and altered cardiac conduction after pre-eclampsia.**

Electrical activity of the heart is regulated by the dynamic interplay of autonomic nervous system (ANS) activity and the efficiency of electrical conduction through the cardiac tissue. Ambulatory monitoring of the surface electrocardiograph (ECG) is an established, non-invasive clinical tool that can provide insight into autonomic imbalance, impairments in cardiac conduction and allude to structural remodeling of the heart. Indeed, deviations of ECG parameters from the norm may be used as early markers of MetS (166), atrial fibrillation (167) and all-cause mortality (168). Atypical ANS modulation of heart rate is observed in women with PE (169) and similar findings prior to and early in pregnancy (170) before the onset of PE is suggestive of underlying
cardiovascular abnormalities that may predate the pregnancy itself. Without data collected postpartum however, it is unknown whether alterations in ECG parameters normalize after afflicted pregnancies or persist into later life. Therefore, we HYPOTHESIZE that changes to the regulation of cardiac cycle will be evident in women with a recent history of PE.

**Main Objectives:**

1. To characterize the effect of normotensive pregnancy on cardiac autonomic control mechanisms by examining parameters of heart rate variability in late pregnancy and postpartum compared to never-pregnant controls.

2. To determine if cardiac autonomic tone differs in women with a recent history of PE compared to time-matched postpartum, and never-pregnant controls through the use of heart rate variability measurements.

3. To characterize the effect of normotensive pregnancy on cardiac conduction, namely P-Wave and QRS duration, and postpartum recovery versus non-pregnant controls.

4. To determine if parameters of cardiac conduction, namely P-Wave and QRS duration, differs in women with a recent history of PE compared to time-matched postpartum, and never-pregnant controls.
Chapter 2

Differential expression of plasma miRNA in pre-eclamptic patients at delivery and one year postpartum

This chapter has been modified from its original published version: Murphy MSQ, Casselman RC, Tayade C, Smith GN. Differential expression of plasma miRNA in pre-eclamptic patients at delivery and one year postpartum. American Journal of Obstetrics and Gynecology, 2015. MS#E15-0034 (in press).
2.1 Abstract

Objectives: Pre-eclampsia (PE) is a hypertensive disorder of pregnancy characterized by widespread maternal endothelial dysfunction. Although clinical signs subside following delivery, long-term risks associated with PE include hypertension, stroke and cardiovascular disease. miRNAs are emerging as critical regulators of biological function, and while alterations to the miRNAome have been described in the context of pregnancy and PE, the postpartum implications of PE on miRNA expression is unknown. The goal of this study was to characterize circulating miRNA profiles at the time of delivery and at one year postpartum for women who did and did not develop PE.

Study Design: Using a targeted RT-PCR approach, selected miRNAs putatively involved in the pathophysiology of PE were examined in 17 normotensive control and 13 PE maternal plasma samples at the time of delivery and one year postpartum. Ingenuity Pathways Analysis was used to map putative mRNA targets of differentially expressed miRNA to global molecular networks based on gene function.

Results: Significant increases ($p<0.05$) in 7 miRNA with anti-angiogenic, inflammatory and apoptotic functions (miR-98-5p, miR-222-3p, miR-210-3p, miR-155-5p, miR-296-3p, miR-181a-5p, miR-29b-3p) were evident in maternal plasma at the time of severe PE compared to time-matched controls. Plasma samples from individuals who developed mild PE exhibited no changes compared to control samples for the subset of miRNAs analyzed here. Differential expression of plasma miRNA at the time of delivery for women with PE were largely resolved at one year postpartum, and reduced expression of only miR-221-3p ($p<0.05$) was evident. Network analysis of putative targets of differentially regulated miRNA identified 11 interacting networks with
enrichment for proteins involved in cardiovascular disease, organ system development and function and cell signalling and interaction.

**Conclusions:** The systemic effect of PE on maternal systems is evident in the circulating miRNAome with substantial alterations in miRNA expression in women who develop severe PE. In addition, we provide novel evidence of disruption to miR-221 expression one year postpartum following a pregnancy complicated by PE compared to normotensive time-matched controls, which may allude to persistent inflammation in these women after delivery.
2.2 Introduction

Pre-eclampsia (PE) is a multisystem disorder of pregnancy arising in 3-5% of pregnancies contributing to significant maternal and neonatal morbidity and mortality worldwide (171, 172). Insufficient remodelling of maternal spiral arterioles by trophoblast cells in the earliest stages of placental development, together with immune maladaptations establishes increasingly hypoxic conditions as feto-placental demands increase with advancing gestation. Hypoxia-induced upregulation of placental mediators of inflammation and apoptosis coupled with reduction in the bioavailability of angiogenic molecules such as Vascular Endothelial Growth Factor (VEGF) and Placenta Growth factor (PGF) may contribute to the maternal response characteristic of PE (50). Maternal constitutional factors (e.g. obesity, diabetes, microvascular disease) also contribute the pathogenesis of PE, and the variable implications of the placental and maternal environments upon which the pregnancy progresses appear to contribute to the heterogeneous nature of the disorder. While hypertension and proteinuria resolve following delivery, it is apparent that the implications of PE extend well beyond that of the peripartum period. Indeed, PE is recognized as one of several pregnancy related complications that are associated with a woman’s increased risk for future hypertension, cardiovascular disease (including but not limited to myocardial infarction and coronary artery disease) and cerebrovascular disease (95, 173).

MicroRNA (miRNA) are non-coding RNA transcripts, ~22 nucleotides long that provide critical post-transcriptional regulation of gene expression in both health and disease through sequence-specific binding to the 3’-untranslated region of target mRNA transcripts (154). Recent research has identified an abundance of miRNA in the healthy term placenta (157, 174, 175), and alterations of the miRNAome in cases of placental insufficiency highlighting a role for miRNA signalling in the development of PE (156, 157, 174-177).

The identification of stable circulating miRNAs existing in plasma and serum (155) has provided promise for minimally invasive biomarkers for disease prediction, diagnosis and prognosis.
However, there is very limited research in the area of maternal circulating miRNA profiles in PE populations, and none that explore the miRNAome beyond the immediate postpartum period. While the association between PE and future development of cardiovascular disease is well established, the reasons underlying this relationship remain largely unclear. In consideration of the diverse role of miRNA in a range of biological processes, the expression levels of miRNA regulating angiogenic, inflammatory and apoptotic pathways in the plasma of women with a history of mild PE, severe PE and uncomplicated pregnancies may provide insight into physiological processes mediating postpartum cardiovascular susceptibility in this population. This study examined targeted circulating miRNA expression profiles by real-time RT-PCR at the time of delivery and at one year postpartum in women who did/did not develop PE.

2.3 Materials and Methods

2.3.1 Participants and sample collection
Archival plasma samples from the Pre-eclampsia New Emerging Team (PE-NET) prospective longitudinal cohort (107) were used in this study, selected on the basis of availability, plasma volume and quantity of data available on each sample. Plasma samples were obtained in the peripartum and at one year postpartum from women who did/did not develop PE who had delivered at either the Kingston or Ottawa General Hospitals, ON, Canada. The PE-NET study was approved by the Queen's University Research Ethics Board (OBGY-108-03), and all participants provided written informed consent prior to sample collection. Inclusion criteria of the participants have been previously described (107). In brief, a diagnosis of PE was made based on PE-NET cohort diagnostic criteria; systolic blood pressure ≥140 mmHg and diastolic blood pressure ≥90 mmHg with proteinuria >300 mg/24 hours or 1+ on repeat dipstick at the time of presentation to clinic or admission/transfer to a participating research hospital. The PE-NET cohort had collected extensive data on the maternal PE symptoms of study participants, and so for
this reason, severity of PE was considered in sub-analysis of the data collected. Severe PE diagnosis criteria were met if in addition to the criteria for PE, an individual presented with any one of the following: two stable measurements of systolic blood pressure 160 mmHg or diastolic blood pressure 110 mmHg a minimum of at least six hours apart, proteinuria of 5 g/24 hours or 3+ on repeat dipstick, oliguria ≤500 mL/24 hours, cerebral or visual disturbances, epigastric pain, thrombocytopenia <150,000/L, AST>46 U/L, ALT>40 U/L, serum creatinine >106 μmol/L, pulmonary cyanosis or intrauterine growth restricted baby. Diagnosis of early (<34 weeks gestation) and late-onset (>34 weeks gestation) PE was also considered for sub-analysis. Maternal non-fasting blood samples were collected into a heparin-containing BD Vacutainer® (#367878, BD Biosciences Franklin Lakes, New Jersey, USA). Plasma was isolated by centrifugation (1000 g for 15 minutes). Aliquots were stored at -80°C until use.

2.3.2 Total RNA extraction, miRNA isolation and heparinase treatment of samples
Total RNA including microRNA was extracted from archival plasma samples using RNeasy® MiniElute® spin columns (Qiagen, CA USA), modifying the manufacturer’s instructions to optimize RNA isolation from heparinized samples. In brief, 100μL of heparinized plasma was diluted in 100 μL RNase-free water and mixed with 1mL of QIAzol® Lysis Reagent. Samples were incubated at room temperature for 5 minutes before adding 3.5 μL diluted C. elegans miR-39 miRNA mimic spike-in control (#219610, Qiagen, CA, USA), and 200 μL chloroform. Samples were vortexed, incubated at room temperature for 3 minutes and subsequently centrifuged at 12 000 g at 4°C for 15 minutes. The upper phase was collected and mixed with 1.5 v/v 100% ethanol and applied to an RNeasy® MinElute® spin column (Qiagen, CA, USA). After washings, RNA was eluted from the column using RNase-free water, and incubated with 0.5 μL human ribonuclease inhibitor (R2520, Sigma Aldrich) and heparinase based on protocols previously described (178, 179) [heparinase I from flavobacterium heparinum, 1U/μL (H2519,
Sigma Aldrich) in 1x reaction buffer (5 mM Tris pH 7.5, 1 mM CaCl₂) for 2 hours at 25°C. After heparinase treatment samples were stored at -80°C until cDNA synthesis.

2.3.3 cDNA preparation and RT-PCR assays for miRNA analysis
Total RNA including miRNA were reverse transcribed using the miScript Reverse Transcription kit (#218161, Qiagen, Mississauga, Canada) according to the manufacturer’s instructions. In brief, 9 μL of RNA plasma preparation was combined with 4 μL 5x miScript HiSpec Buffer, 10x miScript Nucleic Mix, 2 μL miScript Reverse Transcriptase mix and 3 μL RNase-free water for a total volume of 20 μL per reverse transcription reaction. Each reaction was incubated at 37°C for 60 minutes followed by 95°C for 5 minutes to inactivate the reverse transcriptase reaction. cDNA was then diluted in 200 μL RNase-free water and frozen at -20°C until PCR amplification and quantification of gene products.

Custom miScript miRNA PCR Arrays (#CMIHS02264, Qiagen, CA, USA) were used to quantify miRNA gene expression using the manufacturer’s protocol (Qiagen, Mississauga, ON, Canada). In brief, a reaction mix containing 344 μL of 2x QuantiTect SYBR green PCR master mix, 69 μL 10x miScript universal primer, 25 μL diluted template cDNA and 250 μL RNase-free water, was prepared, and 25 μL applied to each well of the custom array containing specific miRNA primer assays.

PCR was carried out on a LightCycler® 480 Real-Time PCR System (Roche Applied Science, Laval, Quebec, Canada) using the cycling conditions set by the manufacturer (Heat Activation 95°C, 15 minutes followed by 45 cycles of denaturation: 94°C, 15 seconds, annealing: 55°C, 30 seconds and extension: 70°C, 30 seconds with a ramp rate of 1°C/second. SYBR Green fluorescence data were collected during each extension cycle, and a dissociation curve analysis run at the end of the cycling program for each array (70°C - 95°C, ramp rate 0.1 °C/second) to confirm the formation of specific amplified products.
2.3.4 Selection of miRNAs

miRNAs for analysis were carefully selected following extensive review of the literature based on relevance to vascular biology and cardiovascular disease; placenta and pregnancy-specific markers were excluded to justify the re-examination of these miRNA at one year postpartum. Those included in the final analysis were those regulating angiogenic, inflammatory and apoptotic pathways: hsa-let-7f-5p, hsa-miR-98-5p, hsa-miR-221-3p, hsa-miR-222-3p, hsa-miR-126-3p, hsa-miR-130a-3p, hsa-miR-210-3p, hsa-miR-155-5p, hsa-miR-17-5p, hsa-miR-18a-5p, hsa-miR-19a-3p, hsa-miR-29a-3p, hsa-miR-92a-3p, hsa-miR-20a-5p, hsa-miR-20b-5p, hsa-miR-15b-5p, hsa-miR-16-5p, hsa-miR-296-3p, hsa-miR-181a-5p, hsa-miR-195-5p and hsa-miR-29b-3p. Primer assays for specific target miRNAs were designed using sequences from the miRBase sequence database (http://www.mirbase.org) version 21, and sent to Qiagen (Mississauga, ON, Canada) for synthesis of a customized miScript PCR array. The miRBase accession numbers for the target miRNA used in this study are listed in Table 2.1.

2.3.5 Selection of putative mRNA target genes

To determine the biological significance of differentially expressed miRNAs, the miRNA-target prediction tool miRecords (http://mirecords.biolead.org/) was used to generate a list of mRNA targets for those miRNA determined to be differentially expressed between PE and control subjects. miRecords integrates results from 11 independent resources (DIANA-microT, MicroInspector, miRanda, miRDB/miRTarget2, miTarget, NBMirTar, PicTar, PITA, RNA22, RNAhybrid, and TargetScan/TargetScanS), each with their own unique target prediction algorithm. Targets were selected based on the following criteria: validated or predicted with agreement from ≥6/11 target prediction resources used by miRecords. For miR-296 these criteria were widened to targets predicted by ≥4/11 prediction tools. Although we initially attempted RT-PCR of validated and highly predicted mRNA targets to determine the biological implications of differentially expressed miRNA in maternal plasma, amplification of mRNA targets from RNA
### Table 2.1. miRBase accession numbers of selected miRNA.

<table>
<thead>
<tr>
<th>Accession Number</th>
<th>Mature miRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>MIMAT0000067</td>
<td>hsa-let-7f-5p</td>
</tr>
<tr>
<td>MIMAT0000096</td>
<td>hsa-miR-98-5p</td>
</tr>
<tr>
<td>MIMAT0000278</td>
<td>hsa-miR-221-3p</td>
</tr>
<tr>
<td>MIMAT0000279</td>
<td>hsa-miR-222-3p</td>
</tr>
<tr>
<td>MIMAT0000445</td>
<td>hsa-miR-126-3p</td>
</tr>
<tr>
<td>MIMAT0000425</td>
<td>hsa-miR-130a-3p</td>
</tr>
<tr>
<td>MIMAT0000267</td>
<td>hsa-miR-210-3p</td>
</tr>
<tr>
<td>MIMAT0000646</td>
<td>hsa-miR-155-5p</td>
</tr>
<tr>
<td>MIMAT0000070</td>
<td>hsa-miR-17-5p</td>
</tr>
<tr>
<td>MIMAT0000072</td>
<td>hsa-miR-18a-5p</td>
</tr>
<tr>
<td>MIMAT0000073</td>
<td>hsa-miR-19a-3p</td>
</tr>
<tr>
<td>MIMAT0000086</td>
<td>hsa-miR-29a-3p</td>
</tr>
<tr>
<td>MIMAT0000092</td>
<td>hsa-miR-92a-3p</td>
</tr>
<tr>
<td>MIMAT0000075</td>
<td>hsa-miR-20a-5p</td>
</tr>
<tr>
<td>MIMAT0001413</td>
<td>hsa-miR-20b-5p</td>
</tr>
<tr>
<td>MIMAT0000417</td>
<td>hsa-miR-15b-5p</td>
</tr>
<tr>
<td>MIMAT0000069</td>
<td>hsa-miR-16-5p</td>
</tr>
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<td>MIMAT0004679</td>
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<td>MIMAT0000256</td>
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<td>MIMAT0000461</td>
<td>hsa-miR-195-5p</td>
</tr>
<tr>
<td>MIMAT0000100</td>
<td>hsa-miR-29b-3p</td>
</tr>
</tbody>
</table>
samples proved insufficient for analysis. Given the long-term instability of mRNA and the archival nature of the current samples however, it is perhaps not surprising that these samples provided negligible mRNA signal (180). Putative targets identified were instead evaluated using QIAGEN’s Ingenuity® Pathway Analysis (IPA®, QIAGEN Redwood City, www.qiagen.com/ingenuity). Selected genes were filtered through the web-based Ingenuity Knowledge Database, a repository of curated biological interactions and functional annotations and subsequently mapped to global molecular networks based on gene function.

2.3.6 Statistical analysis
Continuously distributed demographic variables are presented as mean ± standard deviation. Variables were compared using an unpaired t-test, or Fisher’s exact test where appropriate (GraphPad Prism 5 Software, La Jolla, CA, USA). PCR array data analysis was performed using PCR Array Data Analysis Software (SABiosciences, http://www.sabiosciences.com/dataanalysis.php) with data normalized by geometric mean to the C. elegans miR-39 spike-in control and C_T cut-off at 35 cycles. Enrichment of putative mRNA genes within global networks mapped by IPA® was evaluated using right-tailed Fisher’s exact tests, considering the number of focus genes participating in a given biological process and comparing it to the total number of genes identified by the Ingenuity Knowledge Database to be associated with that process.

2.4 Results
2.4.1 Participants
Of 44 control and 52 PE RNA extractions, 38 and 36 samples, respectively met array quality control criteria. As a result, paired delivery and postpartum samples from a total of 17 normotensive pregnant controls and 13 women who developed PE were available for data analysis. Clinical characteristics of these patients are listed in Table 2.2. PE subjects exhibited
elevated pre-pregnancy body mass indices (BMI, kg/m²) and delivered earlier in gestation with significant hypertension, and smaller infants compared to control subjects. At one year postpartum BMI and systolic and diastolic blood pressures remained slightly higher in subjects with a history of PE. Nearly 50% of PE subjects delivered with a diagnosis of severe PE. These findings are consistent with reported population characteristics of the larger PE-NET cohort (107). Due to a loss of samples derived from patients who had developed early-onset PE after array-based quality control assessment, only 15.4% (n=2) early-onset PE samples were included in the final analysis, preventing reliable sub-analysis of the effect of PE onset on maternal miRNA profiles.

2.4.2 miRNA expression in PE compared to control subjects at delivery

All selected miRNAs were expressed in the plasma samples obtained from both PE and uncomplicated pregnancies at delivery. Of the 21 mature miRNAs examined, we found differences in several miRNAs with 2 to 4 fold change compared to controls: hsa-miR-222-3p (2.45), hsa-miR-210-3p (2.216), hsa-miR-16-5p (-2.526), hsa-miR-296-3p (3.058), hsa-miR-181a-5p (2.000), hsa-miR-29b-3p (2.133). In particular, expression levels of hsa-miR-296-3p and hsa-miR-181a-5p were significantly increased (p<0.05) in PE compared to uncomplicated pregnancies at the time of delivery. Differential expression of an additional three miRNA neared significance (hsa-miR-222-3p, p=0.097; hsa-miR-210-3p, p=0.055; hsa-miR-29b-3p, p=0.098) (Figure 2.1A).

Results from stratification of miRNA expression by patient diagnosis of severe PE or mild PE (as defined by PE-NET cohort criteria) are depicted in Figure 2.1B. Participants with a diagnosis of mild PE (n=7) exhibited no significant differences in the expression of selected miRNA when compared to controls (n=17) at the time of delivery. In contrast, severe PE (n=6) was associated with increased expression of 7 miRNA (hsa-miR-98-5p, hsa-miR-222-3p, hsa-miR-210-3p, hsa-
Table 2.2. Participant characteristics.

<table>
<thead>
<tr>
<th></th>
<th>Control (n=17)</th>
<th>PE (n=13)</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal Age, yrs</td>
<td>28.2±4.1</td>
<td>30.4±7.3</td>
<td>0.0336*</td>
</tr>
<tr>
<td>Smoking, n (%)</td>
<td>1 (5.9)</td>
<td>2 (15.4)</td>
<td>0.4675</td>
</tr>
<tr>
<td>Nulliparous, n (%)</td>
<td>11 (64.7)</td>
<td>8 (61.5)</td>
<td>0.8656</td>
</tr>
<tr>
<td>Pre-Pregnancy BMI, kg/m²</td>
<td>25.2±3.5</td>
<td>28.9±7.6</td>
<td>0.0049</td>
</tr>
<tr>
<td>Gestational Age at Delivery, wks</td>
<td>40.2±1.5</td>
<td>37.0±2.7</td>
<td>0.0305*</td>
</tr>
<tr>
<td>Pre-Delivery Systolic Blood Pressure, mmHg</td>
<td>113.1±10.2</td>
<td>140.7±12.5</td>
<td>&lt;0.0001*</td>
</tr>
<tr>
<td>Pre-Delivery Diastolic Blood Pressure, mmHg</td>
<td>68.9±8.1</td>
<td>85.7±10.3</td>
<td>&lt;0.0001*</td>
</tr>
<tr>
<td>1 Year Postpartum BMI, kg/m²</td>
<td>25.9±3.9</td>
<td>31.3±8.7</td>
<td>0.0036*</td>
</tr>
<tr>
<td>1 Year Postpartum Systolic Blood Pressure, mmHg</td>
<td>111.0±7.8</td>
<td>114.5±10.9</td>
<td>0.3192</td>
</tr>
<tr>
<td>1 Year Postpartum Diastolic Blood Pressure, mmHg</td>
<td>72.1±7.8</td>
<td>77.5±9.5</td>
<td>0.0939</td>
</tr>
<tr>
<td>Infant Birth weight, g</td>
<td>3623.2±351.4</td>
<td>2965.8±965.8</td>
<td>0.0003*</td>
</tr>
<tr>
<td>Severe PE, n (%)</td>
<td>-</td>
<td>6 (46.2)</td>
<td>-</td>
</tr>
<tr>
<td>Early Onset PE &lt;34 weeks, n (%)</td>
<td>-</td>
<td>2 (15.4)</td>
<td>-</td>
</tr>
<tr>
<td>Subjects at high lifetime risk (≥39%) at 1 year postpartum, n (%)</td>
<td>1 (5.9)</td>
<td>5 (38.5)</td>
<td>0.0606</td>
</tr>
<tr>
<td>Subjects with MetS at 1 year postpartum, n (%)</td>
<td>2 (11.8)</td>
<td>2 (15.4)</td>
<td>1.000</td>
</tr>
</tbody>
</table>

Data presented as mean±SD and compared using unpaired t-test or Fisher’s exact test where appropriate. BMI, body mass index; MetS, metabolic syndrome. Lifetime cardiovascular risk scores for each patient were generated based on biophysical factors including; sex, smoking, total cholesterol fasting glucose, systolic blood pressure, diastolic blood pressure, antihypertensive usage - Categorical data for lifetime cardiovascular risk are divided into <39%, low risk, and ≥39%, high risk. A summary of biophysical parameters considered in lifetime cardiovascular risk assessment and diagnosis of MetS is found in 0. *Statistically significant, p<0.05.
Figure 2.1. Differential expression of selected miRNA in pre-eclampsia vs control samples at delivery.

(A) At the time of delivery, PE was associated with the increased expression of 2 miRNA (hsa-miR-296-3p and hsa-miR-181a-5p). (B) Comparison of mild PE (n=7) and severe PE (n=6) to Controls (n=17). Severe PE was associated with the increased expression of 7 miRNA compared to controls and 6 miRNA compared to mild PE. There were no significant alterations in miRNA expression in patients with mild PE compared to controls. The online tool http://pcrdataanalysis.sabiosciences.com/mirna was used for data analysis. *p<0.05, b p<0.01, c p<0.001, d p<0.0001 versus control. *p<0.05 versus mild PE.
miR-155-5p, hsa-miR-296-3p, hsa-miR-181a-5p, hsa-miR-29b-3p) compared to controls and increased expression of 6 miRNA (hsa-miR-98-5p, hsa-miR-130a-3p, hsa-miR-210-3p, hsa-miR-155-5p, hsa-miR-296-3p, and hsa-miR-181a-5p) compared to mild PE.

2.4.3 miRNA expression in PE compared to control subjects at one year postpartum
All selected miRNAs were expressed in the plasma samples obtained from both PE and uncomplicated pregnancies at one year postpartum. Three miRNA exhibited greater than 2 fold change in expression compared to controls: hsa-miR-221-3p (-3.239), hsa-miR-222-3p (2.016) hsa-miR-29b-3p (3.793) (Figure 2.2A). Of these, significant alteration in expression of only hsa-miR-221-3p (p<0.05) was evident in PE compared to controls. Stratification of miRNA expression by diagnosis of mild and severe PE is depicted in Figure 2.2B. Although whole-group analysis of miRNA expression profiles identified the down-regulation of miR-221 at one year postpartum after PE, there were no differences in miRNA expression profiles between control and PE subjects after stratification based on PE severity.

2.4.4 Correlation of differentially expressed miRNA to blood pressure
Differentially expressed miRNA were correlated to pre-delivery and postpartum blood pressures to determine the relationship between blood pressure regulation and miRNA expression (Figure 2.3). Increases in pre-delivery systolic and diastolic blood pressure were significantly correlated with lower relative C_T values for miR-222. In addition, systolic and diastolic blood pressures were weakly correlated to expression of miR-210 and miR-155 respectively. At one year postpartum, miR-221 expression was inversely correlated to both systolic and diastolic blood pressures. Interestingly normalized C_T values for miR-221 were also correlated to BMI at one year postpartum (r=0.4367; p=0.0158) indicating reduced expression of miR-221 with increasing BMI.
Figure 2.2. Differential expression of selected miRNA in pre-eclampsia vs control samples at one year postpartum.

(A) One miRNA, *hsa-miR-221-3p*, was down-regulated in plasma samples obtained from PE subjects at 1 year postpartum. (B) Comparison of mild PE (n=7) and severe PE (n=6) to Controls (n=17). Postpartum expression levels of selected miRNA did not differ after stratification for mild/severe PE versus controls. The online tool http://pcrdataanalysis.sabiosciences.com/mirna was used for data analysis. *p<0.05 versus controls.*
Figure 2.3. Correlation of differentially expressed miRNA to blood pressure.

(A)(B) Increases in systolic and diastolic blood pressure were significantly correlated to miR-222 expression before delivery. Pre-delivery systolic and diastolic blood pressures were also weakly correlated to expression of miR-210 and miR-155 respectively. (C)(D) In contrast, one year postpartum systolic and diastolic blood pressures were inversely correlated to miR-221 expression. miRNA expression interpreted from CT of each miRNA normalized to CT of C. elegans miR-39 (ΔCT = CT miRNA - CT C. elegans miR-39).
2.4.5 Evaluation of validated and highly predicted target mRNA

Evaluation of the putative target mRNA using miRecords identified 438 genes that were either validated or highly predicted targets of the differentially expressed miRNA observed in maternal plasma samples. Of the 383 genes identified by miRecords screening, IPA® was able to map 435 of these genes to the Ingenuity Knowledge Database. The 3 genes not included in IPA® analysis were FLJ20160, FKJ37543, and MAR7. IPA® network analysis identified an enrichment of genes associated with 11 interacting networks. A summary of these networks is provided in Table 2.3.

2.5 Discussion

MicroRNAs are emerging as critical regulators of biological function, and while alterations to the miRNAome have been described in the context of pregnancy and PE, the postpartum implications of PE on miRNA expression remain unexplored. Using RT-PCR we have identified increases in expression of seven maternal plasma miRNA (hsa-miR-98-5p, hsa-miR-222-3p, hsa-miR-210-3p, hsa-miR-155-5p, hsa-miR-296-3p, hsa-miR-181a-5p, hsa-miR-29b-3p) in patients with severe PE. In addition, we have demonstrated that differential expression of these miRNA at the time of pregnancy and PE are largely resolved one year postpartum in the maternal circulation. Samples taken one year postpartum of the index pregnancies in the same group of women exhibited significant alterations in expression of only one miRNA after PE; hsa-miR-221-3p.

Recent identification of placenta-specific miRNAs in the maternal circulation (181-184) has highlighted their potential as predictive markers for syndromes of placental insufficiency. Implicit to the success of pregnancy however includes the maternal response to the metabolic and vascular needs of the conceptus. Indeed variations in angiogenic and inflammatory response miRNA have been described in the maternal circulation of women who develop PE (158, 185-189).
Table 2.3. Target mRNA enrichment in biological networks.

<table>
<thead>
<tr>
<th>Target mRNA in network</th>
<th># Focus Molecules</th>
<th>Network Functions</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADRB2, ANTXR1, ANTXR2, ARRDC3, ATP1B1, CDH1, CYP19A1, DIO2, DNMT3A, DNMT3B, EED, EFN3A, EN1, GOLGA2, HDAC4, IRS2, LPAR1, MAP2K1, PFN2, PRKCD, RBBP4, RBBP7, SIRT1, FAPC2, TUBB2A, WWC1, ZEB2, ZFP36L1</td>
<td>28</td>
<td>Cancer, Cell Morphology, Hair and Skin Development and Function</td>
<td>36</td>
</tr>
<tr>
<td>ATXN1, CAMK2D, CAMK2G, CDC25A, CDKN1B, COL2A1, COL3A1, FBN1, IKBKE, INSIG1, IqGAP1, ITGB8, KLHL2, LGR4, LIN28A, MAP3K3, NGFR, RALA, RIOK3, SENP1, SPRY1, SPRY4, STK40, TARDBP, TIMP3, TRIM37, ZFAND6</td>
<td>27</td>
<td>Organismal Survival, Embryonic Development, Organ Development</td>
<td>34</td>
</tr>
<tr>
<td>ABCC5, CAV2, CCR3, COL4A1, COL4A5, DCLK1, ESR1, GRM3, HOXA11, IL1A, ITGA3, KAT2B, KRAS, MT2A, NRAS, PCDHA4, PCDHA5, PCDHA8, PCDHA12, PCDHAC2, PDGFC, RAD21, RAN, SGK1, TCERG1, TGFBR1</td>
<td>26</td>
<td>Cancer, Skeletal and Muscular Disorders, Tissue Development</td>
<td>32</td>
</tr>
<tr>
<td>ADM, AHR, BCL11A, CLDN2, E2F7, ECT2, HMGCR, IGF2, IGF2BP3, IL6ST, LIN28B, LRRN1, MYCN, NR4A3, PDGFRα, PLAT, PRKCE, RAB11A, SMPD3</td>
<td>19</td>
<td>Cellular Development, Cellular Growth and Proliferation, Cancer</td>
<td>20</td>
</tr>
<tr>
<td>AMER1, BACH1, CCDC8, DERL1, GDF6, H3F3A/H3F3B, HIF3A, HLF, HMGCS1, POSTN, PRKAB2, RFFL, SEL1L, UBE2B</td>
<td>14</td>
<td>Cellular Development, Cellular Growth and Proliferation, Hematological System Development and Function</td>
<td>12</td>
</tr>
<tr>
<td>ACSL1, G3BP2, KPNB1, LEMD3, LRP1B, OSBPL3, PBX3, PRKAG1, SGK1, TET1, VAPB, WEE1, ZNF282</td>
<td>13</td>
<td>Cancer, Cell Cycle, Developmental Disorder</td>
<td>11</td>
</tr>
<tr>
<td>C2CD5, CBX7, CDC34, CLDN1, CTDSPL, FUK, KLF15, PLIN2, PPARC1A, SOX5, SXX2IP, TCF12, UHRF2</td>
<td>13</td>
<td>Cell Cycle, Connective Tissue Development and Function, Cell Death and Survival</td>
<td>11</td>
</tr>
<tr>
<td>ARF4, ARVC, CACNB2, DYNC1I1, GABRA1, GRM5, NOVA1, RAB11FIP2, SLC20A1, ST8SIA4, STC1, ZIC2</td>
<td>12</td>
<td>Cardiovascular Disease, Organismal Injury and Abnormalities, Cell-to-Cell Signaling and Interaction</td>
<td>10</td>
</tr>
<tr>
<td>CDC73, CPD, CUL3, EIF5A2, GPR37, ISL1, LCORL, NGFR, PCDHA2, PDE2A, TBPL1, TOMM20</td>
<td>12</td>
<td>Cell Death and Survival, Cellular Development, Cellular Growth and Proliferation</td>
<td>10</td>
</tr>
<tr>
<td>ADAMTS9, AGPAT4, ANO1, COL5A2, COL5A3, DCP2, GPD1L, HMGN3, LPL, MLF1, MYLIP, PPIC</td>
<td>12</td>
<td>Cardiovascular Disease, Organismal Injury and Abnormalities, Tissue Morphology</td>
<td>10</td>
</tr>
<tr>
<td>ACVR1C, COL19A1, COL9A1, FBXO33, MOBOAT2, NAV2, NREP, PHC1, PPP1R12B, RDH10, TGFBR1</td>
<td>11</td>
<td>Embryonic Development, Organismal Development, Tissue Development</td>
<td>8</td>
</tr>
</tbody>
</table>
Our results indicate that maternal plasma of women with severe PE is enriched for a heterogeneous array of miRNA supporting angiogenic (miR-210, miR-296, miR-222, miR-98), inflammatory (miR-155, miR-181a) and apoptotic (miR-29b) processes. Whether these circulating miRNA have their origins in the placenta, or are derived from maternal systems is unclear, although the putative impacts of these miRNA on biological functions determined from networking analysis are consistent with those described in patients with PE (cardiovascular disease, organ system development and function and cell signaling and interaction). Elevated miR-210 has been consistently identified in both the serum/plasma (185, 188) and placentas (174, 177, 190) of women who develop PE. A critical role for miR-210 expression in regulating these pathophysiological responses to placental hypoxia apparent; miR-210 drives VEGF-mediated endothelial cell migration and tubule formation in vitro and its overexpression reduces trophoblast invasion (185). Although elevated serum miR-221 is shown to be highly predictive of the disease through 2nd trimester plasma sampling (185), high-throughput sequencing of 754 miRNA and RT-PCR validation of 15 miRNA isolated from sera of women in the 1st trimester of pregnancy failed to identify any miRNA with potential for early pregnancy prediction of PE (191). Our findings of altered miR-181a expression in the plasma of women with severe PE are corroborated by others (187) and are also reported in the pre-eclamptic placenta (177, 192). miR-181a appears as a regulator of mesenchymal stem cell (MSC) proliferation in the placenta, and its overexpression impairs MSC immunosuppressive properties by enhancing their expression of IL-6 and VEGF (193).

The novel finding reported here is the down-regulation of miR-221 in the plasma of mothers one year postpartum after PE. In mature human endothelial cells, miR-221, and its paralogue miR-222, exhibit largely anti-angiogenic properties, targeting a vast array of active mRNA targets including c-kit and endothelial nitric oxide synthase (eNOS). Increased expression of miR-221/222 appear largely pro-atherogenic in the early stages of atherosclerosis, down-regulating the
activity of eNOS, an enzyme critical to the recruitment of progenitor cells to sites undergoing active angiogenesis and vascular repair. In non-vascular tissues, chronic inflammation and advance glycation end-product-induced down-regulation of miR-221 (194, 195) underscore its role in cardiometabolic diseases. miR-221 is shown to be down-regulated in adipogenesis (196-198) and levels negatively correlated with tumour necrosis factor-α in adipocytes derived from obese subjects (196). Importantly, decreased expression of miR-221 is seen in sclerotic intimal samples of patients with peripheral arterial disease (199) alluding to a role for this miRNA in mechanisms mediating neointimal plaque formation. Our findings of reduced miR-221, one year postpartum of PE may well reflect lingering inflammation in these women. While we were not able to identify a correlation between miR-221 expression levels and postpartum cardiovascular risk scores (0), the inverse correlation of miR-221 expression to blood pressure and BMI postpartum may suggest lifestyle modification in this population as a suitable avenue of CVD prevention and risk reduction; dynamic changes in circulating miRNA expression, including miR-221/222 have been described after acute exhaustive exercise and sustained aerobic exercise training, indicating a potential of circulating miRNA as biomarkers of exercise induced cardiovascular adaptation (200).

This study is the first to examine alterations in circulating miRNA profiles after PE. Examination of plasma samples taken at the time of delivery indicate that maternal plasma is enriched for angiogenic and pro-inflammatory miRNA in patients with PE. In addition, documented differences in miRNA expression are largely resolved by one year postpartum. Lower circulating miRNA-221 postpartum however is suggestive of persistent inflammation after PE. In the search for minimally invasive markers of disease progression, much work remains to be done before the utility of circulating miRNA profiles in the prediction of PE and associated cardiovascular risk is to be realized.
2.6 Acknowledgements

The authors would like to thank Jessica Pudwell, MPH, Michelle Roddy, RN, BScN, and Heather Ramshaw, BSc, of Kingston General Hospital for their assistance in compilation of patient data. We would also like to acknowledge Dr. Mark Walker and Dr. Shi-Wu Wen of the Ottawa Hospital for their contributions to the original PE-NET cohort sample and data collection.
Chapter 3

Alterations to the maternal circulating proteome after pre-eclampsia

This chapter has been modified from its original submitted version: Murphy MSQ, Bytautiene E, Saade G, Smith GN. Alterations to the maternal circulating proteome after pre-eclampsia. *American Journal of Obstetrics and Gynecology*, 2015. MS#E15-0188.
3.1 Abstract

Objectives: The long-term maternal cardiovascular and metabolic implications associated with preeclampsia (PE) include risk of hypertension, heart disease and metabolic syndrome. The objective of this study was to investigate if a recent history of PE and/or lifetime risk of cardiovascular disease (CVD) was associated with detectable alterations in the circulating maternal proteome.

Materials and Methods: Six month postpartum plasma from PE women (n=12) and women with uncomplicated obstetrical history (n=12) were used for analysis. Depleted maternal plasma was analyzed by label-free liquid chromatography-tandem mass spectrometry assay. Identified peptides were searched against the International Protein Index human database v3.87. Exponentially modified protein abundance indices (emPAI) were used for comparison. Results were analyzed using QIAGEN's Ingenuity® Pathway Analysis.

Results: A total of 126 eligible peptides were identified for analysis; 3 peptides were differentially expressed in the PE proteome, and an additional 5 peptides were unique to control subjects and 7 to PE subjects. PE peptide profiles were more strongly associated with markers of coagulation and complement activation compared to controls and mapped more significantly to CVD functions. Stratification of subjects by low (<39%) and high (≥39%) lifetime risk of CVD rather than by diagnosis produced similar findings. Comparison of low-risk controls (n=6) to low-risk PE subjects (n=6) found that while similar for BMI, blood pressure and fasting lipid profiles at six months postpartum, PE peptide profiles continued to display stronger associations for coagulation and CVD functions. Global network analysis found that unique peptides to low-risk PE subjects were associated with cardiac infarction, CVD and organismal injury and abnormalities.
**Conclusions:** Markers of CVD and dysfunction are evident in the maternal circulating proteome at six months postpartum after PE. Augmentations in circulating peptide profiles occur in previously PE patients who otherwise do not have clinically measurable cardiovascular risk factors. Our data highlight the need for the implementation of postpartum prevention programs in the PE population and identify molecules that may be targeted for screening or therapeutic benefit.
3.2 Introduction

Pre-eclampsia (PE) is a hypertensive disorder of pregnancy that poses significant risk of adverse maternal and fetal outcomes. The pathogenesis underlying the maternal endothelial dysfunction characteristic of PE remains poorly understood, although manifestations of impaired endothelial function, oxidative stress and hypercoagulability bear striking similarity to those observed in states of cardiovascular risk and disease (CVD). Indeed, the long-term maternal cardiovascular and metabolic implications associated with PE are well established and include risk of heart disease, stroke and the metabolic syndrome (95, 109).

A lack of targeted programs providing cardiovascular risk screening based on obstetrical history poses a significant hurdle in the attempts at risk reduction in this population of women (201). Development of risk factors for CVD soon after PE, including persistent hypertension, dyslipidemia, insulin resistance as well as propensity for weight retention, allude to early changes in the biophysical profiles of these women that could be targeted before the development of CVD itself (110, 202). In addition to traditional risk factors, evidence exists for impaired flow-mediated dilation, increased artery intima media thickness and cardiac remodelling after pregnancies complicated by PE (126, 203-205).

The use of non-traditional markers of cardiovascular risk to assess states of cardiovascular health in pre-menopausal women remains important in establishing the short-term and long-term implications of PE and other hypertensive disorders of pregnancy. Recently, induction of PE-like symptoms via overexpression of soluble fms-like tyrosine kinase-1 (sFlt-1) in CD1 mice was shown to induce long-term alterations in maternal peptide profiles after pregnancy (92). Translation of knowledge gained from animal models of PE to human study is critical for the development of preventative strategies aimed at minimizing the burden of PE and CVD on healthcare systems.
Only a small handful of clinical studies have been undertaken in the past 10 years to examine the proteome associated with PE, and the majority has focused their examinations on placental tissue (206-212). Of those studies examining maternal biofluids, none have attempted analysis of the postpartum circulating proteome in women with a history of PE (161, 213-217). Given the evidence for a predisposition toward CVD after PE, we sought to determine if women who had recently developed PE would exhibit detectable alterations in their circulating proteomes at six months postpartum. Furthermore, we aimed to use data on cardiovascular risk profiles to examine whether the presence or absence of classic cardiovascular risk factors impacted differences between control and PE peptide profiles.

3.3 Materials and Methods

3.3.1 Sample collection

This study was approved by the Queen’s University Research Ethics Board. Written informed consent was obtained from all subjects. Plasma samples were collected from women attending the Maternal Health Clinic at Kingston General Hospital (KGH). The Maternal Health Clinic is one of the first clinics in North America designed to provide postpartum cardiovascular risk screening and counselling to all mothers delivering at KGH with select obstetrical complications; PE, eclampsia or HELLP Syndrome, gestational hypertension, gestational diabetes or gestational impaired glucose tolerance, intrauterine growth restricted baby, idiopathic preterm birth or placental abruption leading to delivery (153). Biophysical measurements and fasting blood work collected by the clinic are used to generate lifetime cardiovascular risk scores for each patient to help guide maternal cardiovascular risk counselling at six months postpartum. Plasma samples included for analysis were from women who had experienced pregnancies complicated by PE (n=12) or those with normotensive uncomplicated pregnancies (n=12). PE was defined as the development of de novo hypertension (≥140/90 mmHg) and proteinuria (>300 mg/24 hours or +1
on repeat dipstick). Individuals with a history of hypertension, diabetes (including the development of gestational diabetes), kidney disease, CVD, or current smoking were excluded. Control and PE groups were further stratified based on low (<39%) and high (≥39%) lifetime risk for CVD, as calculated at the Maternal Health Clinic (n=6 for each of 4 groups). Calculations for lifetime cardiovascular risk are based on biophysical factors including; sex, smoking, total cholesterol fasting glucose, systolic blood pressure, diastolic blood pressure, antihypertensive usage (136).

3.3.2 Sample preparation for mass spectrometry

Plasma was analyzed for each subject individually. Whole blood samples were collected into EDTA and centrifuged at 1000 g for 10 minutes within 2 hours of collection. Plasma was isolated and stored in aliquots at -80°C. Samples used for analysis were stored for a maximum of three years prior to analysis.

20 µl of each plasma sample was diluted with 0.7 µl of (10x dilution) protease inhibitor (Sigma-Genosys, The Woodlands, TX). Whole plasma was depleted of 14 highly abundant proteins using Agilent Human 14 Multiple Affinity Removal Columns (Agilent Technologies, Santa Clara, CA) according to manufacturer’s instructions. Flow-through fractions were concentrated and buffer exchanged to 100 mM ammonium bicarbonate by centrifugal filtration through a 5 kDa MWCO Agilent spin concentrator (Agilent Technologies, Santa Clara, CA). High molecular weight fractions for each sample were collected and a small aliquot used to perform a Bradford total protein assays (Bio-Rad, Hercules, CA).

Depleted plasma samples were denatured with 6 M urea in 150 mM Tris HCl, pH 8.0, and reduced with 20 mM DTT at 37°C for 40 minutes. Samples were then alkylated with 40 mM iodacetamide in the dark for 30 min and diluted 10-fold with 50 mM Tris-HCl pH 8.0 prior to overnight digestion at 37°C with trypsin (Promega, Madison, WI). Digestion was terminated with
equal volume 1% formic acid. Samples were desalted with Waters Oasis C18 cartridges (Waters, Milford, MA).

3.3.3 LC/MS/MS analysis
An aliquot of the tryptic digest (in 2% acetonitrile/0.1% formic acid in water) was analyzed by LC/MS/MS on an LTQ-Orbitrap-XL mass spectrometer (Thermo-Fisher Scientific, Bremen, Germany) interfaced with an Eksigent Nano-LC-Ultra-2D plus CHiPLC Nanoflex system (AB SCIEX, Framingham, MA). 0.5 µg of each sample was loaded onto a ChromXP C18-CL trap column (200 µm inner diameter, 0.5 mm length, 3 µm) at flow rate of 3 µL/min. Reversed-phase C18 chromatographic separation of peptides was carried out on a ChromXP C18-CL column (75 µm inner diameter, 15 cm length, 3 µm) at 300 nL/min, column temperature was controlled at 35°C. Gradient conditions were: 3%-8% B, 5 min; 8%-33% B, 120 min; 33%-90% B, 10 min; 90%B, 10 min; 90%-3% B, 5 min (solvent A, 0.1% formic acid in water; solvent B, 0.1% formic acid in acetonitrile). Total run time was 150 min. The LTQ Orbitrap was operated in parallel mode with measurement of the full mass scan at 100 000 resolution in the Orbitrap concurrent with the acquisition of five most intense data dependent MS/MS scans in the ion trap. For each cycle, MS1 was acquired at target value 1E6, and MS2 scanned at 3E4. The spray voltage was 1.35 KV, charge state screening and rejection of singly charged ions were enabled. Ion selection thresholds were 8 000 for MS2: 35% normalized collision energy was used, activation Q was 0.25 and dynamic exclusion was employed for 90 seconds. Each sample was analyzed in duplicate.

3.3.4 Data processing and analysis
Raw data files were processed to generate a Mascot Generic Format with Mascot Distiller and searched against the International Protein Index human database v3.87 (containing 91 464 protein sequences) using the licensed Mascot search engine v2.3.02 (Matrix Science Inc., Boston, MA) run on an in-house server. The spectra were also searched against a decoy database. For trypsin
proteolysis, up to two missed cleavages were allowed. MS tolerance was set 10 ppm; MS/MS tolerance 0.6 Da. Carbamidomethylation on cysteine residues was used as fixed modification; serine, threonine, tyrosine phosphorylation, methionine oxidation and protein N-terminal acetylation were set as variable modifications. The false discovery rate for both proteins and peptides were set at 0.01.

3.3.5 Ingenuity Pathways Analysis
QIAGEN’s Ingenuity® Pathway Analysis (IPA®, QIAGEN Redwood City, CA, www.qiagen.com/ingenuity, v21901358, Sept2014) was used to determine the long-term effect of PE according to methods described in detail by our co-investigators elsewhere (92). Analyses included Canonical Pathways analysis, Biological and Toxicity Functions analyses and examination of network associations. Peptides present in <8/12 samples per subject group (CTRL, PE, High-Risk or Low-Risk) were excluded from analysis. Remaining peptides were averaged by subject group and exponentially modified protein abundance indices (emPAI) were used for comparison. Molecules with intensity values greater than 0.1 were included in the final analysis. IPA is structured on web-based access to QIAGEN’s Ingenuity Knowledge Database, a repository of curated biological interactions and functional annotations sourced from individually modelled biological relationships. Results from the Ingenuity Knowledge Base were filtered for species (human), confidence (experimentally observed) and relationships considered (both direct and indirect). Analyses were run based first on diagnosis (Controls, n=12 versus PE, n=12) and second on lifetime risk CVD (Low-Risk, n=12 versus High-Risk, n=12). In addition, PE/Control protein ratios were calculated to determine the potential effect of exposure to PE on circulating peptides.

3.3.6 Statistical analysis
Demographic variables are presented as mean ± standard deviation (SD). GraphPad Prism 5 Software (La Jolla, CA, USA) was used for statistical comparisons. Unpaired t-test, two tailed,
was used to compare continuously distributed variables (between and subsequently within corresponding risk groups) and a $\chi^2$ comparison was used for categorical measures. Statistical significance was accepted if the null hypothesis could be rejected at $p<0.05$. IPA uses a right-tailed Fisher’s exact test to calculate $p$-values by considering the number of focus peptides participating in a given biological function or process and comparing it to the total number of peptides known to be associated with that process.

3.4 Results

3.4.1 Comparison group profiles
A complete summary of pre-pregnancy, pregnancy and postpartum characteristics of study participants are presented in Error! Reference source not found.. Low (<39% lifetime risk of CVD) and high-risk (≥39% lifetime risk of CVD) control and PE groups were similar for age, and pre-pregnancy and postpartum body mass indices (BMI). High-Risk PE women exhibited elevated systolic and diastolic blood pressures at six months postpartum compared to other subject groups in the absence of anti-hypertensive medication use. While low-risk control and PE groups were similar for biochemical markers, High-Risk Control and PE subjects exhibited variable increases in total cholesterol, triglycerides and LDL cholesterol.

IPA identified a total of 126 peptides eligible for analysis in the dataset (111 Low-Risk Control, 109 High-Risk control, 114 Low-Risk PE, 111 High-Risk PE) associated with 1 596 molecules. Two types of comparisons were run. From the first analysis based on diagnosis (Control n=12 versus PE n=12), IPA determined there to be 101 common peptides between Control and PE groups, with five peptides unique to control profiles, and seven unique to PE (Figure 3.1A). The second comparison based on lifetime cardiovascular risk status (Low-Risk, n=12 versus High-Risk n=12) identified 101 peptides common to both risk groups, and two peptides unique to Low-Risk profiles, and six peptides unique to High-Risk profiles (Figure 3.1B).
Table 3.1. Summary of pre-pregnancy and postpartum biophysical data.

<table>
<thead>
<tr>
<th></th>
<th>Low-Risk Control (n=6)</th>
<th>High-Risk Control (n=6)</th>
<th>Low-Risk PE (n=6)</th>
<th>High-Risk PE (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal age (y)</td>
<td>31.8 (3.97)</td>
<td>30.0 (4.00)</td>
<td>33.0 (5.22)</td>
<td>29.0 (5.37)</td>
</tr>
<tr>
<td>Parity, n (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nulliparous (0)</td>
<td>2 (33.3)</td>
<td>3 (50.0)</td>
<td>4 (66.7)</td>
<td>3 (50.0)</td>
</tr>
<tr>
<td>Multiparous (≥ 1)</td>
<td>4 (66.7)</td>
<td>3 (50.0)</td>
<td>2 (33.3)</td>
<td>3 (50.0)</td>
</tr>
<tr>
<td>Pre-pregnancy weight (kg)</td>
<td>70.3 (14.8)</td>
<td>61.6 (7.02)</td>
<td>62.2 (11.6)</td>
<td>83.5 (29.7)</td>
</tr>
<tr>
<td>Pre-pregnancy BMI (kg/m²)</td>
<td>25.4 (5.44)</td>
<td>23.9 (3.03)</td>
<td>25.3 (4.08)</td>
<td>30.2 (10.1)</td>
</tr>
<tr>
<td>Gestational age at delivery (wks)</td>
<td>40.1 (1.11)</td>
<td>40.2 (1.27)</td>
<td>36.6 (3.63)</td>
<td>34.2 (4.24)</td>
</tr>
<tr>
<td>6 month Postpartum Weight (kg)</td>
<td>76.1 (17.5)</td>
<td>61.8 (8.78)</td>
<td>65.6 (17.6)</td>
<td>84.8 (25.8)</td>
</tr>
<tr>
<td>6 month Postpartum BMI (kg/m²)</td>
<td>27.5 (6.66)</td>
<td>23.9 (3.54)</td>
<td>26.6 (6.62)</td>
<td>30.6 (8.53)</td>
</tr>
<tr>
<td>Pre-pregnancy to 6 month postpartum follow-up change in BMI (kg/m²)</td>
<td>2.11 (2.08)</td>
<td>0.039 (1.86)</td>
<td>1.35 (2.62)</td>
<td>0.368 (3.22)</td>
</tr>
<tr>
<td>6 month Postpartum Blood Pressure (mmHg)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Systolic Pressure</td>
<td>103.7 (8.89)</td>
<td>103.5 (12.1)</td>
<td>109.2 (7.88)</td>
<td>133.8 (17.6)</td>
</tr>
<tr>
<td>Diastolic Pressure</td>
<td>68.2 (7.05)</td>
<td>69.3 (10.8)</td>
<td>74.0 (5.76)</td>
<td>88.5 (15.2)</td>
</tr>
<tr>
<td>Glucose (mmol/L)</td>
<td>4.72 (0.20)</td>
<td>4.23 (0.38)</td>
<td>4.75 (0.45)</td>
<td>4.54 (0.42)</td>
</tr>
<tr>
<td>Total cholesterol (mmol/L)</td>
<td>4.04 (0.51)</td>
<td>5.89 (0.54)</td>
<td>4.06 (0.58)</td>
<td>5.14 (1.14)</td>
</tr>
<tr>
<td>Triglycerides (mmol/L)</td>
<td>0.61 (0.20)</td>
<td>0.65 (0.30)</td>
<td>0.93 (0.28)</td>
<td>1.41 (0.84)</td>
</tr>
<tr>
<td>HDL cholesterol (mmol/L)</td>
<td>1.42 (0.28)</td>
<td>1.83 (0.60)</td>
<td>1.37 (0.40)</td>
<td>1.22 (0.38)</td>
</tr>
<tr>
<td>LDL cholesterol (mmol/L)</td>
<td>2.34 (0.33)</td>
<td>3.76 (0.76)</td>
<td>2.26 (0.46)</td>
<td>3.27 (0.85)</td>
</tr>
<tr>
<td>Microalbumin/creatinine (mg/mmol) (median [lower,upper quartile])*</td>
<td>0.80 (0.40, 1.1)</td>
<td>0.50 (0.3, 1.9)</td>
<td>0.95 (0.65, 1.5)</td>
<td>2.30 (1.85, 15.3)</td>
</tr>
<tr>
<td>hsCRP (median [lower,upper quartile])*</td>
<td>1.00 (0.97, 4.6)</td>
<td>1.05 (0.82, 7.0)</td>
<td>0.95 (0.80, 3.2)</td>
<td>8.00 (1.45, 17.2)</td>
</tr>
</tbody>
</table>

Low-Risk, <39% lifetime risk of CVD; High-Risk, ≥39% lifetime risk of CVD; Comparisons made: Low-Risk PE vs. Low-Risk CTRL, High-Risk PE vs. High-Risk CTRL, Low-Risk CTRL vs. High-Risk CTRL, Low-Risk PE vs. High-Risk PE; NS, non-significant; *Mann-Whitney U Test; a p<0.05 versus Low-Risk group of same obstetrical outcome; b p<0.05 versus control group of same CVR status.
Ingenuity Pathways Analysis software identified 126 peptides eligible for analysis in the dataset (111 Low-Risk Control, 109 High-Risk Control, 114 Low-Risk PE, 111 High-Risk PE) associated with 1,596 molecules. Two types of comparisons were run: Control versus PE and Low-Risk versus High Risk for Cardiovascular Disease. A) 101 peptides were common between Control and PE groups, with five peptides unique to control profiles, and seven unique to PE. B) 101 peptides common to profiles of High and Low lifetime risk of cardiovascular disease. Two peptides were unique to Low-Risk profiles, and six peptides were unique to High-Risk profiles.
3.4.2 Comparison of control and pre-eclamptic profiles

Canonical Pathways: IPA identified 32 canonical pathways associated with our dataset. The top five canonical pathways for each group were Acute Phase Response Signaling, LXR/RXR Activation, FXR/RXR Activation, the Complement System and the Coagulation System. PE profiles presented with more peptides associated with coagulation system activation and Extrinsic and Intrinsic Prothrombin Activation Pathways (Figure 3.2A).

Biological Functions Analysis: Identified peptides were spread across 74 high-level functional categories. The top ten high-level functional categories associated with Control profiles were Developmental Disorder, Hereditary Disorder, Immunological Disease, Metabolic Disease, Neurological Diseases, Psychological Disorders, Cardiovascular Disease, Hematological Disease, Cancer and Gastrointestinal Diseases. For PE, these were Developmental Disorder, Hereditary Disorders, Immunological Disease, Metabolic Disease, Neurological Disease, Psychological Disorders, Cardiovascular Disease, Hematological Disease, Cell-To-Cell Signalling Interaction and Tissue Development. Control profiles were more strongly associated with Metabolic Disease, Psychological Disorders and Cancer (0). In contrast, the PE proteome was more significantly associated with Cardiovascular and Hematological Disease Functions, as well as Cell-To-Cell Signalling and Interactions than control profiles (Figure 3.2B). Lower level functional analysis found that the top 5 diseases/disorders associated with Cardiovascular Disease in the PE dataset were Thrombosis (16 molecules), Infarction (14 molecules), Vascular Disease (27 Molecules), Venous Thromboembolism (6 molecules) and Acute Coronary Syndrome (12 molecules).

Differentially Expressed Peptides: Of the 101 peptides IPA identified as common to both Control and PE profiles, only 3 peptides were differentially expressed (>1.5 fold increase/decrease) (Table 3.2).
Figure 3.2. Functional analysis of control and pre-eclamptic peptide profiles.

Comparison of A) top canonical pathways B) top 5 high-level biological functional categories associated with peptide profiles of Control (CTRL) and pre-eclamptic (PE) groups. The PE proteome mapped more significantly to coagulation system pathways than control proteomes, particularly intrinsic and extrinsic prothrombin activation pathways. Cardiovascular and hematological disease were more significantly implicated in the PE proteome compared to control profiles, as was predicted affected biological functions of cell-to-cell signalling and interaction. *Threshold for significant association, p<0.05.
Table 3.2. Differentially expressed peptides in the pre-eclamptic proteome.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Fold Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>CLEC3B; C-type lectin domain family 3 member B (tetranectin)</td>
<td>-1.712</td>
</tr>
<tr>
<td>HBB; Beta hemoglobin</td>
<td>-1.540</td>
</tr>
<tr>
<td>IGHM; Immunoglobulin heavy constant mu</td>
<td>1.966</td>
</tr>
</tbody>
</table>
Unique Peptides Analysis: IPA was used to identify and map the biological functions associated with unique peptides for each comparison group. Of the 101 peptides that were common to both Control and PE profiles; 5 peptides (ALB, APOC2, CA1, IGFBP3, SERPINA10) were unique to Control subjects while 7 peptides (ACTA1, CASP12, CFHR1, CFL1, F10, HPR, KLKB1) were unique to a history of PE (Table 3.3). 5 of the 7 unique molecules to PE subjects (ACTA1, CASP12, CFL1, F10, HPR) that were common to both Low-Risk and High-Risk PE profiles.

Biological Functions Analysis of Unique Peptides: Functional analysis of unique peptides demonstrated that Cardiovascular Disease scored within the top 5 high-level biological functions associated with PE profiles. The top 5 high-level biological functions to PE profiles were Organismal Injury and Abnormalities, Gene Expression, Cardiovascular Disease, Cell-to-Cell Signalling and Interaction, and Developmental Disorders versus Cellular Development, Skeletal and Muscular System Development and Function, Tissue Development, Inflammatory Disease and Organismal Injury and Abnormalities for controls (Figure 3.3A, Figure 3.3B). Unique peptides were mapped to associated biological networks in the IPA knowledge base. Of interest, F10, a PE specific molecule was mapped to networks (1 of 10 molecules in network) involving cardiovascular system development, function and disease.

Toxicity Functions Analysis of Unique Peptides: Examination of lower-level functions clustered under Cardiovascular Disease for PE found that the top 5 diseases/disorders associated with Cardiovascular Disease signalling involved peptides associated with Mass of Blood Clot, Non-Valvular Atrial Fibrillation, Superficial Venous Thrombosis and Thrombosis. These functions were attributable to the molecules F10 and CFHR1. Unique PE peptides identified strongly with abnormalities in cardiac function and signaling due to the presence of F10 in the PE proteome whereas peptides unique to Control subjects were more consistent with liver and renal dysfunction (Figure 3.3C).
Table 3.3. Unique peptides linked to control and pre-eclamptic profiles.

<table>
<thead>
<tr>
<th>Control</th>
<th>Pre-Eclampsia</th>
</tr>
</thead>
<tbody>
<tr>
<td>Albumin; ALB</td>
<td>α-1 Actin; ACTA1</td>
</tr>
<tr>
<td>Apolipoprotein C-II; APOC2</td>
<td>Caspase 12; CASP12</td>
</tr>
<tr>
<td>Carbonic Anhydrase 1; CA1</td>
<td>Complement Factor H-Related 1; CFHR1</td>
</tr>
<tr>
<td>Insulin-like Growth Factor Binding Protein 3; IGFBP3</td>
<td>Co-filin 1 (non-muscle); CFL1</td>
</tr>
<tr>
<td>Serpin Peptidase inhibitor, clade A, member 10; SERPINA10</td>
<td>Coagulation Factor X; F10</td>
</tr>
<tr>
<td></td>
<td>Haptoglobin related protein; HPR</td>
</tr>
<tr>
<td></td>
<td>Kallikrein B; KLKB1</td>
</tr>
</tbody>
</table>
Figure 3.3. Functional comparison of unique peptides associated with control and pre-eclamptic profiles.

Top 5 high-level biological functions associated with unique A) Control (CTRL) and B) Pre-eclamptic (PE) proteomes at six months postpartum. Included among the top 5 predicted affected biological functions associated with PE profiles was cardiovascular disease. C) Toxicity functions analysis of unique peptides identified for Control and PE proteomes. Unique peptides to the PE proteome were significantly mapped to toxicity functions of cardiac abnormalities. Control peptides mapped heterogeneously to functions of cardiac, renal and liver dysfunction. Threshold for significant association, $p<0.05$. 
3.4.3 Comparison of low-risk and high-risk profiles.

A secondary analysis was performed to examine the impact of lifetime cardiovascular risk profiles on the maternal circulating proteome, regardless of obstetrical history. Lifetime risk of CVD was determined based on biophysical reports generated from the Maternal Health Clinic at KGH as described earlier. Risk was stratified by Low-Risk (<39% risk) and High-Risk (≥39% Risk).

**Canonical Pathways and Biological Functions Analysis:** Results from functional analysis of Low-Risk and High-Risk profiles proved similar to diagnosis-based comparisons. High-Risk profiles were more significantly associated with cardiovascular and hematological disease functions, the coagulation system, and extrinsic and intrinsic prothrombin activation pathways.

**Unique Peptides Analysis:** IPA identified 101 peptides common to both risk groups. Two peptides (CD14, SERPINA10) were unique to Low-Risk groups and six peptides were unique to High-Risk groups (C4BPB, CFHR1, CFL1, F10, KLKB1, SAA4) (Table 3.4).

**Biological Functions Analysis of Unique Peptides:** The top 5 affected high-level biological functions for Low-Risk profiles included carbohydrate metabolism, organismal injury and abnormalities, small molecule biochemistry, cell-to-cell signalling and interaction and cellular compromise. For High-Risk profiles these were hematological system development and function, organismal injury and abnormalities, gene expression, organismal functions, cardiovascular disease (Figure 3.4A, Figure 3.4B).

**Toxicity Functions Analysis of Unique Peptides:** Peptides unique to Low-Risk profiles were minimally associated with cardiac arteriopathy and cardiac infarction, and mapped more significantly to processes involved in liver hyperplasia. High-risk profiles were significantly associated with cardiac dysfunction; cardiac arrhythmia, cardiac pulmonary embolism, cardiac
Table 3.4. Unique peptides linked to low-risk and high-risk profiles.

<table>
<thead>
<tr>
<th>Low-Risk</th>
<th>High-Risk</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD14 molecule; CD14</td>
<td>Complement Component 4 Binding Protein; C4BPB</td>
</tr>
<tr>
<td>Serpin Peptidase inhibitor, clade A, member 10; SERPINA10</td>
<td>Complement Factor H-Related 1; CFHR1</td>
</tr>
<tr>
<td></td>
<td>Co-filin 1 (non-muscle); CFL1</td>
</tr>
<tr>
<td></td>
<td>Coagulation Factor X; F10</td>
</tr>
<tr>
<td></td>
<td>Kallikrein B; KLKB1</td>
</tr>
<tr>
<td></td>
<td>Serum Amyloid A4; SAA4</td>
</tr>
</tbody>
</table>
Figure 3.4. Functional comparison of unique peptides associated with profiles for low- and high-risk for cardiovascular disease.

Top 5 high-level biological functions associated with unique proteins for A) Low-Risk and B) High-Risk profiles based on lifetime cardiovascular risk estimates at 6 months postpartum. Included among the top predicted affected biological functions associated with the High-Risk proteome were Hematological System Development and Function, Organismal Injury and Abnormalities and Cardiovascular Disease. C) Toxicity functions analysis of unique peptides identified for subjects at low-risk and high lifetime-risk for CVD based on cardiovascular risk estimates. The High-Risk proteome was significantly mapped to toxicity functions associated with cardiac abnormalities. *Threshold for significant association, $p<0.05$. 

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stenosis, cardiac infarction as well as kidney failure (Figure 3.4C). All cardiotoxicity functions mapped to High-Risk profiles were attributable to the F10 peptide.

3.4.4 Comparison of low-risk subject groups
Peptide profiles of Low-Risk Control and Low-Risk PE groups were compared to determine if a history of PE had an effect on maternal circulating profiles in individuals who did not otherwise have clinically measurable cardiovascular risk factors.

Canonical Pathways and Biological Functions Analysis: Functional analysis comparison between these groups identified differences consistent with those observed when PE and control groups were compared as a whole. The Low-Risk PE proteome was more strongly associated with canonical pathways involving the coagulation system and prothrombin activation pathways. In addition, peptide profiles of Low-Risk PE subjects were more strongly mapped to biological functions of cardiovascular and hematological disease than those of Low-Risk Controls.

Unique Peptides Analysis: IPA identified 103 peptides that were common to both Low-Risk Control and PE profiles – 8 peptides were unique to Low-Risk Controls (APOC2, ATRN, CA1, CD44, CD5L, CFHR2, ORM1, SELL) and 11 were unique to Low-Risk PE (ACTA1, CASP12, CFHR1, CFL1, F10, HPR, KLKB1, PHN1, TAGLN2, TLN1, YWHAZ). The top ranked network identified for peptides unique to Low-Risk PE involved 5 focus molecules (ACTA1, CFL1, F10, TLN1, YWHAZ) associated with Cardiac Infarction, Cardiovascular Disease, and Organismal Injury and Abnormalities (Figure 3.5).

3.5 Discussion
Exaggerated maternal responses to pregnancy – including excessive inflammation and a hypercoagulable state (218) – are characteristic of PE and bear striking similarity to features of
Figure 3.5. Comparison of control and pre-eclamptic profiles without evidence of cardiovascular risk factors.

103 proteins were common to both Control (CTRL) and Pre-eclamptic (PE) profiles associated with low lifetime risk of cardiovascular disease. 8 peptides were unique to Low-Risk Control profiles, while 11 peptides were unique to Low-Risk PE profiles. The top network linked to PE profiles in individuals at low lifetime risk for cardiovascular disease was associated with Cardiac Infarction, Cardiovascular Disease and Organismal Injury and Abnormalities. 5/11 unique peptides to PE profiles were involved in this network (ACTA1, CFL1, F10, TLN1, YWHAZ).
CVD. Epidemiological data supporting the association between the development of PE in pregnancy and maternal risk of CVD (95) are substantiated by evidence of vascular dysfunction (219), cardiac remodeling (204) and renal dysfunction (220) after affected pregnancies. How early these physiological changes might be identified and effectively targeted after pregnancy remains a significant hurdle in the development and implementation of postpartum cardiovascular screening programs.

We present evidence of alterations to the maternal circulating proteome after pregnancies complicated by PE. Analysis of peptide profiles from women who had developed PE in their most recent pregnancy identified stronger associations with coagulation cascade signaling compared to those women who had experienced uncomplicated pregnancies. Top networks affected by PE profiles included those associated with cardiovascular and hematological diseases, and proteins implicated in processes involving cardiac dysfunction were identified. The influence of PE on peptide profiles proved similar to the influence of high lifetime risk of CVD, and comparison of circulating proteomes from normotensive and PE individuals with no apparent cardiovascular risk factors still identified stronger influences of peptides associated with CVD signalling and coagulation at six months postpartum after PE compared to controls.

Our co-investigators have previously used mouse models to examine the implications of PE both during and after pregnancy (92, 124, 221). Their recent examination of the maternal mouse proteome six months postpartum of sFlt-1 overexpression in murine pregnancy identified a number of alterations in haemostatic molecules (92), corroborating the clinical data presented here. In the current study we have demonstrated a significant enrichment of proteins associated with coagulation and complement cascades in the proteome of formerly PE women. Both coagulation and immune systems are intimately connected, and given that activation of both cascades is highly relevant to both PE and CVD, a persistence of endothelial dysfunction with
ensuing dyslipidemia, hypercoagulability and inflammation likely contributes to CVD susceptibility following maternal hypertensive disorders of pregnancy.

Coagulation Factor X (F10) is the first member of the final common coagulation pathway, and directly promotes thrombus formation through cleavage of prothrombin. Predicted abnormalities in cardiovascular function associated with PE profiles in this study were directly linked the presence of F10 in the postpartum PE proteome. We have previously shown Factor X to be upregulated (x9.4) six months postpartum of sFlt-1-induced PE in mice (92), and clinical studies have corroborated the upregulation of Factor X during pregnancies affected by the maternal hypertensive syndrome (160, 222).

We also demonstrate that expression of tetranectin (CLEC3B) is decreased in the plasma of recently PE women. Tetranectin is an adhesion molecule of the C-type lectin superfamily that binds and activates plasminogen to enhance plasmin formation. Its role in mediating fibrinolytic activity is evident from studies showing lower plasma tetranectin with advancing severity of coronary artery disease (223). Decreased levels of tetranectin are found in patients with acute myocardial infarction and are improved following thrombolytic treatment (224).

In addition to persistent haemostatic perturbations (225), increases in markers of inflammation (226, 227) are evident years to decades after affected pregnancies, and as early as six months postpartum we still find evidence of alterations in proteins implicated in inflammatory processes. Plasma kallikrein (KLKB1), a glycoprotein participating in surface-dependent activation of blood coagulation, kinin generation and inflammation (228), was associated with PE profiles. Bradykinin, a product of high molecular weight kininogen cleavage by plasma kallikrein, increases vascular permeability and is a potent vasodilator. Although endothelial dysfunction of PE is typically associated with impaired vasodilation of large conduit vessels, recent studies indicate increased endothelial-dependent vasodilation in the microvasculature of women both during and soon after PE (129, 131, 229), with mechanisms unknown. The multifaceted role of
the kallikrein-kinin system in mediating both coagulation and inflammation may therefore provide a promising avenue for future research in this area. Lastly, although complement proteins have been well described in the PE proteome (160), in our study only complement factor H related protein, CFHR1, was identified, and any role it may have in potentiating cardiovascular risk in this population is unclear.

Whether PE exacerbates pre-existing, subclinical cardiovascular risk factors, or is a direct trigger for mechanisms potentiating CVD processes remains unclear. In a recent systematic review of 12 mass spectrometry-based proteomic studies comparing pre-eclamptic and noromotensive placental and maternal serum samples, 53 differentially regulated proteins found to be associated with the development of PE. Differentially regulated proteins clustered within pathways involving hemostasis and immune response (230), a finding that we confirm to persist in the postpartum. As an exploratory study we were unable to link our findings to outcomes of metabolic syndrome or CVD. As such, the predictive utility of the PE-associated proteins identified here remains uncertain. Rather, we provide an assessment of the maternal circulating proteome after PE as a means of highlighting the impact of PE on postpartum maternal systems and providing discourse as to how this population may be effectively targeted for cardiovascular risk screening. Although differential expression of peptides in the postpartum maternal proteome was not confirmed in the present study, future work should include exploration of potential biomarkers that could identify women at particularly high risk of developing CVD soon after PE.

In summary, changes in the protein expression of members of the coagulation cascade favouring thrombophilia and inflammation may well provide a link between PE and future development of CVD. Identification of peptides associated with the development of PE remains critical in developing tools not only for disease prediction in pregnancy, but also in determining those that might persist long after PE to affect long term maternal health. In consideration of our findings that alterations to the PE proteome are suggestive of cardiovascular and thrombotic risk in both
symptomatic and asymptomatic individuals as early as six months after PE, we highlight the need for targeted cardiovascular risk counselling in the early postpartum period.

3.6 Acknowledgements

The authors would like to acknowledge Michelle Roddy, RN, BScN, and Jessica Pudwell, MPH of Kingston General Hospital for their work with the Maternal Health Clinic and generation of lifetime cardiovascular risk estimates. In addition, the authors would like to thank Li Li, MSc, Manager of Clinical and Translational Proteomics Service Center, Center for Proteomics and Systems Biology, Medical School, University of Texas Health Science Centre, for her assistance in mass spectrometry experiments.
Chapter 4

Postpartum alterations in circulating endothelial progenitor cells in women with a history of pre-eclampsia.

This chapter has been modified from its original published version: Murphy MSQ, Casselman RC, Smith GN. Postpartum alterations to circulating endothelial progenitor cells in women with a history of pre-eclampsia. *Pregnancy Hypertension: An International Journal of Women’s Cardiovascular Health*, 2013. 3(3): 178-185.
4.1 Abstract

Objective: To characterize persistent postpartum maternal endothelial dysfunction following pre-eclampsia (PE) through the assessment of endothelial progenitor cells as markers of endothelial reparative capacity.

Study Design: Maternal circulating endothelial progenitor cells were measured at two months and six months postpartum in women who had recently experienced pre-eclamptic pregnancies (n=17). Normotensive controls (n=13) with uncomplicated pregnancies served for comparison at the same time points. Progenitor cells were measured by flow cytometry and by colony forming units. Maternal lifetime cardiovascular risk was measured at 6 months postpartum.

Results: CD34+VEGFR-2+ and CD133+VEGFR-2+ cells were elevated in pre-eclamptic subjects at two months postpartum compared to healthy control subjects, although were reduced by six months postpartum compared to controls. Pre-eclampsia was associated with reduced colony forming units at two and six months postpartum. Cardiovascular risk scores were increased after pre-eclampsia compared to normotensive controls although biochemical measures of cardiovascular risk factors were not elevated.

Conclusions: We have demonstrated that there is a physiological alteration in the number and function of circulating progenitor cells following pre-eclamptic pregnancies. Furthermore, this population of women exhibited elevated cardiovascular risk profiles compared to those with uncomplicated pregnancies. Pregnancy and the development of pre-eclampsia identify an early window of opportunity for cardiovascular risk screening in women. Cellular markers of vascular health offer an approach to the investigation of postpartum endothelial dysfunction.
4.2 Introduction

Pregnancy is now recognized as a cardiovascular stress test (74) that has the potential to provide information on a woman’s susceptibility toward metabolic and vascular dysfunction (231). Indeed, the development of pregnancy-related complications – including preterm birth, intrauterine growth restriction, gestational diabetes mellitus, and pre-eclampsia (PE) – identify women at future risk of disease (74, 78). The American Heart Association’s 2011 Update for the Effectiveness-Based Guidelines for the Prevention of Cardiovascular Disease in Women (15) identifies complications of pregnancy as relevant in the determination of cardiovascular risk.

Endothelial dysfunction is thought to underlie the maternal hypertension and proteinuria characteristic of PE. While maternal signs and symptoms largely dissipate following delivery of the feto-placental unit, impaired vascular function is known to persist from months to decades after delivery in women with both early- and late-onset PE (129, 132).

Maintenance of endothelial homeostasis was until recently attributed to the migration and proliferation of locally activated endothelial cells. However, evidence has since emerged for the existence of bone marrow-derived endothelial progenitor cells (EPCs), capable of being incorporated into sites of active angiogenesis to promote re-endothelialization and neovascularization (162, 232). EPCs are released into the circulation in response to a host of chemotactic and cytokine factors arising from vascular trauma and hypoxic stress (233, 234) and are now largely believed to be critical in the maintenance of vascular integrity and function (235, 236).

As a measure of regenerative and angiogenic capacity, EPCs may represent a physiological link underlying the association between the endothelial dysfunction of PE and future cardiovascular disease (CVD). PE and CVD share common risk factors including hyperlipidemia, endothelial dysfunction and lipid deposition in blood vessel walls (237); endothelial dysfunction and long-term risk for CVD following PE pregnancies are well described (96, 113, 238). There exists little
postpartum EPC data in healthy or clinically at-risk populations however, and it remains unclear how pregnancy may affect postpartum maternal EPC physiology. We hypothesized that differences in postpartum EPC physiology would serve as a marker of endothelial dysfunction and provide insight into the postpartum recovery following PE. The objective of this study was to assess circulating EPC number and function in women who developed PE, compared with those who did not.

4.3 Materials and Methods

4.3.1 Study population
This study was approved by the Queen’s University Research Ethics Board (OBGY-108-03). Written informed consent was obtained from all participants. Women aged 18-40 years, presenting with a singleton pregnancy were approached to participate. Normotensive women were recruited at the time of presentation to general obstetrical clinics for routine third trimester check-ups. Pre-eclamptic women were identified following delivery at Kingston General Hospital. PE was defined as maternal hypertension $\geq 140/90$ mmHg and proteinuria $\geq 300$ mg/24 hours or $\geq 1+$ on repeat dipstick after 20 weeks gestation. Patients with a history of hypertension, diabetes (including the development of gestational diabetes), renal disease, or CVD were excluded. Controls with previous pregnancy complications, including PE, were also excluded. Six month postpartum follow-up involved a physical examination (blood pressure, waist circumference, BMI calculation) and biochemical assessment for cardiovascular risk factors in the Maternal Health Clinic at the Kingston General Hospital. Blood was obtained for measurement of fasting glucose, high-sensitivity C-reactive protein (hsCRP), triglycerides, total cholesterol, low-density and high-density lipoprotein cholesterol (LDL and HDL, respectively), and first morning urine for microalbumin and creatinine. Data collected at six-month follow-up were used to calculate the lifetime risk estimates for CVD (145) and existence of metabolic
syndrome in both normotensive and PE groups. Variables included in cardiovascular risk scoring calculations were age, total and HDL cholesterol levels, current smoking status, and blood pressures.

4.3.2 Blood collection
Maternal peripheral venous blood was drawn via venipuncture into ethylenediaminetetraacetic acid (EDTA) into evacuated blood collection tubes (BD Vacutainer®, EDTAK2). All blood samples were processed within 2 hours. Plasma was stored at -80°C until further analysis.

4.3.3 Flow cytometry
Vascular endothelial growth factor receptor-2 (VEGFR-2) positive cells co-expressing either CD34 or CD133 were quantified as previously described (163, 239). Briefly, erythrocytes were eliminated from whole blood using an erythrocyte lysing solution [155 mM NH₄Cl, 10 mM KHCO₃, 0.1mM EDTA, pH 7.2-7.3] and leukocytes were maintained in flow cytometry buffer [2% fetal bovine serum (FBS), 2mM EDTA in phosphate buffered saline]. Non-specific binding to the Fc receptor was prevented through incubation with Fc Receptor Blocker (Miltenyi Biotech Inc., CA, USA). Mouse anti-human monoclonal antibodies (mAbs) for flow cytometry included anti-CD45-FITC (0.625 µg/mL), anti-CD34-PerCPefluor710 (1.25 µg/mL) and anti-CD133-APC (1.25 µg/mL) all from eBioscience (San Diego, CA, USA) and anti-VEGFR-2-PE (1 µg/mL) from Miltenyi Biotech (Auburn, CA, USA). Isotype-matched mAbs diluted to equivalent immunoglobulin concentrations served as negative controls. Antibody incubation was for 15 minutes at 4°C in darkness. Cells were washed and fixed in 2% paraformaldehyde (PFA). Samples were stored in darkness at 4°C until analysis.

Cells were analyzed on a Beckman Coulter FC500 flow cytometer using CXP Software (Beckman Coulter, Mississauga, Ontario, Canada). For each sample 10⁶ events were collected. CD34+VEGFR-2+ and CD133+VEGFR-2+ cells were determined in the mononuclear gate, where EPCs are normally found, based on CD45 antigen positivity and Forward Scatter/Side
Scatter profiles. Post-acquisition analyses were performed using FlowJo software (Tree Star, Inc., Ashland, Oregon, USA). Maternal EPCs were enumerated as a percentage of total mononuclear cells.

### 4.3.4 Colony forming unit-EPC assay

Blood samples for Colony forming unit-EPC (CFU-EPC) assays were provided at 2 months (Ctrl n=5, PE n=7) and 6 months (Ctrl n=13, PE n=17) concurrent to those taken for flow cytometry assessment. Mononuclear cells were cultured by adherence depletion, as previously described (235). Briefly, isolated peripheral blood mononuclear cells were cultured in Medium 199 (M3769, Sigma-Aldrich Canada Ltd., Oakville, ON, Canada) supplemented with 20% FBS (v/v). Cells were seeded at 5x10^6 cells/well of a Fibronectin 6-well Multiwell Plate (BD BioCoat™ Cellware) and incubated at 5% CO₂, 37°C, for 48 hours. Non-adherent cells were collected and re-seeded at 1x10^6 cells/well of a Fibronectin-coated 4-well Culture Slide or 24-well Multiwell Plate (both BD BioCoat™ Cellware) and cultured as before for 72 hours. CFU-EPCs were quantified based on distinct morphological characteristics; true colonies appeared with a central core of rounded cells, with elongated spindle-like cells radiating from the central colony surface (0). CFU-EPCs were scored manually by two independent investigators, one of whom was blinded to clinical conditions.

### 4.3.5 Immunocytochemistry

Cytoplasmic accumulation of acetylated low-density lipoprotein (acLDL) and lectin staining were assessed to confirm endothelial characteristics in cultured colonies. On the final day of culture, cells were incubated for 4 hours with 10ug/mL 1,1’-dioctadecyl-3,3,3,3’-ß-tetramethylindocarbocyanine perchlorate-labeled acLDL (Invitrogen, Burlington, ON, Canada). Cells were washed and fixed in 2% PFA. Cells were counterstained with 10 μg/mL Lectin-GSII-Alexa488 griffonia simplicifolia (GS-Lectin) (Invitrogen, Burlington, ON, Canada) for 1 hour.
Human umbilical vein endothelial cells (HUVEC) seeded at passages 6-8 served as positive controls for immunoreactivity.

4.3.6 Statistical analysis
Continuous demographic variables are presented as mean±standard deviation (SD). An unpaired t-test or one-way analysis of variance (ANOVA) with Bonferroni post hoc test was used to compare continuously distributed factors and a $\chi^2$ comparison was used for categorical measures. The Wilcoxon matched pairs signed-rank test was used to compare cellular data across experimental time-points in each patient group. The Mann-Whitney test was used to compare cellular data between experimental time-points of PE and uncomplicated pregnancies. Statistical significance was accepted if the null hypothesis could be rejected at $p<0.05$. Statistical analyses for longitudinal data are not presented. The statistical software package of GraphPad Prism v5.0 was used for all analyses.

4.4 Results

4.4.1 Baseline characteristics
In total, thirteen (n=13) normotensive and eighteen (n=18) PE women were recruited into the study. One PE woman was lost to follow-up. Thirteen normotensive women and seven PE women participated in this study over two early postpartum time-points: Two months postpartum, and six months postpartum. An additional ten women were identified upon presentation to a Maternal Health Clinic for 6 month postpartum sampling.

Baseline characteristics are summarized in Table 4.1. There were no statistical differences in baseline characteristics amongst controls or PE subjects when stratified by time-point of participation (Appendix E). When compared to a larger established cohort (Pre-Eclampsia-
Table 4.1. Comparison of maternal baseline characteristics.

<table>
<thead>
<tr>
<th></th>
<th>Normotensive Control (n=13)</th>
<th>Pre-eclampsia (n=17)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal age (y)</td>
<td>31.8±3.7</td>
<td>31.5±5.4</td>
<td>NS</td>
</tr>
<tr>
<td>Parity, n (%)&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Primiparous</td>
<td>3 (23.1)</td>
<td>15±88.2</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Multiparous</td>
<td>10 (76.9)</td>
<td>2±11.8</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Maternal BMI (kg/m&lt;sup&gt;2&lt;/sup&gt;)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre-pregnancy</td>
<td>25.8±4.9</td>
<td>24.7±3.6</td>
<td>NS</td>
</tr>
<tr>
<td>Delivery</td>
<td>29.8±4.3</td>
<td>31.4±4.3</td>
<td>NS</td>
</tr>
<tr>
<td>BP (mmHg)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Systolic</td>
<td>119.8±13.1</td>
<td>164.0±21.7</td>
<td>&lt;0.0001*</td>
</tr>
<tr>
<td>Diastolic</td>
<td>69.8±6.3</td>
<td>104.4±11.8</td>
<td>&lt;0.0001*</td>
</tr>
<tr>
<td>GA at delivery (wks)</td>
<td>39.9±1.2</td>
<td>35.9±3.6</td>
<td>&lt;0.001*</td>
</tr>
</tbody>
</table>

Abbreviations: BMI, body mass index; BP, blood pressure; GA, gestational age; NS, not significant. *Fisher’s exact test. *p<0.05, significant.
New Emerging Team [PE-NET] (107), participants proved similar for baseline characteristics (Appendix F).

4.4.2 Flow cytometry
Analysis of women completing both postpartum assessments demonstrated that circulating CD34+VEGFR-2+ and CD133+VEGFR-2+ cells were increased in the PE group at two months postpartum compared to time-matched controls (p<0.005 and p<0.0005, respectively). CD34+VEGFR-2+ and CD133+VEGFR-2+ cells did not differ between study groups at six months postpartum (p>0.05 for both). Paired analysis of controls showed that CD34+VEGFR-2+ and CD133+VEGFR-2+ levels were elevated at six months postpartum compared to two months postpartum (p<0.05 and p<0.0005 respectively). Conversely, PE subjects demonstrated peak levels of both cellular subsets at approximately two months postpartum, with differences reaching statistical significance amongst CD34+VEGFR-2+ EPCs (p<0.05). These results are summarized in Figure 4.1. The addition of data obtained from subjects who gave blood samples at six months postpartum only for un-paired analysis demonstrated similar results, as shown in Figure 4.2, with differences in CD133+VEGFR-2+ EPCs between normotensive and PE patients also reaching significance (p<0.05).

4.4.3 CFU-EPC assay and immunocytochemistry
Cultured HUVEC were positive for DiI-ac-LDL uptake and GS-lectin surface staining (Appendix G). Dual DiI-Ac-LDL and GS-lectin immunoreactivity was confirmed in CFU-EPC obtained from controls (n=4) and PE subjects (n=3) (Figure 4.3). As shown in Figure 4.4, CFU-EPC from PE subjects were reduced at both two months and six months postpartum (both p<0.05) compared to time-matched controls.
Figure 4.1. Paired assessment of maternal EPCs by phenotype.

Scatter plots of (A), (C) CD34+VEGFR-2+ and (B), (D) CD133+VEGFR-2+ cells progenitor cell distributions from women providing blood samples at both postpartum time-points (Ctrl n=13; PE n=7). EPC were increased at 6 months postpartum in normotensive women compared to 2 months postpartum. CD34+VEGFR-2+ cells were reduced at 6 months postpartum in PE subjects. Both cellular subsets were elevated in PE patients compared to controls at two months postpartum. CD34+VEGFR-2+ and CD133+VEGFR-2+ cells at six months postpartum follow-up were not statistically different between the two patient groups. **p<0.005, ***p<0.001 compared to comparison subjects. †p<0.05, †††p<0.001 versus six months postpartum of same patient group. Abbreviations: 2M PP, 2 months postpartum; 6M PP, 6 months postpartum.
Figure 4.2. Cross-sectional assessment of maternal EPCs at 6 months postpartum.

Scatter plots of (A) CD34+VEGFR-2+ and (B) CD133+VEGFR-2+ cells progenitor cell distributions at 6 months postpartum (Ctrl n=13; PE n=17). CD34+VEGFR-2+ EPCs did not differ amongst the two patient groups. CD133+VEGFR-2+ cells at six months postpartum were reduced in formerly PE women, *$p<0.05$. Δ, PE subjects participating at 6-month postpartum only.
Figure 4.3. CFU-EPC characterization.

(A) Colonies appeared with a central core of rounded cells, with elongated spindle-like cells radiating from the periphery, perpendicular to the central colony surface. CFU-EPC displayed positive (B) uptake of DiI-ac-LDL (red) and (C) binding of AlexaFluor 488-conjugated *griffonia simplicifolia*-lectin (green). (D) Images are combined to demonstrate dual positivity and distribution of staining.
Figure 4.4. Number of CFU-EPC following seven day culture.

Results are reported as the number of colonies formed per $10^6$ non-adherent cells re-plated after day 2 of culture. Women with a recent history of PE yielded statistically fewer CFU than normotensive controls at 2-months (Ctrl=5, PE=7) and 6-months postpartum (Ctrl=13, PE=17). Error bars denote standard error mean. *$p<0.05$ versus normotensive controls. Error bars denote standard error mean. Abbreviations: ☐, normotensive controls; ■, pre-eclamptic subjects.
4.4.4 Cardiovascular risk at six-month follow-up

Comparison of traditional cardiovascular risk factors in control (n=8) and PE subjects (n=13) who completed cardiovascular risk screening at six months postpartum identified increased lifetime cardiovascular risk scores amongst individuals with a history of PE (>39% lifetime risk). 53.8% of PE subjects demonstrated elevated cumulative cardiovascular lifetime risk versus 0% of controls (p<0.05). Cardiovascular risk variables are summarized in Table 4.2.

4.5 Discussion

Our study demonstrates that PE is associated with elevated levels of circulating CD34+VEGFR-2+ and CD133+VEGFR-2+ EPCs at two months postpartum. Levels of both cell types were subsequently reduced six months postpartum compared to controls. In contrast to PE women, alterations of circulating EPCs in normotensive women saw a progressive rise in number of these cells from two months to six months postpartum follow-up. These cellular alterations were associated with reduced CFU-EPC in the postpartum amongst PE women compared to controls.

Endothelial dysfunction, unmasked or exaggerated by the normal adaptations of pregnancy, plays an integral role in the development of PE. The metabolic and vascular abnormalities that manifest in PE closely resemble those seen in CVD (240), and the long-term cardiovascular implications of PE are well recognized. Large population-based studies now relate PE to increased risk of hypertension (241, 242), ischemic heart disease (96, 100, 113) and stroke (96, 113, 242), ranging from 8 to 32 years post-delivery. Our group has demonstrated that a large number of mothers who develop PE, by one year postpartum, exhibit metabolic and biochemical cardiovascular risk factors (107, 109). Despite this, short-term follow-up of this susceptible population has suggested that many women with a history of PE would not be considered for primary prevention based on conventional risk scoring for CVD (108, 243).
Table 4.2. Cardiovascular risk variables at six months postpartum.

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Normotensive Control (n=8)</th>
<th>Pre-eclampsia (n=13)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Family history, n (%)(^a)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cardiovascular disease</td>
<td>6 (75)</td>
<td>10 (76.9)</td>
<td></td>
</tr>
<tr>
<td>Hypertension</td>
<td>7 (87.5)</td>
<td>10 (76.9)</td>
<td>NS</td>
</tr>
<tr>
<td>Hypertension during pregnancy</td>
<td>0 (0)</td>
<td>1 (7.7)</td>
<td></td>
</tr>
<tr>
<td>Maternal BMI (kg/m(^2))</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre-Pregnancy</td>
<td>25.2±4.9</td>
<td>24.2±3.1</td>
<td>NS</td>
</tr>
<tr>
<td>6 months postpartum</td>
<td>25.0±4.4</td>
<td>26.6±5.6</td>
<td>NS</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>105.9±8.2</td>
<td>117.8±10.7</td>
<td>&lt;0.05(^*)</td>
</tr>
<tr>
<td>Diastolic Blood Pressure (mmHg)</td>
<td>70.5±6.4</td>
<td>79.7±8.1</td>
<td>&lt;0.05(^*)</td>
</tr>
<tr>
<td>Lifetime Risk Scores, n (%)(^a)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;39% Risk</td>
<td>8 (100)</td>
<td>6 (46.2)</td>
<td>&lt;0.05(^*)</td>
</tr>
<tr>
<td>≥39% Risk</td>
<td>0 (0)</td>
<td>7 (53.8)</td>
<td>&lt;0.05(^*)</td>
</tr>
</tbody>
</table>

Lifetime cardiovascular risk scores for each patient were generated based on biophysical factors including; sex, smoking, total cholesterol fasting glucose, systolic blood pressure, diastolic blood pressure, antihypertensive usage - categorical data for lifetime cardiovascular risk are divided into <39%, low risk, and ≥39%, high risk. Biochemical cardiovascular risk factors are included in 0. \(^*\)Fisher’s exact test; NS, not significant; \(^a\) Statistically significant, \(p<0.05\).
Endothelial Progenitor cells have emerged as reliable cellular markers of vascular reparative capacity and predictors of adverse cardiovascular events. Although no specific EPC surface antigen has been identified to date, combinations of CD34, CD133 and VEGFR-2 are routinely employed (232, 239, 244, 245). While pregnancy poses a considerable physiological challenge to the vascular system, the literature on EPCs in pregnancy and related complications remains sparse.

Pregnancy-based evidence suggests that numbers of circulating EPCs increase with advancing trimester in healthy pregnancy (246, 247) whereas numbers are reduced in pre-eclamptic women at the time of delivery (163-165, 248). Investigations into maternal EPC subsets in PE populations have thus far been small with sample sizes ranging from 8 to 12 in only 4 studies (163-165, 248) all of which have been cross-sectional in nature. A lack of uniform methods for identification and isolation of EPCs hinder appropriate comparison of findings, and are further confounded by contradictory results.

Matsubara and colleagues, 2006 (164), using flow cytometry found no difference in a rarer subset of triple positive CD34+CD133+VEGFR-2+ EPCs amongst PE and normotensive subjects before delivery. In contrast, Luppi et al., 2010 (163) demonstrated decreased levels of CD34+VEGFR-2+ and CD133+VEGFR-2+ EPCs in PE women compared to matched controls. Preliminary data from our group however, using comparable phenotypic subsets to Luppi et al., indicate that PE patients exhibit elevated circulating EPC levels although blood sampling of PE subjects by Luppi et al. occurred much later in gestation (35±3.9 weeks versus 29.1±3.9 weeks in the present study), which may account for these differences.

Proliferation and colony formation of EPCs in culture lend not only to analysis of absolute numbers of cells, but also reflect overall cellular activity. The colony-forming potential of EPCs and characterization of these cells in vitro therefore provides critical insight into the capacity of
isolated cells for endothelial repair. CFU-EPCs are well correlated to flow-mediated brachial reactivity measurements for endothelial function and positive Framingham cardiovascular risk scores in healthy individuals (235). Reduced CFU in PE women has provided insight into the endothelial dysfunction associated with the maternal syndrome (165, 248). While neither pre-pregnancy, nor pregnancy data were examined in the current paper, postpartum comparisons yielded similar data, and were associated with increased maternal cardiovascular risk. Our findings suggest that poor cellular function previously reported in pregnancy continues postpartum, and may in fact contribute to the vascular dysfunction known to persist following PE. Chemokine production at sites of hypoxia is critical to promote recruitment of bone-marrow progenitor cells that are essential in vascularization processes. Induction of VEGF and SDF-1α in particular promotes neovascularization of injured ischemic tissues (249, 250). Elevated concentrations of these cytokines were previously reported in patients with various degrees of heart failure in association with CD34+CD133+VEGFR-2+ cells (251). Other aspects of function, including recruitment and incorporation into the endothelium are thought to play a role in EPC dysfunction (252, 253), but were not examined in this study.

Our study is the first to attempt longitudinal assessment of postpartum changes in maternal EPCs following either normotensive or PE pregnancies. Here, thirteen control and seven PE subjects completed two successive study visits. Furthermore, the concurrent assessment of EPC number by flow cytometry and function by CFU-EPC formation at all postpartum visits lends significantly to the value of the longitudinal results presented here.

Although cells examined by flow cytometry and cell culture likely represent distinct mononuclear progenitor subsets, both assays are indicative of angiogenic potential and correlate to brachial artery reactivity measurements for endothelial function. Taken together, the data suggest that PE serves as a stimulus to activate compensatory pathways that promote the mobilization of circulating EPCs following periods of critical endothelial stress. The hemostatic and metabolic
changes that occur during normal pregnancy typically normalize 4-6 weeks postpartum, such that by the second month following delivery, a mother has returned to her pre-conceptual physiological state. Peak levels of EPCs observed at two months postpartum in PE patients may indeed be indicative of the activity of such compensatory mechanisms during the puerperium. Our in vitro results however suggest that despite an increase in mobilization of these cells, the relative ability of these cells to promote healing and repair may be impaired.

Using lifetime-risk estimations for CVD, we found that reduced EPC levels at six months postpartum amongst PE women were associated with elevated lifetime risk scores for CVD compared to women who had experienced uncomplicated pregnancies. These women demonstrated elevated clinical markers of cardiovascular risk at six months follow-up, without the presence of altered cholesterol, fasting glucose, or triglycerides. This may imply that aberrant cardiovascular status might be detectable early in the postpartum period before the onset of critical biochemical changes through measured alterations in circulating EPC levels. Pre-pregnancy and late pregnancy EPC data were not collected, and it remains possible that the differences observed here may be existent pre-pregnancy, rather than having been altered by pregnancy itself. Increasing CFU-EPC are reported however with advancing gestational age, suggesting a distinct effect of pregnancy on EPC physiology. In light of modest sample sizes, the direct effect of maternal risk factors on the reported cellular alterations remains to be determined, and a more comprehensive assessment of the PE effect on EPC function is needed. While migration and tubulogenesis potential were not assessed here, we believe that this preliminary study offers critical insight into postpartum endothelial dysfunction and cardiovascular risk following PE.

In summary we have demonstrated that there exists a physiological alteration in the number and function of maternal circulating EPCs following pregnancies complicated by PE. Furthermore, we have shown that this population of young women exhibits elevated cardiovascular risk
profiles compared to those with uncomplicated pregnancies. Given the importance of EPCs in vascular homeostasis, we suggest that EPCs may offer an important avenue of investigation into the early postpartum cardiovascular effects of PE.

4.6 Acknowledgements

The authors thank members of the Queen’s Perinatal Research Unit, Department of Obstetrics and Gynecology, Queen’s University, Kingston, ON, Canada (Jessica Pudwell, MPH, Heather Ramshaw, BSc, and Michelle Roddy, RN) for their excellent help with participant recruitment, and statistical analysis. We also thank Dr. Carl Hubel of the Magee-Women’s Research Institute, University of Pittsburgh, PA, USA for his invaluable provision of the methods for CFU-EPC culture.
Chapter 5

Increased microvascular vasodilation and cardiovascular risk following a pre-eclamptic pregnancy.

This chapter has been modified from its original published version: Murphy MSQ, Vignarajah M, Smith GN. Increased microvascular vasodilation and cardiovascular risk following a pre-eclamptic pregnancy. *Physiological Reports*, 2014. Vol 2: e12217.
5.1 Abstract

**Objectives:** Women who develop pre-eclampsia are at high-risk for premature cardiovascular disease and death. The aim of this study was to assess microvascular function and cardiovascular risk in the early postpartum period for women who did or did not have a pregnancy complicated by pre-eclampsia.

**Study Design:** Peripheral microvascular function was assessed in women in the third trimester of uncomplicated pregnancies, with re-evaluation at six weeks and six months postpartum. The effect of pre-eclampsia on postpartum microvascular function was assessed two and six months after delivery. Never-pregnant, naturally cycling women served for comparison. Cutaneous microvascular reactivity to acetylcholine and sodium nitroprusside, delivered locally by iontophoresis, was measured by laser Doppler flowmetry. 30-year and lifetime risk estimates for cardiovascular disease were established.

**Results:** Acetylcholine-mediated vasodilation was enhanced by normotensive pregnancy, and declined to non-pregnant levels by six months postpartum. Acetylcholine-mediated vasodilation remained high in pre-eclamptic subjects from six weeks to six months postpartum compared to normotensive and never-pregnant controls. Pre-eclamptic subjects exhibited elevated 30-year and lifetime risk at six months postpartum.

**Conclusions:** This study provides in vivo evidence of microvascular and cardiovascular risk implications of pre-eclampsia as early as six months postpartum, and suggests that the development of pre-eclampsia may be used to identify women at risk and eligible for risk screening and intervention.
5.2 Introduction

Pre-eclampsia (PE) is a complication of pregnancy characterized by widespread maternal endothelial dysfunction and *de-novo* onset of hypertension and proteinuria after 20 weeks gestation. Endothelial dysfunction is common to the pathophysiology of both PE and cardiovascular disease (CVD), and it is now well established that the development of PE identifies women at increased risk for hypertension, ischemic heart disease, stroke and premature death from CVD compared to women with an uncomplicated obstetrical history (95). While the symptoms of PE typically remit following delivery of the placenta, maternal vascular dysfunction has been shown to persist decades into the postpartum period (132). The risk associated with PE may be measured as early as a few months after the affected pregnancy (107, 109), although the degree of recovery of maternal vascular function in the early postpartum period has not been well examined.

States of cardiovascular risk and endothelial dysfunction are classically associated with reduced or impaired endothelial-dependent vasodilation in the larger resistance vessels, a matter that has been confirmed in PE subjects (219, 238). The means by which the vascular endothelium responds to pharmacologic stimuli has been demonstrated to vary by vascular bed, vessel size and by disease state, however. Indeed, examination of microvascular function during pregnancy suggests a negative correlation in vasodilator responses between the macro- and microcirculation and endothelial-dependent vasodilation is increased in the cutaneous micro vessels of PE subjects (129-131). Despite a growing understanding of the importance of PE in identifying long-term maternal cardiovascular health risks, over what time course these findings return to “normal” postpartum, if at all, is unknown. It has long been stated that the cardiovascular system whether following a normal pregnancy, or one complicated by PE, normalizes to the pre-pregnant state by six weeks postpartum. Little work has been done to study this, however. Multiple studies have now been done postpartum that report variable vascular dysregulation in formerly PE women.
(126, 131, 132, 219), however all have been performed at a single time-point ranging from months to years postpartum. Even within studies, large ranges of time are used for analysis, likely accounting for the significant variability in reported findings. There has been no attempt at focused assessment of changes in vascular function over the postpartum, either in formerly PE women or controls. Given that the microcirculation is likely the initial site of development of vascular disease, we sought to determine whether previously documented increases in micro vessel vasodilator responses in PE subjects would persist into the postpartum period.

5.3 Materials and Methods

5.3.1 Subject recruitment
This study was approved by the Queen’s University Research Ethics Board. Written informed consent was obtained from all participants. Women experiencing normotensive singleton pregnancies were identified in the third trimester upon presentation for routine antenatal appointments at the Kingston General Hospital (KGH). PE subjects were identified through chart review, and defined as blood pressures ≥140/90 mmHg and proteinuria (≥300 mg/24 hours or ≥30 mg/mmol albumin:creatinine random urine or ≥1+ on repeat dipstick). To determine if naturally cycling hormones or hormonal contraceptive use was relevant to postpartum measurements, microvascular function was assessed in never-pregnant subjects. Never-pregnant controls were studied three times over the course of a single menstrual cycle to correspond with menses (M), follicular (F) and luteal (L) phases. Estimation of phase was completed based on onset of menses and regular length of each cycle. The medicated phase of oral contraceptive use was measured twice to correspond with time points of assessment in naturally cycling individuals. All individuals with a history of chronic hypertension, diabetes (including the development of gestational diabetes), renal disease, CVD, or current smoking were excluded.
Normotensive pregnant control subjects were examined in the third trimester and at six weeks and six months postpartum. Due to the variable effects of anti-hypertensive and anti-seizure medications on vascular function it was not feasible to perform measurements in the time leading up to delivery for women with PE. For this reason PE subjects were only assessed at six weeks and six months postpartum. Participants still taking anti-hypertensive or anti-seizure medications at follow-up were excluded. At each visit blood pressure was measured and measures of obesity (waist circumference and body mass index (BMI)) were made.

At six months postpartum, normotensive and PE subjects were invited to attend the Maternal Health Clinic at KGH. Information captured included, weight, blood pressure, physical activity level, breastfeeding status, pregnancy history, family medical history of CVD and current medication use. Blood requisitions were given for fasting blood and urine samples for core lab analysis of glucose, high-sensitivity C-reactive protein, lipid profiles and albumin:creatinine ratio. All information from the Maternal Health Clinic was used to generate CVD risk scores (152).

5.3.2 Laser Doppler flowmetry

Measurements were taken in a temperature-regulated environment with subjects lying in a semi-supine position. Subjects were asked to abstain from caffeine consumption and use of over-the-counter medications the morning prior to testing. Cutaneous perfusion was measured by laser Doppler flowmetry (Moor Instruments Ltd., Axminster, UK). Two combined temperature and laser Doppler fluximetry probes, surrounded by an iontophoresis Perspex chamber were secured to the volar aspect of the forearm. Iontophoresis chambers were adhered 4 cm apart, avoiding areas with broken skin and superficial veins. Continuous recordings of cutaneous perfusion and skin temperature were collected using a laser Doppler flow monitor (moorVMS-LDF, Moor Instruments Ltd, Axminster, UK) with data recorded in arbitrary perfusion units of flux (PU).
5.3.3 Iontophoresis

Iontophoresis is a technique used for the non-invasive delivery of drug solutions across the skin. The principle is based on the movement of charged ions across the skin in the presence of an applied electrical field. The magnitude of charge (Q) is dependent on size of the current (I), and corresponds to the amount of drug delivered. 1% solutions of acetylcholine (ACh; Miochol®-E, Bausch & Lomb Inc.) and sodium nitroprusside (SNP; Nipride, Hospira Inc.) were introduced into the anodal and cathodal chambers for assessment of endothelial-dependent and independent function, respectively. The vehicle for drug delivery was de-ionized sterile water. Following a 10-minute period of stable baseline perfusion recordings, dose-response curves to ACh and SNP were obtained by the step-wise application of currents (130) (5 μA, 10 μA, 15 μA, 20 μA, 50 μA, and three applications of 100 μA) by an iontophoresis controller (MIC2, Moor Instruments Ltd, Axminster, UK). Currents were applied for 10 seconds followed by 2 minute recording periods for a total charge delivery of 4 mC. Corresponding changes in cutaneous blood flow were assessed using a laser Doppler flow monitor (moorVMS-LDF, Moor Instruments Ltd, Axminster, UK) (Figure 5.1). Vasodilation was taken as the ratio of peak flux to an average of the total 10 minute baseline perfusion for each drug administered per iontophoretic dose.

5.3.4 Statistical analysis

Demographic variables are presented as mean ± standard deviation (SD). An unpaired t-test or one-way analysis of variance (ANOVA) with Bonferroni post hoc test was used to compare continuously distributed variables and a χ² comparison was used for categorical measures. Dedicated software (moorVMS-PC V3.1, Moor Instruments Ltd, Axminster, UK) was used to analyze individual vascular responses to delivery of ACh and SNP by iontophoresis. Vasodilation was calculated as the ratio between the maximum achieved perfusion in response to each iontophoretic dose. Data were then exported to GraphPad Prism 5 for statistical analyses and
Figure 5.1. Laser Doppler flowmetry and iontophoresis recordings.

A representative depiction of dose-response recordings generated from the application of laser Doppler flowmetry and iontophoresis. Linear graphs correspond to changes in blood flow in response to (A) 1% SNP and (B) 1% ACh diluted in de-ionized water.
comparison within and between subject groups. Comparison within subject groups, across experimental time-points were analyzed by matched two-way ANOVA. Comparisons between subject groups, by time-point of measurement were achieved by unpaired two-way ANOVA. Statistical significance was accepted if the null hypothesis could be rejected at $p<0.05$.

5.4 Results

Baseline characteristics of all participants are summarized in Table 5.1. Never-pregnant subjects were younger than parous subjects, although did not differ by BMI when compared to pre-pregnant indices of both normotensive pregnant and PE groups. Baseline perfusion and cutaneous temperature measurements did not differ significantly between subject groups or across time (0).

5.4.1 Microvascular function

*Effect of naturally cycling hormones and oral contraceptives on microvascular function.* Twenty-five (n=25) never-pregnant subjects participated in the study. Of these fifteen (n=15) were naturally cycling and ten (n=10) individuals reported use of monophasic oral contraceptive medication for a minimum of four months. Measurements taken across the course of a natural menstrual cycle demonstrated no effect by phase on microvascular vasodilation. Similarly, oral contraceptive use did not alter microvascular measurements compared to naturally cycling individuals (0). For this reason, data from normotensive and PE subjects were compared to the mid-follicular phase of never-pregnant subjects in subsequent figures.

*Effect of normotensive pregnancy on microvascular function.* Microvascular measurements taken in twenty-three (n=23) normotensive pregnant controls demonstrated that the third trimester of pregnancy was associated with enhanced endothelial-dependent vasodilation in response to ACh.
Table 5.1. Characteristics at time of recruitment or diagnosis of pre-eclampsia.

<table>
<thead>
<tr>
<th></th>
<th>Never-pregnant</th>
<th>Normotensive</th>
<th>Pre-eclampsia</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n=15</td>
<td>n=23</td>
<td>n=25</td>
</tr>
<tr>
<td>Age (y)</td>
<td>23.5±3.5</td>
<td>30.4±4.2</td>
<td>32.1±6.1</td>
</tr>
<tr>
<td>Parity, n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Primiparous</td>
<td>-</td>
<td>9 (39)</td>
<td>12 (48)</td>
</tr>
<tr>
<td>Multiparous</td>
<td>-</td>
<td>14 (61)</td>
<td>13 (52)</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>22.2±2.7</td>
<td>23.5±3.5</td>
<td>26.1±7.4</td>
</tr>
<tr>
<td>Pregnancy Weight Gain (kg)</td>
<td>-</td>
<td>14.8±6.1</td>
<td>16.6±8.6</td>
</tr>
<tr>
<td>Blood Pressure (mmHg)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Systolic</td>
<td>103.8±6.2</td>
<td>112.5±6.5</td>
<td>175.6±21.6</td>
</tr>
<tr>
<td>Diastolic</td>
<td>68.2±7.6</td>
<td>72.3±7.4</td>
<td>103.8±8.2</td>
</tr>
<tr>
<td>GA Delivery (wks)</td>
<td>-</td>
<td>39.8±1.1</td>
<td>36.0±3.8</td>
</tr>
</tbody>
</table>

BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure. *p<0.0001 versus Normotensive group.
Microvascular reactivity returned to levels similar to those of never-pregnant controls by six months postpartum ($p > 0.05$). Microvascular responses to SNP were unchanged in pregnancy and in the postpartum ($p > 0.05$) (Figure 5.2).

Effect of PE on postpartum microvascular function. Microvascular reactivity to ACh and SNP was unchanged from six weeks to six months postpartum in fifteen (n=15) PE subjects. Maximal perfusion responses to ACh were not different at six weeks postpartum, but were significantly elevated in PE subjects compared to controls at six months postpartum. Postpartum vascular responses to SNP did not differ ($p > 0.05$). To better assess the effect of PE on microvascular function at six months postpartum, data from an additional 10 PE subjects was included. Baseline characteristics and microvascular measurements for these additional subjects did not differ from the initial PE group. For this reason, the additional data are included in Table 5.1 and Figure 5.3.

5.4.2 Cardiovascular risk at six months postpartum
Normotensive (n=21) and PE (n=22) subjects provided complete biophysical profiles at 6 months postpartum (CTRL 26.1±3.8 weeks vs PE 28.1±2.6 weeks; $p > 0.05$). Measurements of obesity at six months postpartum did not differ between subject groups, although fewer PE subjects were breastfeeding at that time. 30-year (144) and lifetime (145) cardiovascular risk scores were based on physical and biochemical parameters of cardiovascular risk (sex, age, smoking, total cholesterol, fasting glucose, systolic blood pressure, antihypertensive use; and sex, smoking, total cholesterol fasting glucose, systolic blood pressure, diastolic blood pressure, antihypertensive usage respectively) and calculated following the six months postpartum clinic assessment. A summary of findings is found in Table 5.2.

Our PE group included 5 women with early-onset PE, and 20 with late-onset PE. Additionally 20 women experienced severe PE, while 5 experienced mild PE. Stratification of subjects revealed
Figure 5.2. Microvascular reactivity after uncomplicated pregnancy.

(A) Endothelial-dependent responses to acetylcholine, ACh, in normotensive women (n=23) in the third trimester (3TM), 6 weeks postpartum (6W PP) and 6 months postpartum (6M PP) compared to never-pregnant (NP, n=15) subjects. (B) Endothelial-independent responses to sodium nitroprusside, SNP in the same subjects. Data are presented as mean±SEM. Posthoc comparisons *p<0.001 3TM vs 6M PP; † p <0.05 3TM vs NP; ‡p<0.05, 6W PP vs 6M PP.
Figure 5.3. Microvascular reactivity after pre-eclampsia.

Comparison of microvascular responses at six weeks postpartum and six months postpartum to acetylcholine, ACh, and sodium nitroprusside, SNP. Data are presented as mean±SEM. NP, never-pregnant (n=15); CTRL, normotensive control, (n=23); PE at six weeks postpartum (n=15), PE at six months postpartum (n=25); post hoc comparisons *p<0.05; **p<0.01.
Table 5.2. Cardiovascular risk variables at six months postpartum.

<table>
<thead>
<tr>
<th></th>
<th>Normotensive (n=21)</th>
<th>Pre-eclampsia (n=22)</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weeks postpartum (wks)</td>
<td>26.1±3.8</td>
<td>28.1±2.6</td>
<td>0.0844</td>
</tr>
<tr>
<td>BMI (kg/m(^2))</td>
<td>25.4±4.7</td>
<td>29.4±8.4</td>
<td>0.0674</td>
</tr>
<tr>
<td>Weight Retention (kg)</td>
<td>5.6±5.9</td>
<td>6.1±9.5</td>
<td>0.8377</td>
</tr>
<tr>
<td>Waist Circumference (cm)</td>
<td>85.7±11.7</td>
<td>91.78±18.74</td>
<td>0.2166</td>
</tr>
<tr>
<td>Breasfeeding, n (%)</td>
<td>17 (80.9)</td>
<td>12 (54.5)</td>
<td>0.0001*</td>
</tr>
<tr>
<td>Blood Pressure (mmHg)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Systolic</td>
<td>106.2±8.99</td>
<td>124.0±13.2</td>
<td>0.0001*</td>
</tr>
<tr>
<td>Diastolic</td>
<td>70.14±7.44</td>
<td>85.27±9.76</td>
<td>0.0001*</td>
</tr>
<tr>
<td>Fasting Glucose (mmol/L)</td>
<td>4.50±0.38</td>
<td>4.68±0.37</td>
<td>0.1345</td>
</tr>
<tr>
<td>Total Cholesterol (mmol/L)</td>
<td>4.78±0.98</td>
<td>4.72±0.83</td>
<td>0.8010</td>
</tr>
<tr>
<td>Triglycerides (mmol/L)</td>
<td>0.66±0.31</td>
<td>1.332±1.2</td>
<td>0.0170</td>
</tr>
<tr>
<td>HDL Cholesterol (mmol/L)</td>
<td>1.65±0.4</td>
<td>1.37±0.38</td>
<td>0.0232*</td>
</tr>
<tr>
<td>LDL Cholesterol (mmol/L)</td>
<td>2.83±0.88</td>
<td>2.77±0.71</td>
<td>0.8238</td>
</tr>
<tr>
<td>Albumin:Creatinine (mg/mmol)</td>
<td>0.49±0.59</td>
<td>3.8±7.8</td>
<td>0.0589</td>
</tr>
<tr>
<td>C-Reactive Protein</td>
<td>2.69±3.1</td>
<td>5.8±10.46</td>
<td>0.993</td>
</tr>
<tr>
<td>Metabolic Syndrome, n (%)</td>
<td>0 (0)</td>
<td>6 (27)</td>
<td>&lt;0.00001*</td>
</tr>
<tr>
<td>30 Year Risk</td>
<td>5.0±1.85</td>
<td>10.0±4.42</td>
<td>0.0002*</td>
</tr>
<tr>
<td>Lifetime Risk, n(%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low Risk (&lt;39%)</td>
<td>13 (62)</td>
<td>9 (40)</td>
<td>0.0029*</td>
</tr>
<tr>
<td>High Risk (&gt;39%)</td>
<td>8 (38)</td>
<td>13 (60)</td>
<td></td>
</tr>
</tbody>
</table>

BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; HDL, high density lipoprotein; LDL, low density lipoprotein. Lifetime cardiovascular risk scores for each patient were generated based on biophysical factors including; sex, smoking, total cholesterol fasting glucose, systolic blood pressure, diastolic blood pressure, antihypertensive usage - Categorical data for lifetime cardiovascular risk are divided into <39%, low risk, and ≥39%, high risk. *p<0.05, statistically significant.
no significant differences in ACh-mediated vasodilation by onset and severity of PE (0). Conversely, high-risk PE subjects had elevated vasoreactivity to ACh compared to high-risk normotensive controls at six months postpartum (PE n=13, vasodilation: 15.17±2.207 vs CTRL n=8, vasodilation: 8.282±1.671; p<0.05). This difference was not observed when vasodilation was compared between low-risk groups (PE n=9, vasodilation: 17.41±3.540 vs CTRL n=13, vasodilation: 12.12±2.210; p>0.05).

5.5 Discussion

Here we describe for the first time prospective assessment of postpartum recovery of microvascular function following uncomplicated and PE pregnancies. Our findings demonstrate that pregnancy-enhanced endothelial-dependent vasodilation returns to non-pregnant levels by six months postpartum. In contrast, ACh-stimulated vasodilation remains persistently increased in the cutaneous micro vessels of women with a recent history of PE such that by six months postpartum endothelial-dependent vasodilation exceeds levels seen in women with uncomplicated obstetrical history or never-pregnant controls. Our findings in postpartum PE women are not consistent with those reported in larger vessels. Attenuated vascular responses to flow-mediated dilation and venous occlusion plethysmography are reported months to years after afflicted pregnancies (126, 203, 219, 238). A single evaluation of microvascular function 15-25 years after PE described reduced endothelial-dependent and – independent responses typical of the larger conduit vessels (132). Comparison of endothelial function in conduit arteries with that in small resistance vessels should be made with caution. Macro- and microvascular properties of vasodilation often poorly correlate, and even still the use of pharmacological (e.g. ACh, SNP) and physiological (shear stress) stimuli likely induce different contributions from the endothelium to prompt vasodilation (254). Evaluation of microvascular function in response to pharmacologic stimuli in PE and the early
postpartum are few, but indicate that microvascular vasodilator responses to ACh are increased in women with PE compared to those with uncomplicated pregnancy (129-131). Khan et al. performed serial measurements from 22 weeks gestation to six weeks beyond delivery in 54 control women and 15 women who developed PE (131). The authors reported increased vasodilator responses to ACh in PE women before delivery, which subsequently declined to levels comparable to controls by six weeks postpartum. In contrast, Blaauw and colleagues reported enhanced microvascular responses to ACh in subjects examined between 3 and 11 months postpartum (129). Our findings both corroborate and extend these observations by taking consecutive measurements of microvascular function into the postpartum period at well-defined time-points. In consideration of previous reports, we suggest that pregnancy-mediated increases in endothelium-dependent vasodilation declines postpartum following uncomplicated pregnancies, whereas this postpartum “recovery” of vascular function is altered in PE subjects. At what time microvascular responses transition from enhanced to attenuated, as reported by 15-25 year follow-up of this population (132) is unclear however and the mechanisms underlying these changes require further investigation. Even further, paired measurements of large and micro vessel function in this population would be beneficial to enhance our understanding of micro vessel physiology in health and disease.

Differences in microvascular function are unlikely the result of increased sensitivity to nitric oxide at the level of vascular smooth muscle at the early points postpartum studied here, as responses to SNP were not altered in PE subjects. Rather, this points to changes in vasodilator synthesis or dependence, and further, perhaps in the permeability of the endothelium to vasoactive substances. The relative contributions of each of endothelial nitric oxide synthase (eNOS), cyclooxygenase (COX) and endothelium derived hyperpolarizing factor pathways to endothelial-dependent vascular responses in the forearm microcirculation has been debated, although in vivo experimentation with eNOS and COX inhibitors suggests that cutaneous
microvascular responses to ACh are likely the result of contributions from both NO and prostaglandin pathways (255-259). Demonstrated plasticity of endothelial function within the coronary circulation indicates a shift in dependence of vasodilatory mediators in adult health versus disease (260). Therefore, there is the potential for similar response mechanisms modulating endothelial-dependent activity at the level of the cutaneous microvessels in states of cardiovascular risk that may explain inconsistencies in correlation between brachial and cutaneous function (133, 254).

Laser Doppler flowmetry as used in the present study provides a useful, non-invasive tool for assessment of microvascular function as it enables the evaluation of microvascular blood flow continuously over time, and response to a given stimulus (261, 262). The inherent variability in Doppler and iontophoresis measurements however must be considered. Reported inter-individual and day-to-day variation of flowmetry data may vary significantly and must be reported for each study (263, 264). The major source of variation is the site of measurement however, and when spatial variability is minimized, day-to-day reproducibility of Doppler flowmetry data compares well with traditional measurements of vascular function (<10%) (261). This technique in our laboratory has shown good reproducibility (CV%30.5; ICC0.64), although the prolonged nature of follow-up in our experiments made spatial standardization difficult. Given that variability in baseline flow in our subjects was minimal, the significant alterations in vasodilator responses detected in our cohort, despite the high degree of variability in single-point Doppler measurements in forearm micro vessels, are substantial.

Abnormalities in endothelial function contribute substantially to cardiovascular risk and outcome, and whether endothelial dysfunction is present before, or incurred as a result of pregnancies affected by PE remains an important question that needs to be addressed. It has been suggested that the development of PE may be in part due to the subclinical physiological state of a mother’s endothelium prior to pregnancy, and its ability to meet the hemodynamic and metabolic demands
of the growing conceptus. Indeed, subclinical endothelial dysfunction prior to a diagnosis of PE has been well described (121, 127, 131). Although our sample sizes were small, stratification of our data by cardiovascular risk lends support to this hypothesis. Differences in endothelial function between PE and control subjects were persistent only in women with high lifetime risk for CVD, suggesting that biophysical profiles play an important role in the persistence or progression of altered resistance vessel function in women with a history of PE.

Risk of CVD is modifiable with lifestyle intervention, and modest modifications to dietary and exercise habit have demonstrable effects on blood pressure, lipid profiles and inflammatory and hemostatic regulators (68). A recent study from the Netherlands examined the benefits of 12 week exercise training in 6-12 month postpartum PE subjects. In addition to benefits on blood pressure, parameters of obesity and cholesterol, exercise training reduced carotid artery intima media thickness, and improved brachial and femoral artery flow mediated dilation responses. Autonomic cardiac activity quantified by spectral analysis of heart rate variability was also improved following training. Interestingly, while training improved all parameters of endothelial function and autonomic activity in formerly PE patients, these parameters did not normalize to levels comparable to those observed in trained controls (265). Pharmacologic intervention in non-hypertensive postpartum PE women with demonstrated risk for CVD may also prove a promising option for CVD risk intervention. One- and five-year follow-up of newly diagnosed hypertensive patients prescribed daily use of angiotensin converting enzyme (ACE) inhibitors demonstrates long-term improvements in endothelial progenitor cell function, inversely correlated to carotid intimal media thickness (266). Additionally, prescription of ACE inhibitors reduces albuminuria and progressive kidney failure in type 1 diabetes and improves endothelial-dependent function (267, 268).

In conclusion, this study compares longitudinal in vivo microvascular reactivity following normotensive and PE pregnancies. ACh-mediated vasodilation was elevated in the postpartum PE
period and PE was associated with increased endothelial-dependent vasodilation compared to normotensive pregnancies at six months postpartum. While the resulting sample sizes were small, we feel that the findings presented here provide important evidence of the functional changes taking place in the maternal endothelium in the early postpartum period following PE. These findings provide support for the need for targeted early postpartum cardiovascular risk screening in women who have experienced pregnancies complicated by PE.

### 5.6 Acknowledgements

The authors would like to thank Michelle Roddy, RN, BScN, and Jessica Pudwell, MPH of Kingston General Hospital, for their assistance in subject identification and compilation of patient data. These findings have been presented at the 61st Annual Meeting, Society for Gynecologic Investigation in Florence, Italy (March 26-29 2014). The abstract was published in Reproductive Sciences: Murphy MSQ, G Smith. (2014) Pre-eclampsia is associated with early postpartum endothelial dysfunction as measured by laser Doppler flowmetry and iontophoresis (O-138). Reproductive Sciences. 2014; 21(3) Supplement. This study was funded by the Canadian Institutes of Health Research (#299823).
Chapter 6

Reduced heart rate variability and altered cardiac conduction after pre-eclampsia

This chapter has been modified from its original submitted version: Murphy MSQ, Seaborn EJ, Redfearn DP, Smith GN. Reduced heart rate variability and altered cardiac conduction after pre-eclampsia. Reproductive Sciences, 2015. MS#RSCI-15-122.
6.1 Abstract

**Objectives:** Pre-eclampsia (PE) is a hypertensive disorder of pregnancy that is associated with elevated maternal risk for cardiovascular disease. Although women with a history of PE exhibit markers of endothelial dysfunction, postpartum cardiac function after PE has been poorly described. The aims of this study were to determine the effect of normal pregnancy on postpartum parameters of the electrocardiogram, and furthermore how PE may affect postpartum cardiovascular recovery.

**Study Design:** Fifteen naturally-cycling never-pregnant controls, twenty normotensive pregnant controls and twenty women with a recent history of PE completed the study. Normotensive women were measured at 40.0±1.06 weeks gestation, and returned for follow-up at six weeks and six months postpartum. PE women were assessed at six months postpartum. Ten-minute high-resolution (1000 Hz) orthogonal Holter electrocardiogram recordings were used to measure heart rate variability (HRV). Signal-averaged P-wave and QRS complex durations were determined.

**Results:** Uncomplicated pregnancy was associated with reduced HRV, which returned to non-pregnant levels by six months postpartum. Time-domain parameters of HRV were significantly reduced in PE women at six months postpartum compared to controls. Frequency-domain indices of HRV were not altered by a diagnosis of PE. Pre-eclamptic women presented with significantly longer P-Wave and QRS complex duration compared to control groups. Only QRS duration was independent of differences in blood pressure.

**Conclusions:** Pre-eclampsia is associated with alterations in heart rate variability and cardiac conduction at 6 months postpartum. Whether these changes are the direct result of PE or are inherent in individuals at risk of developing PE remains to be determined.
6.2 Introduction

Pre-eclampsia (PE) is a common hypertensive disorder of pregnancy that affects 6-10% of pregnancies worldwide. In addition to posing severe maternal and neonatal complications in the peripartum period, PE is associated with increased maternal risk of cardiovascular disease and stroke in later life (95). The American Heart Association’s Effectiveness-Based Guidelines for the Prevention of Cardiovascular Disease in Women now recommends including the development of pregnancy-related complications, including PE, in risk screening practices for heart disease and stroke (15). In addition, a recent evidence-based review has called for the implementation of cardiovascular risk screening clinics designed to target postpartum women with a history of indicated pregnancy complications (269). While it remains uncertain whether PE exacerbates previously undiagnosed or underlying cardiovascular risk factors, or if cardiovascular risk associated with PE is the direct result of the manifestation of the disorder itself, developing an understanding of the early postpartum implications of PE on the maternal cardiovascular system remains important if targeted prevention and screening are to be successful.

Although increased sympathetic activity in PE has been well-described (270-272), postpartum cardiac electrophysiology and function have been infrequently examined in this population (273-276). As women with PE are at significantly increased risk of heart failure and both atrial and ventricular dysrhythmia in the years after pregnancy (277) assessment of the cardiac state in the earliest times postpartum remains critical in identifying those individuals at greatest risk. The aims of this study were to determine the effects of normal pregnancy on postpartum parameters of autonomic tone and furthermore how PE may affect cardiovascular recovery in the early times postpartum by use of short-term electrocardiogram (ECG) recordings.
6.3 Methods

6.3.1 Subject identification and follow-up

Approval for this study was obtained from the Queen’s University Health Sciences Research Ethics Board (OBGY-232-12, OBGY-233-12). Written informed consent was obtained from all participants. Fifteen naturally-cycling individuals served as never-pregnant controls (NP). Twenty healthy women experiencing uncomplicated normotensive pregnancies were identified upon routine presentation to low-risk obstetrical clinics and examined in the third trimester, at six weeks and six months postpartum.

Due to the variable effects of anti-hypertensive and anti-seizure medications on cardiovascular function it was not feasible to perform measurements in the time leading up to delivery for women with PE. For this reason fifteen women with a diagnosis of PE were recruited in the few days following delivery at Kingston General Hospital and consented for follow-up at six weeks and six months postpartum. A diagnosis of PE was confirmed by chart review (blood pressure $\geq 140/90$ mmHg; proteinuria $\geq 300$ mg/24 hours or $\geq 30$ mg/mmol albumin:creatinine random urine or $\geq 1^+$ on repeat dipstick). Patients with hemolysis, elevated liver enzymes, low platelets (HELLP) were not included in the study. All individuals with a pre-pregnancy history of hypertension, diabetes (including the development of gestational diabetes), renal disease, cardiovascular disease, or who currently were smoking were excluded.

6.3.2 Electrocardiography

Experiments were performed in a quiet temperature-controlled room with the subjects seated in a semi-supine position. All subjects were asked to abstain from caffeine intake and over the counter medication use the morning of the study visit. Skin was cleansed with 70% isopropyl alcohol prior to positioning of electrodes in an orthogonal manner. Ten-minute high-resolution (1000 Hz) Holter ECG recordings were collected using a 3 lead SpiderView™ digital ECG Holter recorder (ELA Medical, Montrouge, FR). All non-sinus beats were excluded from analysis by operator
inspection and all R-wave detection errors were corrected. A summary of ECG parameters assessed in this study are presented in Table 6.1.

### 6.3.3 P-wave and QRS duration

Signal-averaged P-wave (SAPW) and QRS (SAQRS) analyses were performed using custom P-Wave Averaging Software (278) using the same raw ten-minute high-resolution (1000Hz) Holter ECG recordings used for HRV analysis. As previously described (279), ECG signals were amplified 10 000 times and band-pass filtered between 1Hz and 300 Hz. The lead exhibiting the clearest P-wave or QRS-complex was further filtered between 20 Hz and 50 Hz, and used as a trigger to align subsequent P-waves for signal averaging. The analogue data were sampled at 1 kHz with 12-bit resolution and a minimum of 100 beats were used to produce an averaged value of P-wave and QRS-complex duration.

### 6.3.4 Statistical analysis

Demographic variables are presented as mean±standard deviation (SD) unless otherwise stated. An unpaired t-test or one-way analysis of variance (ANOVA) with Bonferroni post hoc test was used to compare continuously distributed variables and a \( \chi^2 \) comparison was used for categorical measures. GraphPad Prism 5 Software (La Jolla, CA, USA) was used for statistical analyses and comparison within and between subject groups. Normality of data was determined using the D’Agostino and Pearson omnibus normality test, and parametric or non-parametric statistical analysis was completed accordingly. Comparison of normotensive pregnancy control data across experimental time-points were analyzed by matched two-way ANOVA. Comparisons between subject groups, by time-point of measurement were achieved by unpaired one-way ANOVA. Multivariable regression was performed using SPSS software (IBM SPSS Version 22.0, Armonk, New York). Due to the known impact of blood pressure on P-wave and QRS duration, regression models were generated to control for systolic and diastolic blood pressure in the comparison of P-
Table 6.1. Summary of ECG parameters.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Application</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Time-Domain Variables of HRV</strong></td>
<td>Time-domain HRV indices mathematically describe the variability in duration between successive RR intervals. Reduced variability of time-domain indices is reflective of mortality risk in a variety of disease states.</td>
</tr>
<tr>
<td>Mean RR</td>
<td>Average duration of successive R-R intervals.</td>
</tr>
<tr>
<td>SDNN</td>
<td>Standard Deviation of Normal-Normal RR Intervals.</td>
</tr>
<tr>
<td>RMSSD</td>
<td>Root Mean Square Successive Difference.</td>
</tr>
<tr>
<td>pNN50</td>
<td>Proportion of R-R intervals differing from their directly adjacent R-R intervals &gt;50ms.</td>
</tr>
<tr>
<td><strong>Frequency-Domain Variables of HRV</strong></td>
<td>Non-linear analysis of R-R interval series using Fast-Fourier Transformation techniques. Frequency-domain indices of HRV provide an evaluation of the contributions of pre-determined frequency ranges to the overall variability in the R-R interval signal.</td>
</tr>
<tr>
<td>LF</td>
<td>Low Frequency (0.04-0.15Hz); regarded as a marker of both sympathetic and parasympathetic modulation.</td>
</tr>
<tr>
<td>HF</td>
<td>High Frequency (0.15-0.4 Hz); reflects parasympathetic activity</td>
</tr>
<tr>
<td>LF:HF</td>
<td>A ratio used as a measure of sympathovagal balance</td>
</tr>
<tr>
<td><strong>P-Wave Duration</strong></td>
<td>P-wave represents atrial depolarization. Long P wave duration indicates a slowing of electrical conduction throughout the atrium, and may occur in left atrial enlargement.</td>
</tr>
<tr>
<td><strong>QRS Duration</strong></td>
<td>QRS complex corresponds to ventricular depolarization. Broad QRS complexes indicate aberrant conduction of supraventricular complexes.</td>
</tr>
</tbody>
</table>
Wave and QRS duration across subject groups. Statistical significance was accepted if the null hypothesis could be rejected at $p<0.05$.

### 6.4 Results

Characteristics of the enrolled subjects are summarized in Table 6.2. Fifteen naturally-cycling NP controls, twenty women with uncomplicated pregnancy and fifteen women with a recent history of PE were initially recruited into the study. ECG parameters were unchanged from six weeks to six months postpartum in the fifteen (n=15) original PE subjects. To better assess the effect of PE on ECG parameters at 6 months postpartum, an additional five women with a diagnosis of PE were recruited from the Maternal Health Clinic at Kingston General Hospital when attending for a routine six month postpartum check-up. For this reason, data from twenty PE women are included in Table 6.2 and in subsequent figures. ECG parameters taken at six weeks postpartum in PE subjects are summarized in the text.

Women experiencing uncomplicated pregnancy were measured at 40.0±1.06 weeks gestation, and returned for follow-up measurements at six weeks postpartum (7.37±1.07 weeks) and six months postpartum (26.50±3.64 weeks). PE women were assessed at 26.13±3.64 weeks postpartum. PE subjects exhibited elevated systolic and diastolic blood pressures at six months postpartum compared to NP and time-matched parous controls. Seventeen (85%) of parous control subjects and eleven (55%) of PE women were still breastfeeding at 6 months postpartum ($p=0.082$). Six (30%) of parous controls and fourteen (70%) of PE subjects had resumed their normal menstrual cycles at six months postpartum ($p=0.056$). In addition, mean RR (average duration of RR intervals) in PE subjects was significantly reduced six months postpartum compared to time-matched and NP controls. Although uncomplicated pregnancy was associated with reduced mean RR compared to never pregnant controls, these differences were resolved postpartum. No patients were using anti-hypertensive medications at the time of follow-up. Preliminary work determined
Table 6.2. Subject characteristics at time of examination.

<table>
<thead>
<tr>
<th></th>
<th>NP n=15</th>
<th>Uncomplicated Pregnancy n=20</th>
<th>PE n=20</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age (y)</strong></td>
<td>23.5±3.5</td>
<td>29.95±3.72a</td>
<td>32.12±7.16a</td>
</tr>
<tr>
<td><strong>Primiparity n (%)</strong></td>
<td>-</td>
<td>8 (40)</td>
<td>8 (40)</td>
</tr>
<tr>
<td><strong>GA delivery (wks)</strong></td>
<td>-</td>
<td>39.94±1.06</td>
<td>35.06±3.46c</td>
</tr>
<tr>
<td><strong>BMI (kg/m²)</strong></td>
<td>22.2±2.7</td>
<td>23.67±3.81</td>
<td>24.52±4.89</td>
</tr>
<tr>
<td><strong>SBP (mmHg)</strong></td>
<td>103.8±6.2</td>
<td>112.4±6.86</td>
<td>105.2±8.64</td>
</tr>
<tr>
<td><strong>DBP (mmHg)</strong></td>
<td>68.2±7.6</td>
<td>72.35±7.27</td>
<td>69.45±7.83</td>
</tr>
</tbody>
</table>

GA, gestational age; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; GA, gestational age; 3TM, third trimester; 6W PP, 6 weeks postpartum; 6M PP, 6 months postpartum. a p<0.01 versus never-pregnant; b p<0.01 versus time-matched uncomplicated pregnancy.
there to be no significant effect of natural hormonal cycling and oral contraceptive use on heart rate variability (0). For this reason, data collected during the mid-cycle of naturally-cycling NP controls were used for comparison to parous subject groups. In acknowledgement of the limited sample sizes postpartum, PE data was not stratified by onset (early/late) or severity (mild/severe) PE.

6.4.1 Time domain parameters of HRV
All time-domain indices of HRV were significantly reduced in the third trimester of uncomplicated pregnancy compared to NP controls and returned to non-pregnant levels by six months postpartum. SDNN (standard deviation of the normal-normal RR interval durations), root mean square successive difference (RMSSD), and pNN50 (proportion of intervals differing from directly adjacent R-R intervals by >50 ms) however were significantly reduced in PE women at six months postpartum. In addition, PE subjects exhibited significantly reduced SDNN, RMSSD and pNN50 compared to time-matched subjects after uncomplicated pregnancy. Time-domain parameters are summarized in Figure 6.1. Comparison of available paired data from 15 PE subjects at both six weeks and six months postpartum indicated no changes in time-domain parameters across the early postpartum period after PE (PE six weeks postpartum vs six months postpartum: Mean RR (ms), 851.0±128.0 vs 794.2±86.24; SDNN (ms), 55.35±17.84 vs 50.17±15.28; RMSSD (ms), 49.59±25.09 vs 40.67±15.30; pNN50 (%), 27.16±21.43 vs 19.55±17.67, all \( p>0.05 \)). Time domain parameters of HRV were all highly correlated to mean RR duration Figure 6.2A.

6.4.2 Frequency domain parameters of HRV
Frequency domain indices were unaltered by normotensive uncomplicated pregnancy. PE subjects exhibited similar degrees of LF (low frequency spectral components; reflects a combination of vagal/parasympathetic and sympathetic activity), HF (high frequency spectral
Mean RR and time domain indices of HRV were reduced in the 3rd trimester of uncomplicated pregnancy, UP. Time domain parameters of HRV return to never-pregnant levels by 6 months postpartum, whereas in PE subjects these variables are reduced compared to time-matched and never-pregnant controls. 3TM, 3rd trimester; 6W PP, 6 weeks postpartum; 6M PP, 6 months postpartum.

Figure 6.1. Time domain indices of HRV.
Figure 6.2. Correlation of heart rate variability parameters to mean RR.

All (A) time domain; SDNN, RMSSD, pNN50 and (B) frequency domain; LF, HF, LF/HF parameters were significantly correlated to corresponding mean RR duration in study subjects.
components; reflects vagal/parasympathetic activity) and LF:HF modulation of heart rate at six months postpartum compared to time-matched women after uncomplicated pregnancy, although frequency domain indices from both six month postpartum groups displayed significant deviations from NP controls. Frequency domain parameters are summarized in Figure 6.3. Comparison of available paired data from 15 PE subjects at both six weeks and six months postpartum demonstrated a recovery of autonomic tone similar to that observed after uncomplicated pregnancy (PE six weeks postpartum vs six months postpartum: LF\textsubscript{norm} (nu) 54.29±19.84 vs 65.51±14.80; HF\textsubscript{norm} (nu) 45.71±19.84 vs 34.49±14.80; both \(p>0.05\)). As with time domain parameters, frequency domain parameters of HRV were all highly correlated to mean RR duration Figure 6.2B.

6.4.3 P-wave and QRS Duration

Not all heart rate recordings were suitable for signal-averaged analysis. For this reason sample sizes for P-Wave and QRS duration data were minimally reduced. P-wave and QRS duration were unaffected by uncomplicated pregnancy. PE subjects presented with significantly longer P-wave and QRS complex durations compared to NP and normotensive time-matched controls. Data are summarized in Figure 6.4. Available paired data from 11 PE subjects indicated that P-wave and QRS duration remained consistent across the early postpartum period (PE six weeks postpartum vs six months postpartum: P-Wave (ms), 122.1±6.68 vs 124.4±11.54; QRS (ms), 114.7±12.20 vs 115.0±13.56; both \(p>0.05\)).

After multiple regression controlling for systolic and diastolic blood pressures, signal averaged P-wave durations at six months postpartum were not significantly different between PE subjects, time-matched controls and never-pregnant controls. In brief, using PE as the reference group, never-pregnant subjects had P-wave durations an average of 2.2 ms longer (\(p=0.743, 95\%\text{CI} -11.13, 15.48\)), while women with a history of uncomplicated pregnancy had P-wave durations
Figure 6.3. Frequency domain indices of HRV.

Frequency domain indices of HRV were not significantly altered by uncomplicated pregnancy, UP. Six month postpartum PE subjects and time-matched controls had significantly increased LF and reduced HF components compared to NP controls. 3TM, 3rd trimester; 6W PP, 6 weeks postpartum; 6M PP, 6 months postpartum.
Uncomplicated pregnancy, UP, was not associated with alterations in P-Wave or QRS duration compared to never-pregnant controls, NP. PE subjects at 6 months postpartum exhibited increased duration in both P-wave and QRS complexes. 3TM, 3rd trimester; 6W, 6 weeks postpartum; 6M, 6 months postpartum.

Figure 6.4. P-Wave and QRS complex duration.
that were on average 6.1 ms shorter ($p=0.363, 95\% \text{CI} -19.50, 7.29$) at six months postpartum. In contrast, QRS duration remained significantly longer for the PE versus comparison groups after adjustment for blood pressures. Again with PE as the reference group, never-pregnant subjects had QRS durations on average 8.8 ms shorter ($p=0.026, 95\% \text{CI} -16.57, 1.08$), and subjects with a history of uncomplicated pregnancy had QRS durations that were on average 10.7 ms shorter ($p=0.008, 95\% \text{CI} -18.50, -2.92$) at six months postpartum.

6.5 Discussion

Using short-term electrocardiogram recordings, here we demonstrate that the dominant parasympathetic mechanisms regulating cardiac control are reduced by normotensive uncomplicated pregnancies and are subsequently ameliorated as early as six months postpartum. Furthermore, women who have experienced a PE pregnancy exhibit reduced time domain parameters of HRV at six months postpartum compared to time-matched parous and nulliparous controls. In large, these differences appear to be driven by reductions in mean RR duration. Examination of P-wave and QRS complex duration in our study cohort further revealed abnormalities of cardiac electrophysiology in PE subjects at six months postpartum, although differences in P-Wave duration were resolved after adjustment for blood pressure. As uncomplicated pregnancy appeared to have no effect on electrical conduction through the heart, prolonged QRS duration six months postpartum of PE suggests that significant increases in myocardial conduction times may have been present before pregnancy itself. Indeed secondary analysis of paired data at six weeks and six months postpartum in this group of women indicate that these parameters remain stable from the earliest times after pregnancy.

It has long been recognized that fluctuations in heart rate can be used to indirectly assess autonomic control of the heart (280), with high degrees of variability in heart rate a sign of adaptability, and well-functioning autonomic control mechanisms. Conversely, reductions in
HRV are often indicative of physiological abnormalities in autonomic systems regulating the heart. Analysis of HRV is becoming increasingly used as a non-invasive clinical tool, as HRV has emerged as a reliable indicator of risk related to adverse cardiac events in otherwise normal healthy subjects (281).

While major reductions in maternal HRV parameters are likely established as early as six weeks after conception (282), our findings confirm those of Chamchad et al. (283) who found that time domain indices of HRV were reduced by late gestation of normotensive pregnancies while frequency domain indices remained unaffected. We have extended these results and shown that alterations at six weeks postpartum remain stable later into the postpartum period. Reduced HRV and increased cardiac sympathetic activity in PE have been well-described (270-272), although postpartum cardiac electrophysiology and function has been infrequently examined in this population (273-275). 24-hour ambulatory spectral indices of HRV indicate reduced vagal modulation of heart rate at 3-6 months postpartum in PE subjects compared to time-matched controls (273), and such alterations may play an important role in the potentiation of cardiovascular risk after PE.

Observations of persistently increased blood pressure, prolonged P-wave and QRS complexes prompt concern over the severe cardiovascular risks associated with these measures (168, 284, 285). Increased P-wave and QRS duration are strongly associated with the development of arrhythmia, and combined with the pro-arrhythmic effect of reduced HRV and hypertension, this study provides mechanistic insights and possible markers of cardiovascular risk in this population. Although differences in P-wave duration were resolved after adjustment for blood pressure in our study, slightly elevated blood pressure in this population is cause for concern given the impact of even small increases in blood pressure on risk of adverse cardiovascular events (20).
Reverse remodeling of P-wave duration has been described in some situations, however the presence of significant QRS conduction delay some six months postpartum speaks to structural atrial remodeling that persists and confers an increased risk of cardiac arrhythmia. Through examination of a large retrospective cohort, the HAD MPS study showed a two-fold increased risk of not only atrial or ventricular arrhythmia, but also hospitalization for heart failure a median of 7.8 years postpartum after pre-eclampsia compared to parous women without PE (277).

Our data is supported by recent findings of cardiac remodeling and changes in cardiac function postpartum of PE. Left ventricular hypertrophy and concentric remodeling is shown at 1 year postpartum following pre-term development of PE, with increased likelihood of developing asymptomatic stage B heart failure compared to PE acquired at term, and control pregnancies (204). As left ventricular dysfunction may persist 13-18 years postpartum beyond the index pregnancy (286), identification of cardiac dysfunction soon after delivery provides an opportunity to identify high-risk individuals before other risk factors become apparent. Exercise motivated improvements offer a viable route of intervention; 12 week aerobic exercise and dietary programs are shown to improve retrograde shear rate, intima media thickness, conduit artery flow mediated dilation, and LF:HF specifically in this population, at 6-12 months postpartum (274, 275). Such data provides evidence that long-term improvements in cardiac function may be achievable through education and exercise-based intervention.

Reduced time domain parameters of heart rate variability were likely driven by the reductions in mean RR observed in PE patients at six months postpartum, and increased P Wave duration was largely affected by increases in blood pressure. Given the small sample size of our cohort however, the robustness of multivariable modelling is limited and so this does not rule out the possibility of interplay between unfavourable biophysical parameters and endothelial dysfunction described in PE subjects in the early postpartum period. Although HRV in women fell to within non-pregnant limits after uncomplicated pregnancy, it was not determined whether these
parameters were similar or different from pre-pregnant values for those same women. Similarly, pre-conception, and gestational data for PE women were not compared in this study. It remains unclear why LF:HF ratios were elevated in both postpartum groups compared to the non-pregnant state, and furthermore why LF:HF was slightly negatively correlated with systolic blood pressure. Given that non-pregnant recordings were obtained from never-pregnant subjects, our data suggest a possible long-term effect of pregnancy itself on sympathovagal balance, although this is merely speculation.

Pre-eclampsia has been likened to a cardiovascular stress test capable of identifying women at risk of hypertension, ischemic heart disease and stroke. We have provided novel evidence of joint autonomic imbalances and abnormalities in the cardiac conduction system in women as early as six weeks postpartum that remain stable at six months postpartum after PE. These findings provide evidence of subclinical alterations in cardiovascular health after PE and lend support for the value of targeted postpartum cardiovascular risk screening clinics for young women exhibiting pregnancy-related cardiovascular risk indicators (152).

6.6 Acknowledgements
The authors would like to thank Meera Vignarajah, BScH for her support in preliminary studies requiring recruitment and collection of ECG recordings for never-pregnant controls. We would also like to thank Michelle Roddy, RN, BScN, and Jessica Pudwell, MPH of Kingston General Hospital, for their assistance in subject identification.
Chapter 7

General Discussion

and Future Directions
7.1 Overall Summary

Awareness of cardiovascular disease (CVD) in women has grown substantially in recent years and has become a major public health and research focus (287, 288). Advocating for lifestyle modification to support risk management and CVD prevention has been the driving message of major heart associations, although formal risk screening is still rarely undertaken in premenopausal women. A vast amount of epidemiological data has emerged over the past few decades implicating the association between hypertensive disorders of pregnancy and future maternal risk of adverse cardiovascular outcome (95). It is only recently however, that the contribution of obstetrical history, and complications thereof, to the course of a woman’s cardiovascular health have been formally recognized (15). While current recommendations prompt inclusion of obstetrical history in formal cardiovascular risk assessments, risk management soon after the development of obstetrical “cardiovascular risk indicators” is still wanting (201).

To provide impetus and discourse for the implementation of postpartum cardiovascular health clinics, this thesis presents a series of studies examining subclinical and non-clinical parameters of cardiovascular health in women with a recent history of pre-eclampsia (PE). Thorough assessment of cardiovascular risk factors in the patients enrolled in the present studies identified a propensity for increased blood pressure as far as uncontrolled hypertension, weight retention after pregnancy and dyslipidemia in women as early as 6-12 months postpartum after PE. In addition, our use of long-term (lifetime) cardiovascular risk estimates, confirmed elevated risk for CVD in this population compared to women with uncomplicated obstetrical history.

*Beyond these observations the main findings of this thesis are the identification of alterations in subclinical markers of cardiovascular health in women with a recent history of PE. We have provided novel evidence of changes to circulating factors – namely microRNAs (miRNAs), peptides and progenitor cells – in the early postpartum period after PE compared to postpartum.*
controls. In addition we report vascular dysfunction, as well as changes in cardiac autonomic tone and conduction in women within six months after developing PE. Of particular importance, we report some of the described changes independent of apparent cardiovascular health.

7.1.1 Circulating factors and cardiovascular health

As a primary sensor for physical and chemical stimuli, the endothelium is a critical regulator of vascular tone, coagulation and inflammation (289). Degrees of endothelial activation impose a phenotype characterized by vasoconstriction with exaggerated pro-coagulant and inflammatory responses and chronic disruptions to endothelial integrity provide pivotal mechanisms for the development and progression of vascular dysfunction and disease. Whereas the state of endothelial dysfunction in PE has been well reviewed (290, 291), prior to this thesis assessment of longitudinal changes to cardiovascular homeostasis within the first year postpartum had not been previously studied.

Cardiovascular risk factors profoundly affect endothelial function due to their influence on circulating factors and fluctuating haemodynamics. In the earliest stages of atherosclerosis, coordinated changes to endothelial protein expression favours increases in oxidative stress, elevated levels of inflammatory cytokines and the up-regulation of cell surface adhesion molecules (292). Activation of the endothelium is also linked to the production of coagulation factors and triggers vasomotor responses, smooth muscle cell contraction and proliferation (292). It is this cascade of events that supports the progression of endothelial dysfunction, chronic low-grade inflammation and pro-coagulant tendencies at the blood-endothelial interface.

In Chapter 2, we reported unique patterns of miRNA expression in the plasma of severe pre-eclamptic women that were largely resolved by one year postpartum. The expression of only one miRNA, miR-221, was significantly altered at this time, and a trend of decreased miR-221 expression was observed in both mild and severe PE subjects. miR-221 and its paralogue miR-222 are implicated as important modulators of vascular biology, in particular for their positive
effect on smooth muscle cell proliferation and negative regulation of vessel formation (293, 294). Down-regulation of mi-R221 is reported however in endothelial cells (194) and adipocytes (295) exposed to inflammatory stimuli, and also in the sera of patients with advanced atherosclerosis (199). The reason for discrepancies in the direction of miR-221 (-3.2 fold expression, \( p=0.047 \)) and miR-222 expression (2.0 fold expression, \( p=0.431 \)) reported in Chapter 2 are unclear, however may be the result of differences downstream of their common seed sequence. Certainly, the complex role of the miR-221/222 cluster in inflammation mediated vascular remodeling remains to be explored and the impact of inflammation and cardiovascular risk factors on miRNA biology are still under investigation. An inverse correlation of miR-221 expression to postpartum systolic and diastolic blood pressure in our study suggests an effect of modifiable risk factors on miRNA physiology.

Inflammatory interactions influence the coagulation system through enhanced synthesis and activation of pro-coagulant proteins and compromising fibrinolysis. In keeping with our speculation that differential expression of the miR-221/222 cluster at one year postpartum in PE subjects (Chapter 2) is the result of persistent low-grade inflammation in this population, we also reported an enrichment of proteins associated with the coagulation and complement cascades in the plasma of women who had recently experienced PE (Chapter 3). Importantly, we identified alterations to the plasma proteome favouring coagulation and cardiovascular dysfunction in formerly PE women without apparent cardiovascular risk factors. Of note however, not only does inflammation activate coagulation, but coagulation in turn perpetuates the inflammatory response, initiating a vicious cycle that worsens endothelial dysfunction in the absence of intervention. As such, in subsequent chapters of this thesis, the effects of PE on other aspects of maternal postpartum physiology were explored.
7.1.2 Endothelial progenitor cells in vascular health and homeostasis after pre-eclampsia

In the modern field of vascular biology, the discovery of endothelial progenitor cells (EPCs) has been a significant advancement. Implication of these cells in mediating vascular repair and angiogenesis leaves the study of EPC physiology openly applicable to research in cancer biology, atherosclerosis, diabetes and placental development. As it pertains to pregnancy, maternal circulating levels of EPCs increase with advancing gestation (163, 246), whereas variable EPC dysfunction in women with PE (163-165) likely contributes to the impaired placentation and maternal endothelial dysfunction associated with the disorder. Whether EPC dysfunction in PE was unique to pregnancy, or persisted beyond the clinical resolution of symptoms was previously unknown however.

In Chapter 4, we described alterations to maternal EPC physiology after pregnancies complicated by PE. Exacerbated inflammation and coagulation in a pregnancy complicated by PE likely contribute to EPC dysfunction. There is increasing evidence that a transient acute inflammatory response stimulates EPC mobilization, whereas chronic inflammatory stimuli in the form of obesity, diabetes mellitus and arthritis have deleterious effects on EPC number and function (296). Endothelial cells and EPCs exposed to circulating factors, be it miRNAs, proteins, or other signaling molecules may thus influence the overall function and health of the endothelium, and eventually contribute to cardiovascular risk progression. Findings from Chapters 2 and 3 indicate that within one year postpartum PE women exhibit profiles consistent with inflammation and a hyper-coagulable state, and thus may explain the EPC dysfunction we observed in Chapter 4. Our findings of elevated levels of circulating EPCs shortly after PE, at two months postpartum, is consistent with EPC physiology after acute vascular syndromes (297). In contrast, reduced EPC number and function by six months postpartum after PE relative to controls likely reflect prolonged postpartum recovery after pregnancy and unaddressed risk factors shown to plague this population.
As a rationale for implicating EPCs in the link between PE and future risk of disease, we postulate that depleted EPC supply within or mobilization from, the bone marrow with or without impaired functional capacity leaves the endothelium without the necessary means of repair. Indeed, CFU-EPC formation \textit{in vitro} is inversely correlated to brachial artery flow mediated dilation and Framingham risk scores (235). More importantly, circulating progenitor cells independently predict incident cardiovascular events. Although only a few studies to date have evaluated the prognostic value of EPCs, patient groups as large as 500 and follow-up ranging from ten months to two years report the predictive value of EPCs in patients at low risk and with stable or acute coronary syndromes for composite cardiovascular endpoints (236, 298) and all-cause survival (299, 300). Circulating endothelial cell and EPC levels correlate well to conduit artery flow mediated dilation and cardiovascular risk (235), and our observations of reduced EPC number at six months postpartum may provide an explanation for findings of variable vascular dysfunction reported after PE.

\textbf{7.1.3 Microvascular function after pre-eclampsia}

Inactivation of nitric oxide (NO) by super-oxides and other reactive oxygen species (ROS) occurs in hypertension, type II diabetes, and heart failure (301), and manifest in attenuated endothelial-dependent vasodilation. Reduced vasodilator bioavailability and excess reactive oxygen species (ROS) lend toward states of inflammation and coagulation perpetuating feed forward mechanisms of worsening endothelial dysfunction. These findings are consistent with observations made in PE subjects who exhibit impaired endothelial-dependent vasodilation in the conduit vessels although, impairments in macro vessel function are also reported to manifest or persist after pregnancies complicated by PE (126, 219, 238).

The microcirculation is hypothesized to be the initial site of endothelial damage in subjects at risk of CVD, and dysfunction at the level of the micro vessels may precede large artery stiffening. Interestingly, cutaneous vessels of women with PE are instead \textit{hypersensitive} to pharmacological
stimulation by acetylcholine (ACh); a finding we have confirmed to persist as far as six months postpartum (Chapter 5). The cause for this discrepancy is unclear, and studies from our group have confirmed that hormonal fluctuations due to the menstrual cycle or hormonal contraceptive use are unlikely culprits (0).

One other study performed in 25 women with a recent history of PE ranging from 3-11 months postpartum reported similar findings to those we describe in Chapter 5 and in the absence of mechanisms to explain their findings, the authors proposed a compensatory response of the microcirculation to an impaired vasodilatory response of the larger vessels as a unique form of “(micro)angiopathy” associated with PE (129). Underlying microvascular dysfunction in PE subjects both during pregnancy and the postpartum may be disruptions to circulating factors including miRNAs (Chapter 2), proteins of the coagulation-complement cascades (Chapter 3) and EPC mobilization and function (Chapter 4). In keeping with findings from Chapter 2 demonstrating no differences in postpartum miRNA expression between women who developed mild and severe PE, we report in Chapter 5, no differences in the effect of early/late onset or mild/severe PE on the degree of microvascular dysfunction after pregnancy (0). Acknowledging that sample sizes in the study were small, the exact role of these factors in mediating microvascular function, either in health or disease, however, has yet to be established.

In summary, the literature suggests that the microcirculation transitions from being hyper-reactive to circulating vasodilators during and soon after PE, as shown by our group (229) and others (129-131), to hypo-reactive decades postpartum (132), in consensus with observations of dysfunction in the larger conduit vessels. We postulate that this “transition” may reflect a limited capacity of the microvasculature to cope with worsening local oxidant stress and inflammation long-term.
7.1.4 Implications to alterations in the electrocardiogram after pre-eclampsia

Through prospective longitudinal follow-up of 64 PE women, Melchiorre et al. found evidence of subclinical left ventricular dysfunction and hypertrophy by one year postpartum and global myocardial impairment (204). The effect of early-onset development of PE was greater than late-onset of the disorder, consistent with the greater risk of CVD associated with severe and early-onset PE (94). Importantly, the authors found that the left ventricular alterations they observed were consistent with a diagnosis of stage B (asymptomatic) heart failure (204). Indeed, 70% of women who developed early onset PE were found to have stage B heart failure, whereas the prevalence of this asymptomatic heart failure in adults >45 years of age is only 34% (302).

Although measures of cardiac function per se, were not examined in Chapter 6, we used non-invasive recordings of heart rate to identify stable alterations in heart rate variability (HRV) and signal averaged P wave and QRS durations – parameters strongly associated with risk of dysrhythmias – in women as early as six weeks and six months postpartum. Reductions in HRV of PE subjects indicated increases in sympathetic input to the heart relative to controls; autonomic regulation in PE characterized by sympathetic dominance is supported by previous reports of not only reduced HRV in the time of pregnancy and PE (169, 271), but also vascular sympathetic over-activity and reduced compliance to volume load (303-305). Clinically, P-wave and QRS deflections correspond to the coordinated depolarization of the atria and ventricles, respectively. Prolongation of these intervals along the electrocardiograph (ECG) reflects delayed depolarization of the atrial and ventricular myocardium and commonly occurs with left atrial abnormalities and ventricular hypertrophy (306).

Interestingly, in addition to its effects on EPC mobilization, and endothelium-dependent vasodilation, reduced NO production and bioavailability also appear to play a role in cardiac remodeling. Mice deficient in endothelial nitric oxide synthase (eNOS) exhibit a greater severity of left ventricular dysfunction and remodeling after acute myocardial infarction compared to wild-type controls (307). Conversely, eNOS overexpression promotes recovery of cardiac
dysfunction after myocardial infarction (308). In this regard, in women who develop PE, dysfunction of circulating factors (e.g. miRNA; Chapter 2) affecting EPC and vascular function (Chapters 4, 5) incurred as a result of PE that persist after delivery may contribute to alterations in cardiac tone and conduction (Chapter 6) and ultimately cardiac dysfunction. Although the changes observed in Chapter 6 were accompanied by elevated blood pressure – specifically, prolongation of P-wave duration was dependent on blood pressure – this demonstrates an end organ effect of blood pressure on cardiac physiology and only underscores the importance of appropriate postpartum risk factor management.

Increased risk of premature heart failure and dysrhythmias after placental syndromes of pregnancy has been demonstrated as early as eight years after the complications (277). Left ventricular and atrial alterations are evident 6 months after index pregnancies by ECG (309), and asymptomatic heart failure based on left ventricular hypertrophy or left ventricular diastolic or systolic dysfunction is evident at just one year postpartum (204) – all are risk factors for dysrhythmias. Further, the presence of the metabolic syndrome (MetS) in women with a history of PE is shown to increase the risk of cardiac diastolic dysfunction by a factor of four (310). Given the high risk of MetS after PE, these findings highlight the importance of reduction and control of modifiable risk factors to the prevention of CVD progression.

Stability of reduced HRV and prolonged signal-averaged P-wave and QRS durations from six weeks to six months postpartum in the subjects examined in Chapter 6 support a hypothesis of subclinical dysfunction persisting beyond PE to affect later maternal cardiovascular risk. In consideration that we have also provided evidence that ECG readings are not affected by hormonal contraceptive treatment, or by cyclic hormonal fluctuations (0), findings from Chapter 6 provide important information regarding the potential utility of ECG measurements as a non-invasive, cost-effective means of risk stratification in women with a recent history of PE.
7.2 Summary of original contributions

Our studies are among the first to examine longitudinal changes in maternal cardiovascular health in the earliest times after PE. In doing so we highlight the early postpartum period as a window of opportunity to provide targeted screening, education and management of cardiovascular risk factors in women after hypertensive disorders of pregnancy. Although recent surveys of women with a history of PE identify them as a highly motivated population (311), it appears that the timing and nature of postpartum intervention plays a key role in their receptivity to lifestyle change (312). We propose that it is within the first year postpartum that a woman is highly motivated towards lifestyle improvements and also when risk reduction may be most beneficial both for subsequent pregnancy and long term maternal health outcomes. In addition to confirming increased risk for CVD as early as six months after index pregnancies, the studies presented in this thesis revealed that minimally invasive biomarkers of subclinical dysfunction may be used to demonstrate distinct postpartum differences between women with a recent history of PE, and those without obstetrical complications (Figure 7.1). In answer to the original presented hypothesis, our studies examining early postpartum cardiovascular health and risk after PE have revealed:

- Differential expression of the miR-221/miR222 cluster (Chapter 2).
- Enrichment of plasma proteins associated with coagulation and inflammation (Chapter 3).
- Lower reserves of EPCs in maternal circulation (Chapter 4).
- Alterations to vascular function in the form of increased microvascular reactivity (Chapter 5).
- Sympathetic dominance of cardiac autonomic regulation and delayed atrio-ventricular depolarization (Chapter 6).
Figure 7.1. Postpartum dysfunction after pregnancies complicated by PE.

The implications of PE extend beyond that of the two-stage model. The background upon which PE develops exacerbates underlying risk factors, that if unaddressed postpartum may perpetuate the characteristics of endothelial dysfunction after delivery. Alterations to circulating factors and microvascular and cardiac abnormalities may be detected within the first year postpartum, and likely contribute to long-term maternal outcomes of cardiovascular disease.
7.3 Future Directions

7.3.1 Assessment of proliferative and vasculogenic potential of ECFCs

Findings in Chapter 4 indicated reduced circulating levels of EPCs with impaired angiogenic capacity at six months postpartum after PE. EPCs represent a heterogeneous population of cells however, and cells generated \textit{in vitro} by short term colony forming unit assay represent but one sub-population of EPCs that cannot be clonally expanded. Colony forming unit endothelial cells (CFU-ECs), derived from circulating angiogenic cells (CACs), as generated in Chapter 4 are shown to increase blood vessel perfusion and capillary density in mouse hind limb models of ischemia (313) although appear incapable of forming \textit{de novo} blood vessels when seeded in Matrigel or \textit{in vivo} (314). There exists a second distinct EPC sub-population that does not arise in culture until several weeks after seeding. This population of highly proliferative endothelial-lineage cells arises as a cobblestone monolayer, similar to patterns observed from cultured human umbilical vein endothelial cells. Aptly termed ‘endothelial colony forming cells’ (ECFCs), these cells form capillary-like tubules in three-dimensional culture and incorporate into newly forming blood vessels both \textit{in vitro} and \textit{in vivo}. As such, this population likely represents a class of ‘true’ endothelial progenitors.

As the phenotypes and functions of these “early” and “late” outgrowth cells continue to be clarified, it is becoming apparent that CACs and ECFCs work in concert to facilitate vascular remodeling and repair in response to physical trauma and environmental stressors. Indeed CACs and ECFCs have been shown to synergistically act to promote vascular repair and/or neovascularization beyond that achieved by injection of each alone into animal models of vascular trauma (315).

We have made preliminary attempts at the isolation of ECFC from maternal blood. Although ECFC colonies were successfully derived \textit{in vitro} from samples obtained in postpartum mothers, maintenance of these cells long-term for subsequent phenotypic and functional analysis proved
unfruitful. Given the cooperative functions of these two EPC populations in promoting angiogenesis and endothelial repair however, we maintain that the assessment of ECFC populations in these women will lead to a better understanding of PE-associated endothelial dysfunction. Currently there exists no data on maternally-derived ECFCs in PE either during pregnancy or postpartum.

**7.3.2 Assessment of macro- and microvascular function in pre-eclampsia**

Unlike assessment of flow-mediated dilation of the brachial artery by ultrasonography, microvascular end function studies have not been standardized. Induction of vasodilation in the cutaneous circulation induces redundant mechanisms that rely on the activation of multiple vascular signaling pathways including NO, prostaglandin and endothelial derived hyperpolarizing factor (EDHF)-dependent pathways (316). Given this, micro vessel function may be uniquely equipped to respond to the residual effects of acute inflammation associated with PE compared to those of larger vessels by changing the magnitude of involvement of a single vasodilation pathway based on impairment of another in a disease-specific manner. Plasticity of the microvascular endothelium in cases of pathology has been previously described (260), although not in PE.

Given the unique presentation of microvascular dysfunction associated with PE (129, 131, 229), the mechanisms underlying both macro- and microvascular dysfunction in this population require exploration. First, measurements of brachial artery and cutaneous micro vessel vasodilation in response to shear-stress and/or pharmacological stimuli should be examined to determine whether these parameters are correlated in PE and normotensive subjects. Second, examination of the effect of NO synthase, cyclooxygenase inhibitors or EDHF (e.g. NG-monomethyl-L-arginine, aspirin, or ouabain respectively) on vascular responses should be performed to shed light on the pathways contributing to vasodilation responses in the PE population.
7.3.3 Examination of miRNA expression patterns in ECFCs and correlation with vascular function and cardiovascular risk

Involvement of miRNAs in the pathogenesis of PE has been recently demonstrated and is an emerging field of investigation (177, 187, 192). Although much progress has been made toward establishing miRNAs as important regulators in PE and vascular dysfunction, how miRNAs regulate target genes to contribute to PE and future development of CVD remains unclear. Observations from Chapters 2 and 3 implicate persistent inflammation and coagulation after PE as a background upon which endothelial dysfunction is exacerbated. EPCs are shown to be enriched for miRNA and dysregulation of miRNA implicated in angiogenesis and inflammation, including the miR-221/222, are observed in patients with coronary artery disease (317).

Examination of miRNA profiles and their effects on target gene expression in ECFCs isolated from women who have developed PE will provide insight into the miRNA regulation of endothelial function in these women. Furthermore, given the implications of EPC physiology on vascular function, the correlation of differentially expressed miRNAs in ECFCs to clinical manifestations of vascular dysfunction and cardiovascular risk in recently PE subjects may provide insight into the mechanisms underlying increased postpartum microvascular reactivity observed in Chapter 5.

7.3.4 Assessment of exercise intervention on ECFCs, miRNA expression patterns and vascular function

Although EPCs and miRNA signaling are unlikely to be a viable therapeutic option to individuals in the earliest stages of cardiovascular risk progression, their utility may lie instead as biomarkers of risk and risk improvement. It is well known that exercise improves endothelial function in patients with CVD (318, 319). Indeed, exercise increases bone-marrow EPC reserves and upregulates EPC release into peripheral blood (318, 320), and studies have demonstrated exercise-induced alterations to miRNAs – specifically those known to play key mechanistic roles in regulating inflammatory function. eNOS activation promotes not only NO-mediated
vasodilation of blood vessels, but also induces up-regulation of matrix metalloproteinases and release of soluble Kit ligand that confer signals to enhance mobilization of EPCs into the vascular niche. Sustained exercise training increases vascular NO concentrations, and so it is perhaps unsurprising with regular physical exercise there is an improvement in circulating EPC levels, and also vascular function in patients. This effect has also been described in individuals with CAD (321) and MetS (322). In addition, the use of 3-Hydroxy-3-methylglutaryl coenzyme A reductase inhibitors, or statins, affects miRNA expression and stimulates EPC mobilization and improves cardiac and vascular function through increasing endothelial NO production, reducing inflammation and decreasing platelet deposition (323-325). The beneficial impact of exercise on vascular health may therefore be attributable to improved mobilization and function of EPCs through alterations in miRNA signaling.

Any significant findings of the proposed studies should be considered for reassessment after structured exercise intervention to determine their utility as biomarkers of risk improvement. In 2014, under the Mothers’ Health Education, Research and Screening (MotHERS) program at Kingston General Hospital, a pilot study was initiated to examine the impact of education and exercise-based intervention on women after pregnancy. The Health Improvement after Pregnancy (HIP) Program is a mobile website-based program that provides daily and weekly motivation for physical activity and nutritional guidance through interactive videos. Women are randomized to the HIP Program or a control group that does not receive guided lifestyle interventions, and changes in biophysical profiles are monitored over the course of a six month period. As a structured short-term lifestyle modification program, the HIP program in particular provides an opportunity to monitor the effect of exercise on microvascular and ECG parameters women with a recent history of PE. Should notable differences in the measured parameters be identified at the end of the intervention period, follow-up studies involving ECFC culture and circulating and cellular miRNA analysis would be of interest.
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Appendix A

Additional cardiovascular risk parameters taken at 1 year postpartum in select PE-NET patients included in Chapter 2

Table A.1. Additional postpartum cardiovascular risk variables taken in Chapter 2.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Pre-eclamptics</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum triglycerides (mmol/L)</td>
<td>1.28±1.29</td>
<td>1.87±2.12</td>
<td>0.3532</td>
</tr>
<tr>
<td>Serum total cholesterol (mmol/L)</td>
<td>4.15±0.63</td>
<td>4.84±0.66</td>
<td>0.0069*</td>
</tr>
<tr>
<td>Serum HDL cholesterol (mmol/L)</td>
<td>1.51±0.25</td>
<td>1.33±0.42</td>
<td>0.1542</td>
</tr>
<tr>
<td>Serum LDL cholesterol (mmol/L)</td>
<td>2.19±0.59</td>
<td>2.92±0.71</td>
<td>0.0001*</td>
</tr>
<tr>
<td>Serum fasting glucose (mmol/L)</td>
<td>4.89±0.33</td>
<td>5.08±0.36</td>
<td>0.1441</td>
</tr>
<tr>
<td>Microalbumin:creatinine (mg/mmol)</td>
<td>0.63±0.77</td>
<td>2.71±2.98</td>
<td>0.0289*</td>
</tr>
<tr>
<td>Maternal waist circumference (cm)</td>
<td>85.53±11.48</td>
<td>97.46±14.69</td>
<td>0.0186*</td>
</tr>
<tr>
<td>Maternal hip circumference (cm)</td>
<td>103.44±10.71</td>
<td>112.2±15.58</td>
<td>0.0786</td>
</tr>
</tbody>
</table>

Data presented as mean±standard deviation. Parameters included in the above table contributed to calculations of lifetime cardiovascular risk and considered in the diagnosis of the metabolic syndrome. *Statistically significant, p<0.05.
Appendix B

Correlation of miR-221 expression to obesity parameters and lifetime cardiovascular risk

Figure B.1. Correlation of postpartum miR-221 expression levels to obesity parameters and cardiovascular risk.

Expression of miR-221 was significantly correlated to parameters of obesity at one year postpartum; (A) body mass index, BMI; (B) waist circumference and (C) hip circumference. (D) Stratification of patients by lifetime estimates of cardiovascular risk identified no correlation of miR-221 expression to categorical estimates of cardiovascular risk, CVR; <39% = low risk, ≥39% = high risk.
Appendix C

Top ten high-level biological functional categories associated with Control and PE proteome profiles

Figure C.1. The top ten high-level functional categories associated with Control and PE proteome profiles.
Appendix D

Morphology and Quantification of CFU-EPC

Figure D.1. Colony forming unit characterization.

(A) True colonies appear with a central core of rounded cells, with elongated spindle-like cells radiating from the periphery, perpendicular to the central colony surface. (B) Colonies with disorganized spindle cell organization or (C) without spindle cell association around the central colony were recorded, but not counted as true CFU-Hill.
## Appendix E

### Comparison of patient baseline characteristics by time-point of participation in Chapter 4

#### Table E.1. Stratification of patient characteristics by time-point of participation.

<table>
<thead>
<tr>
<th></th>
<th>PD (n=6)</th>
<th>2M PP (n=13)</th>
<th>6M PP (n=13)</th>
<th>P-Value</th>
<th>PD (n=5)</th>
<th>2M PP (n=8)</th>
<th>6M PP (n=12)</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal age (y)</td>
<td>31.5±4.3</td>
<td>31.8±3.7</td>
<td>31.8±3.7</td>
<td>NS</td>
<td>34.0±5.5</td>
<td>30.4±4.4</td>
<td>31.6±5.6</td>
<td>NS</td>
</tr>
<tr>
<td>Maternal race, n (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>White</td>
<td>6 (100)</td>
<td>12 (92.3)</td>
<td>12 (92.3)</td>
<td>NS</td>
<td>5 (100)</td>
<td>8 (100)</td>
<td>13 (86.7)</td>
<td>NS</td>
</tr>
<tr>
<td>Other</td>
<td>0 (0)</td>
<td>1 (7.7)</td>
<td>1 (7.7)</td>
<td></td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>2 (13.3)</td>
<td></td>
</tr>
<tr>
<td>Parity, n (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Primiparous</td>
<td>2 (33.3)</td>
<td>3 (23.1)</td>
<td>3 (23.1)</td>
<td>NS</td>
<td>5 (100)</td>
<td>8 (100)</td>
<td>13 (86.7)</td>
<td>NS</td>
</tr>
<tr>
<td>Multiparous</td>
<td>4 (66.6)</td>
<td>10 (76.9)</td>
<td>10 (76.9)</td>
<td></td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>2 (13.3)</td>
<td></td>
</tr>
<tr>
<td>Maternal BMI (kg/m²)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre-Pregnancy</td>
<td>29.8±4.7</td>
<td>25.8±4.9</td>
<td>25.8±4.9</td>
<td>NS</td>
<td>27.7±4.7</td>
<td>23.6±3.4</td>
<td>24.7±3.6</td>
<td>NS</td>
</tr>
<tr>
<td>Delivery</td>
<td>32.4±3.5</td>
<td>29.8±4.3</td>
<td>29.8±4.3</td>
<td>NS</td>
<td>34.6±5.8</td>
<td>30.4±4.5</td>
<td>31.1±4.1</td>
<td>NS</td>
</tr>
<tr>
<td>Pregnancy weight gain (kg)</td>
<td>8.5±5.8</td>
<td>10.9±5.8</td>
<td>10.9±5.8</td>
<td>NS</td>
<td>19.6±6.9</td>
<td>19.2±5.7</td>
<td>17.1±6.7</td>
<td>NS</td>
</tr>
<tr>
<td>Appropriate weight gain, n (%)</td>
<td>4 (66.7)</td>
<td>9 (69.2)</td>
<td>9 (69.2)</td>
<td>NS</td>
<td>2 (40.0)</td>
<td>3 (37.5)</td>
<td>7 (46.7)</td>
<td>NS</td>
</tr>
<tr>
<td>Excess weight gain, n (%)</td>
<td>2 (33.3)</td>
<td>4 (30.8)</td>
<td>4 (30.8)</td>
<td></td>
<td>3 (60.0)</td>
<td>5 (62.5)</td>
<td>8 (53.3)</td>
<td></td>
</tr>
<tr>
<td>Systolic Blood Pressure (mmHg)</td>
<td>118.7±9.9</td>
<td>119.8±13.1</td>
<td>119.8±13.1</td>
<td>NS</td>
<td>175.8±27.0</td>
<td>161.4±13.3</td>
<td>164.7±23.7</td>
<td>NS</td>
</tr>
<tr>
<td>Diastolic Blood Pressure (mmHg)</td>
<td>67.3±7.1</td>
<td>69.8±6.3</td>
<td>69.8±6.3</td>
<td>NS</td>
<td>107.0±11.0</td>
<td>104.9±7.5</td>
<td>104.4±12.7</td>
<td>NS</td>
</tr>
<tr>
<td>GA at delivery/follow-up (wks)</td>
<td>40.3±1.1</td>
<td>39.9±1.2</td>
<td>39.9±1.2</td>
<td>NS</td>
<td>29.1±3.9</td>
<td>35.4±5.0</td>
<td>36.0±3.9</td>
<td>*</td>
</tr>
</tbody>
</table>

Data presented as mean±standard deviation. PD, pre-delivery; 2M PP, 2 months postpartum; 6M PP, 6 months postpartum; BMI, body mass index. Fisher’s exact test. Patient characteristics did not differ significantly when stratified by time-point of participation. Data compared by one-way ANOVA within patient groups. *p<0.05.
### Appendix F

**Comparison of Chapter 4 patient characteristics with the PE-NET cohort**

Table F.1. Comparison of baseline characteristics to PE-NET Cohort.

<table>
<thead>
<tr>
<th></th>
<th>Pre-eclampsia</th>
<th>Normotensive Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PE-EPC (n=19)</td>
<td>PE-NET (n=99)</td>
</tr>
<tr>
<td>Maternal age (y)</td>
<td>32.2±5.4</td>
<td>30.3±5.7</td>
</tr>
<tr>
<td>Maternal race, n (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>White</td>
<td>17 (89.5)</td>
<td>95 (96.0)</td>
</tr>
<tr>
<td>Other</td>
<td>2 (10.5)</td>
<td>4 (4.0)</td>
</tr>
<tr>
<td>Maternal education level, n (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>High school or less</td>
<td>1 (5.3)</td>
<td>16 (16.1)</td>
</tr>
<tr>
<td>Postsecondary incomplete</td>
<td>0 (0)</td>
<td>9 (9.1)</td>
</tr>
<tr>
<td>Postsecondary complete</td>
<td>18 (94.7)</td>
<td>74 (74.8)</td>
</tr>
<tr>
<td>Parity, n (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Primiparous</td>
<td>17 (89.5)</td>
<td>64 (64.6)</td>
</tr>
<tr>
<td>Multiparous</td>
<td>2 (10.5)</td>
<td>35 (35.4)</td>
</tr>
<tr>
<td>Maternal BMI (kg/m²)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre-Pregnancy</td>
<td>24.9±3.8</td>
<td>26.7±5.7</td>
</tr>
<tr>
<td>Delivery</td>
<td>31.2±4.2</td>
<td>33.2±6.2</td>
</tr>
<tr>
<td>Pregnancy weight gain, n (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Appropriate weight</td>
<td>9 (47.4)</td>
<td>35 (35.4%)</td>
</tr>
<tr>
<td>Excess weight gain</td>
<td>10 (52.6)</td>
<td>64 (64.6%)</td>
</tr>
<tr>
<td>GA at delivery (wks)</td>
<td>34.5±4.8</td>
<td>35.7±3.7</td>
</tr>
</tbody>
</table>

Data presented as mean±standard deviation. PE-EPC, pre-eclampsia endothelial progenitor cell cohort; CTRL-EPC, control (uncomplicated pregnancy) endothelial progenitor cell cohort; BMI, body mass index; GA, gestational age; NS, not significant. †Fisher’s exact test. ‡Versus diagnosis-matched PE-NET cohort.
Figure G.1. Endothelial phenotypic characterization of HUVEC.

Human umbilical vein endothelial cells (HUVEC) display characteristics typical of mature endothelial cells; (A) positive uptake of Dil-conjugated acetylated low density lipoprotein and (B) binding of AlexaFluor 488-conjugated Griffonia Simplicifolia Lectin. (C) Images are combined to demonstrate dual positivity.
Appendix H
Additional cardiovascular risk parameters taken at six months postpartum in patients included in Chapter 4

Table H.1. Additional postpartum cardiovascular risk variables taken in Chapter 4.

<table>
<thead>
<tr>
<th></th>
<th>Normotensive Control (n=8)</th>
<th>Pre-eclampsia (n=14)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>hsCRP (mg/L)</td>
<td>3.2±5.8</td>
<td>3.4±4.8</td>
<td>NS</td>
</tr>
<tr>
<td>Fasting glucose (mmol/L)</td>
<td>4.6±0.3</td>
<td>4.7±0.3</td>
<td>NS</td>
</tr>
<tr>
<td>Triglycerides (mmol/L)</td>
<td>0.8±0.3</td>
<td>1.1±0.6</td>
<td>NS</td>
</tr>
<tr>
<td>Total cholesterol (mmol/L)</td>
<td>4.0±0.9</td>
<td>4.9±0.9</td>
<td>&lt;0.05*</td>
</tr>
<tr>
<td>HDL Cholesterol (mmol/L)</td>
<td>1.3±0.3</td>
<td>1.5±0.3</td>
<td>NS</td>
</tr>
<tr>
<td>LDL Cholesterol (mmol/L)</td>
<td>2.2±0.7</td>
<td>2.8±0.6</td>
<td>NS</td>
</tr>
<tr>
<td>Microalbumin/Creatinine (mg/mmol)</td>
<td>0.7±0.5</td>
<td>2.7±4.5</td>
<td>NS</td>
</tr>
</tbody>
</table>

Data presented as mean±standard deviation. Parameters included in the above table contributed to calculations of lifetime cardiovascular risk and considered in the diagnosis of the metabolic syndrome. *Fisher’s exact test; NS, not significant; †Statistically significant, p<0.05.
Appendix I

Comparison of baseline cutaneous temperature and blood flow in study subjects included in Chapter 5

Table I.1. Baseline cutaneous blood flow and temperature.

<table>
<thead>
<tr>
<th></th>
<th>Normotensive control</th>
<th>PE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3TM</td>
<td>6W</td>
</tr>
<tr>
<td>Baseline Flux (PU)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACh Probe</td>
<td>10.83±4.51</td>
<td>11.82±5.48</td>
</tr>
<tr>
<td>SNP Probe</td>
<td>9.58±3.71</td>
<td>13.84±8.75</td>
</tr>
<tr>
<td>Skin Temperature (°C)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACh Probe</td>
<td>28.26±1.60</td>
<td>28.25±1.3</td>
</tr>
<tr>
<td>SNP Probe</td>
<td>28.30±1.44</td>
<td>28.26±1.10</td>
</tr>
</tbody>
</table>

Data are presented as mean±standard deviation. PU, perfusion units; ACh, acetylcholine; SNP, sodium nitroprusside; 3TM, third trimester; 6W, six weeks postpartum; 6M, six months postpartum. Data were analyzed by paired one-way ANOVA and t-test within control and PE data respectively. Baseline perfusion and skin temperatures did not significantly change across patient visits. Comparison between patient groups of matched time points by un-matched t-test identified no significant differences in baseline perfusion or skin temperature.
Appendix J

Examination of the effect of the menstrual cycle and monophasic oral contraceptives on laser Doppler flowmetry measurements

J.1 Methods and Materials

J.1.1 Subject identification

The study was approved by the Queen’s University ethics committee. All subjects gave written, informed consent. Twenty-five, never-pregnant women were recruited for the study based on volunteer participation from the general student and staff community of Queen’s University, at Kingston, Ontario. Participants were included in the study based on hormonal contraceptive use: 1) no hormonal contraceptive use (CTRL) and 2) using monophasic oral contraceptives (OC). Fifteen CTRL women with self-reported regular menstrual cycles participated in the study, each indicating a normal cycle length ranging between 26 – 30 days. Ten additional subjects were included based on voluntary use of monophasic combination oral contraceptives. A monophasic combination pill type includes the same dose of estrogen ethinyl and progesterone over a 21 day period followed 7 day allotment of placebo pills. Details of combination pills consumed by study participants are given in Error! Reference source not found..

Subjects who had stopped using oral contraceptive drugs within the preceding four months, or who had not yet achieved regular menses since stopping oral contraceptive use were not included in the study. Similarly, OC subjects were included only if they had been adhering to their current prescription for a minimum of 4 months, and who achieved regular menses with their monthly abstention from the prescribed oral contraceptives.
Table J.1. Summary of oral contraceptives used by participants.

<table>
<thead>
<tr>
<th>Oral contraceptive brand name</th>
<th>Number of Subjects</th>
<th>Ethinyl Estradiol Dose</th>
<th>Progestin Formulation and Dose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alesse</td>
<td>6</td>
<td>0.02mg</td>
<td>0.10 mg levonorgestrel</td>
</tr>
<tr>
<td>Yasmin</td>
<td>3</td>
<td>0.03mg</td>
<td>3mg drospirenone</td>
</tr>
<tr>
<td>Marvelon</td>
<td>1</td>
<td>0.03mg</td>
<td>0.15mg desogestrel</td>
</tr>
</tbody>
</table>
Individuals with a body mass index (BMI) greater than 30kg/m², irregular menstrual cycles, history of hypertension, cardiovascular disease, and use of cardiovascular medications, history of smoking, diabetes, or currently using an intrauterine device or other hormonal contraceptive options were excluded from the study.

J.1.2 Calculation of cycle phase
Each subject was studied three times over the course of a given menstrual cycle. Cycle phase was calculated based on normal cycle duration, and onset of menses. For CTRL subjects, this included menses, follicular (proliferative) and luteal (secretory) phases. Follicular and luteal phases were defined based on self-reported 26, 28 or 30 day cycles: menses (days 2-4 after spotting), follicular (days 8-12, days 10-14, days 12-16 respectively), luteal (days 18-22, days 20-24, days 22-28 respectively). These phases were determined by comparing previously published studies with similar phase distinctions and consultation with an OB/GYN clinician. Cycle phase and timing of measurements were further confirmed by tracking the onset of consecutive menses periods over the investigatory period. OC subjects were assessed once during menses (days 2-4 after spotting), and twice over the course of their medicated phases (Early Medicated Phase, Late Medicated Phase) to correspond with the time points of assessment used for CTRL subjects.

J.1.3 Laser Doppler flowmetry
Measurements were taken in a temperature regulated environment with subjects lying in a semi-supine position with forearms supported on a pillow. Subjects were asked to abstain from caffeine consumption and use of over-the-counter medications the morning prior to testing. Cutaneous perfusion was measured by laser Doppler flowmetry (Moor Instruments Ltd., Axminster, UK). Two combined temperature and laser Doppler fluximetry probes, surrounded by an iontophoresis Perspex chamber were secured to the volar aspect of the forearm using double-sided adhesive tape. Iontophoresis chambers were adhered 4 cm apart, and 4-5 cm from the antecubital fossa. Areas with broken skin and superficial veins were avoided. Continuous recordings of cutaneous
perfusion and skin temperature were collected using a laser Doppler flow monitor (moorVMS-LDF, Moor Instruments Ltd, Axminster, UK) with data recorded in arbitrary perfusion units of flux (PU).

**J.1.4 Iontophoresis**

1% solutions of acetylcholine (ACh; Miochol\textsuperscript{®}-E, Bausch\&Lomb Inc., Vaughn, Canada) and sodium nitroprusside (SNP; Nipride, Hospira Inc., Saint-Laurent, Canada) were introduced into the anodal and cathodal chambers for assessment of endothelial-dependent and -independent function, respectively. The vehicle for drug delivery was de-ionized sterile water. Following a 10-minute period of baseline perfusion recordings, dose-response curves to ACh and SNP were obtained by the step-wise application of currents (5 μA, 10 μA, 15 μA, 20 μA, 50 μA, and three applications of 100 μA) by an iontophoresis controller (MIC2, Moor Instruments Ltd, Axminster, UK). Currents were applied each for 10 seconds followed by 2 minute recording periods for a total charge delivery of 4mC. Corresponding changes in cutaneous blood flow was assessed using the laser Doppler flow monitor (moorVMS-LDF, Moor Instruments Ltd, Axminster, UK).

**J.1.5 Statistical analysis**

Demographic variables are presented as mean ± standard deviation (SD). An unpaired t-test or one-way analysis of variance (ANOVA) with Bonferroni post hoc test was used to compare continuously distributed variables. Dedicated software (moorVMS-PC V3.1, Moor Instruments Ltd, Axminster, UK) was used to analyze individual vascular responses to delivery of ACh and SNP by iontophoresis. Vasodilation was calculated as the ratio between the maximum achieved perfusion for each iontophoretic dose to the averaged 10-minute baseline perfusion. Data was then exported to GraphPad Prism 5 for statistical analyses and comparison within and between subject groups. Comparison within subject groups, across experimental time-points were analyzed by matched two-way ANOVA. Comparisons between subject groups, by time-point of
measurement were achieved by unpaired two-way ANOVA. Statistical significance was accepted if the null hypothesis could be rejected at $p<0.05$.

**J.2 Results**

**J.2.1 Participants**
A summary of physical characteristics of study participants is provided in Table J.2. CTRL and OC participants were similar for age, height, weight and BMI. Systolic and diastolic blood pressures were significantly elevated amongst users of oral contraceptives, although baseline cutaneous blood flow at each probe site of measurement remained similar by cycle phase and between groups.

**J.2.2 Microvascular reactivity**
Microvascular responses to ACh did not differ by phase of menstrual cycle amongst CTRL subjects. In addition, there were no fluctuations in microvascular vasodilation to ACh in OC subjects during medicated or un-medicated phases. Comparison of CTRL and OC groups found no alterations in microvascular reactivity to ACh at matched time-points of assessment. Similarly, microvascular responses to the delivery of SNP did not differ by phase of cycle or oral contraceptive use. Findings are summarized in Figure J.1.
### Table J.2. Physical characteristics of study participants.

<table>
<thead>
<tr>
<th></th>
<th>CTRL (n=15)</th>
<th>OC (n=10)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age, yrs</strong></td>
<td>23.5±3.50</td>
<td>22.2±1.75</td>
<td>0.2787</td>
</tr>
<tr>
<td><strong>Height, cm</strong></td>
<td>164.7±6.55</td>
<td>166.2±4.44</td>
<td>0.5658</td>
</tr>
<tr>
<td><strong>Weight, kg</strong></td>
<td>60.4±8.48</td>
<td>60.3±8.45</td>
<td>0.9605</td>
</tr>
<tr>
<td><strong>BMI, kg/m²</strong></td>
<td>22.2±2.69</td>
<td>21.8±2.84</td>
<td>0.7230</td>
</tr>
<tr>
<td><strong>Systolic Blood Pressure</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phase 1</td>
<td>102.2±8.06</td>
<td>109.8±5.884</td>
<td>0.0177</td>
</tr>
<tr>
<td>Phase 2</td>
<td>103.8±6.25</td>
<td>110.6±6.87</td>
<td>0.0174</td>
</tr>
<tr>
<td>Phase 3</td>
<td>100.3±5.12</td>
<td>111.4±4.12</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td><strong>Diastolic Blood Pressure</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phase 1</td>
<td>64.8±5.76</td>
<td>75.6±6.53</td>
<td>0.0002</td>
</tr>
<tr>
<td>Phase 2</td>
<td>68.2±7.57</td>
<td>74.6±8.53</td>
<td>0.0610</td>
</tr>
<tr>
<td>Phase 3</td>
<td>65.5±7.09</td>
<td>73.6±5.60</td>
<td>0.0058</td>
</tr>
<tr>
<td><strong>Reported Cycle Length, days</strong></td>
<td>29.3±0.976</td>
<td>28±0</td>
<td>-</td>
</tr>
<tr>
<td><strong>Baseline perfusion at ACh probe, (PU)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phase 1</td>
<td>12.0±7.47</td>
<td>9.69±5.18</td>
<td>0.8107</td>
</tr>
<tr>
<td>Phase 2</td>
<td>9.39±4.14</td>
<td>8.78±5.28</td>
<td>&gt;0.9999</td>
</tr>
<tr>
<td>Phase 3</td>
<td>8.75±4.14</td>
<td>8.44±3.75</td>
<td>&gt;0.9999</td>
</tr>
<tr>
<td><strong>Baseline perfusion at SNP probe, (PU)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phase 1</td>
<td>8.20±4.01</td>
<td>8.24±2.55</td>
<td>&gt;0.9999</td>
</tr>
<tr>
<td>Phase 2</td>
<td>10.4±4.03</td>
<td>9.21±4.57</td>
<td>&gt;0.9999</td>
</tr>
<tr>
<td>Phase 3</td>
<td>8.17±2.98</td>
<td>8.62±2.88</td>
<td>&gt;0.9999</td>
</tr>
</tbody>
</table>

CTRL, naturally cycling control; OC, monophasic oral contraceptive user; Phase 1, menses; Phase 2, follicular phase of CTRL, early medicated phase of OC; Phase 3, luteal phase of CTRL, late medicated phase of OC. Unpaired t-test, two-tailed comparison. Comparison of blood pressure using two-way ANOVA, with Bonferroni post-hoc test revealed no changes across cycle phases. *Statistically significant, p<0.05.
Figure J.1. Microvascular reactivity by menstrual cycle phase and oral contraceptive use.

Vasodilation responses to ACh and SNP in CTRL (n=15) and OC (n=10) subject groups. Microvascular reactivity remained unchanged by phase of menstrual cycle and by oral contraceptive use. CTRL, no oral contraceptive use; OC, monophasic oral contraceptive user; Phase 1, menses; Phase 2, follicular phase of CTRL, early medicated phase of OC; Phase 3, luteal phase of CTRL, late medicated phase of OC. Data presented as mean±SEM. Unpaired t-test, two-tailed comparison.
Appendix K

Stratification of microvascular reactivity findings by onset and severity of pre-eclampsia

Figure K.1. Stratification of microvascular reactivity by onset and severity of pre-eclampsia.

Stratification of subjects by onset of pre-eclampsia (PE; early-onset, EO, diagnosis <34 weeks gestation; late-onset, LO, diagnosis ≥34 weeks gestation) found no differences in microvascular reactivity to neither ACh nor SNP at 6 months postpartum. Stratification by PE severity (Mild PE, BP <160/100 mmHg; Severe PE, BP ≥160/100 mmHg) yielded no significant difference in microvascular responses to ACh and SNP. Data compared by one-way ANOVA, with Bonferroni post-hoc comparisons. EO PE, n=5; LO PE, n=20; Mild PE, n=5; Severe PE, n=20; CTRL, n=20.
Appendix L

Examination of the effect of the menstrual cycle and monophasic oral contraceptives on electrocardiography measurements

L.1 Materials and Methods

L.1.1 Subject identification
The study was approved by the Queen’s University ethics committee. All subjects gave written, informed consent. Thirty (n=30), never-pregnant women were recruited for the study based on volunteer participation from the general student and staff community of Queen’s University, at Kingston, Ontario. Participants were included in the study based on contraceptive use: 1) no hormonal contraceptive use (CTRL) and 2) using monophasic oral contraceptives (OC). Study participants and phases of menstrual cycles were identified as described in 0. Details of combination pills included in this study are given in Table L.1.

L.1.2 Electrocardiography
Electrocardiography recordings (ECG) were performed in a quiet temperature-controlled room with the subjects seated in a semi-supine position. All subjects were asked to abstain from caffeine intake and over-the-counter medication use the morning of the study visit. Skin was cleansed with 70% isopropyl alcohol prior to positioning of electrodes in an orthogonal manner. Ten-minute high-resolution (1000Hz) Holter ECG recordings were collected using a 3 lead SpiderView™ digital ECG Holter recorder (ELA Medical, Montrouge, FR). All non-sinus beats were excluded from analysis by operator inspection and all R-wave detection errors were corrected.
Table L.1. Summary of oral contraceptives used by participants.

<table>
<thead>
<tr>
<th>Oral contraceptive brand name</th>
<th>Number of Subjects</th>
<th>Ethinyl Estradiol Dose</th>
<th>Progestin Formulation and Dose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alesse</td>
<td>6</td>
<td>0.02mg</td>
<td>0.10 mg levonorgestrel</td>
</tr>
<tr>
<td>Yasmin</td>
<td>4</td>
<td>0.03mg</td>
<td>3mg drospirenone</td>
</tr>
<tr>
<td>Yaz</td>
<td>2</td>
<td>0.02mg</td>
<td>3mg drospirenone</td>
</tr>
<tr>
<td>Diane 35</td>
<td>1</td>
<td>0.035mg</td>
<td>2mg cyproterone acetate</td>
</tr>
<tr>
<td>Marvelon</td>
<td>1</td>
<td>0.03mg</td>
<td>0.15mg desogestrel</td>
</tr>
</tbody>
</table>
L.1.3 Statistical analysis
Demographic variables are presented as mean±standard deviation (SD) unless otherwise stated. An unpaired t-test or one-way analysis of variance (ANOVA) with Bonferroni post hoc test was used to compare continuously distributed variables and a $\chi^2$ comparison was used for categorical measures. GraphPad Prism 5 Software (La Jolla, CA, USA) was used for statistical analyses and comparison within and between subject groups. Normality of data was determined using the D’Agostino and Pearson omnibus normality test, and parametric or non-parametric statistical analysis was completed accordingly. Comparison of normotensive pregnancy control data across experimental time-points were analyzed by matched two-way ANOVA. Comparisons between subject groups, by time-point of measurement were achieved by unpaired one-way ANOVA.

L.2 Results
L.2.1 Participants
A summary of physical characteristics of study participants is provided in Table L.2. CTRL and OC participants were similar for age, height, weight and body mass index (BMI). Systolic and diastolic blood pressures were significantly elevated amongst users of oral contraceptives.

L.2.2 Heart rate variability
Mean R-R intervals, time domain parameters, and frequency domain parameters of HRV were similar across the time-frames measured for each study group. In addition, comparison of corresponding phases in CTRL and OC groups demonstrated not significant differences in the ECG parameters assessed. These findings are summarized in Error! Reference source not found.
Table L.2. Physical characteristics of study participants.

<table>
<thead>
<tr>
<th></th>
<th>CTRL (n=15)</th>
<th>OC (n=15)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age, yrs</strong></td>
<td>23.5±3.50</td>
<td>21.9±1.55</td>
<td>0.1155</td>
</tr>
<tr>
<td>Height, cm</td>
<td>164.7±6.55</td>
<td>165.9±4.76</td>
<td>0.5706</td>
</tr>
<tr>
<td>Weight, kg</td>
<td>60.4±8.48</td>
<td>65.1±12.9</td>
<td>0.2532</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>22.2±2.69</td>
<td>23.7±4.72</td>
<td>0.3028</td>
</tr>
<tr>
<td><strong>Systolic Blood Pressure</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phase 1</td>
<td>102.2±8.06</td>
<td>110.2±5.09</td>
<td>0.0029*</td>
</tr>
<tr>
<td>Phase 2</td>
<td>103.8±6.25</td>
<td>111.3±8.22</td>
<td>0.0089*</td>
</tr>
<tr>
<td>Phase 3</td>
<td>100.3±5.12</td>
<td>110.5±4.16</td>
<td>&lt; 0.0001*</td>
</tr>
<tr>
<td><strong>Diastolic Blood Pressure</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phase 1</td>
<td>64.8±5.76</td>
<td>74.4±6.83</td>
<td>0.0002*</td>
</tr>
<tr>
<td>Phase 2</td>
<td>68.2±7.57</td>
<td>72.9±10.75</td>
<td>0.1771</td>
</tr>
<tr>
<td>Phase 3</td>
<td>65.5±7.09</td>
<td>73.9±4.91</td>
<td>0.0008*</td>
</tr>
<tr>
<td><strong>Reported Cycle Length, days</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>29.3±0.976</td>
<td>28±0</td>
<td>-</td>
</tr>
</tbody>
</table>

CTRL, no oral contraceptive use; OC, monophasic oral contraceptive user; Phase 1, menses; Phase 2, follicular phase of CTRL, early medicated phase of OC; Phase 3, luteal phase of CTRL, late medicated phase of OC. Unpaired t-test, two-tailed comparison. Comparison of blood pressure using matched two-way ANOVA, with Bonferroni post-hoc test revealed no changes across cycle phases. *Statistically significant, p<0.05.
**Table L.3. HRV by menstrual cycle phase and monophasic oral contraceptive use.**

<table>
<thead>
<tr>
<th>HRV Parameter</th>
<th>CTRL (n=15)</th>
<th>OC (n=15)</th>
<th>OC (n=15)</th>
<th>Medicated Phase I</th>
<th>Medicated Phase II</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Menses</td>
<td>Follicular Phase</td>
<td>Luteal Phase</td>
<td>Un-mediated Phase</td>
<td></td>
</tr>
<tr>
<td>Mean RR (ms)</td>
<td>941.9±192.2</td>
<td>908.8±197.7</td>
<td>904.5±166.4</td>
<td>851.8±102.9</td>
<td>824.1±89.31</td>
</tr>
<tr>
<td>SDNN (ms)</td>
<td>78.07±36.29</td>
<td>74.02±35.35</td>
<td>78.65±38.23</td>
<td>61.81±24.09</td>
<td>64.04±25.57</td>
</tr>
<tr>
<td>RMSSD (ms)</td>
<td>80.98±47.00</td>
<td>87.32±54.30</td>
<td>82.34±50.47</td>
<td>61.02±28.66</td>
<td>61.94±28.10</td>
</tr>
<tr>
<td>pNN50 (%)</td>
<td>45.89±21.42</td>
<td>38.48±21.93</td>
<td>45.92±23.49</td>
<td>35.41±21.72</td>
<td>35.41±18.03</td>
</tr>
<tr>
<td>LF_norm</td>
<td>417.4±0.1535</td>
<td>478.7±0.1338</td>
<td>461.7±131.8</td>
<td>458.1±173.4</td>
<td>498.4±181.9</td>
</tr>
<tr>
<td>HF_norm</td>
<td>582.6±0.1535</td>
<td>52.12±0.1338</td>
<td>538.3±131.8</td>
<td>541.8±173.4</td>
<td>501.6±181.9</td>
</tr>
<tr>
<td>LF/HF</td>
<td>892.9±0.7477</td>
<td>1069.0±657.2</td>
<td>978.0±546.2</td>
<td>109.0±780.0</td>
<td>1253.0±854.9</td>
</tr>
</tbody>
</table>

*CTRL*, naturally cycling control; *OC*, monophasic oral contraceptive user; *mean RR*, average time between successive R-R intervals; *SDNN*, standard deviation of normal-normal R-R intervals; *RMSSD*, root mean square of the difference between successive R-R intervals; *pNN50*, proportion of R-R intervals differing from directly adjacent R-R intervals >50ms; *LF*, low frequency (0.04-0.15Hz); *HF*, high frequency (0.15-0.4Hz). Data presented as mean±SEM. Comparisons were made by two-way ANOVA, with Bonferroni post-hoc test.