THE EFFECT OF PREECLAMPSIA ON RENAL FUNCTION: CROSS-SECTIONAL STUDIES OF POSTPARTUM PREECLAMPTICS AND WOMEN WHO WERE DESTINED TO DEVELOP PREECLAMPSIA

by

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Abstract

Preeclampsia (PE) is a pregnancy complication that is new-onset of hypertension and proteinuria after 20 weeks of gestation. However, subclinical renal dysfunction may be apparent earlier in gestation prior to the clinical presentation of PE. Although the maternal syndrome of PE resolves early postpartum, women with a history of PE are at higher risk of renal dysfunction later in life. Mineral metabolism, such as phosphate balance is heavily dependent on renal function, yet, phosphate handling in women with a history of PE is largely unknown.

To investigate whether women with a history of PE would exhibit changes in phosphate metabolism compared to healthy parous women, phosphate loading test was used. Women with or without a history of PE, who were 6 months to 5 years postpartum, were recruited for this study. Blood and urine samples were collected before and after the oral dosing of 500mg phosphate solution. Biochemical markers of phosphate metabolism and renal function were evaluated. In order to assess the difference in renal function alteration between first trimester women who were or were not destined to develop PE, plasma cystatin C concentration was analysed.

After phosphate loading, women with a history of PE had significantly elevated serum phosphate at both 1- and 2-hour, while controls had higher urine phosphate:urine creatinine excretion ratio at 1-hour than women with a history of PE. Women with a history of PE had no changes in intact parathyroid hormone (iPTH) concentration throughout the study period, whereas controls had elevated iPTH at 1-hour from baseline. In terms of renal function in the first trimester, there was no difference in plasma cystatin C concentration between women who were or were not destined to develop PE.
The elevation of serum phosphate in women with a history of PE could be due to the delay in phosphate excretion. Prolong elevation of serum phosphate can have serious consequences later in life. Thus, oral phosphate challenge may serve as a useful method of early screening for altered phosphate metabolism and renal function.
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<td>AKI</td>
<td>acute kidney injury</td>
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<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
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<td>AUC</td>
<td>area under the curve</td>
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<td>BMI</td>
<td>body mass index</td>
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<td>CI</td>
<td>confident interval</td>
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<tr>
<td>CKD</td>
<td>chronic kidney disease</td>
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<td>CKD-EPI</td>
<td>Chronic Kidney Disease Epidemiology Collaboration</td>
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<tr>
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<td>cardiovascular disease</td>
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<td>DMP1</td>
<td>dentin matrix acidic phosphoprotein 1</td>
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<td>eGFR</td>
<td>estimated glomerular filtration rate</td>
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<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
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<td>ERPF</td>
<td>effective renal plasma flow</td>
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<td>ESKD</td>
<td>end-stage kidney disease</td>
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<td>FE</td>
<td>fractional excretion</td>
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<td>FGF</td>
<td>fibroblast growth factor</td>
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<td>FGF23</td>
<td>fibroblast growth factor 23</td>
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<tr>
<td>GFR</td>
<td>glomerular filtration rate</td>
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<td>HELLP</td>
<td>hemolysis elevated liver</td>
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<td>HRP</td>
<td>horseradish peroxidase</td>
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<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
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<tr>
<td>OaK</td>
<td>Ottawa and Kingston birth cohort</td>
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<td>OBGY</td>
<td>obstetrics and gynecology</td>
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<td>OD</td>
<td>optical density</td>
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<td>OR</td>
<td>odd ratio</td>
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<tr>
<td>PE</td>
<td>preeclampsia</td>
</tr>
<tr>
<td>PHEX</td>
<td>phosphate regulating endopeptidase, X-linked</td>
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<td>PTH</td>
<td>parathyroid hormone</td>
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<tr>
<td>RR</td>
<td>relative risk</td>
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<tr>
<td>RU</td>
<td>relative unit</td>
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<tr>
<td>SD</td>
<td>standard deviation</td>
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<tr>
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<td>standard error of the mean</td>
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<td>sFlt-1</td>
<td>soluble fms-like tyrosine kinase 1</td>
</tr>
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<td>VEGF</td>
<td>vascular endothelial growth factor</td>
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<td>VEGFR</td>
<td>vascular endothelial growth factor receptor</td>
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Chapter 1

Introduction

1.1 Preeclampsia

1.1.1 Epidemiology
Preeclampsia (PE) is a pregnancy complication that is defined as new-onset hypertension and proteinuria after 20 weeks of gestation.\textsuperscript{1,2} Estimated to affect 3 to 8 percent of all pregnancies,\textsuperscript{3} PE continues to be one of the leading causes of maternal and neonatal morbidity and mortality worldwide, especially in developing countries.\textsuperscript{4,5} It is also the most common cause of medically indicated preterm birth (<35 weeks) in the United States.\textsuperscript{6} The current incidence rates of PE are higher compared to a few decades ago,\textsuperscript{7,8} increasing prevalence of obesity, diabetes mellitus and smoking cessation amongst young women are suspected to be some of the contributing factors.\textsuperscript{7}

1.1.2 Risk Factors
Numerous risk factors have been associated with PE including, but not limited to: nulliparity and obesity.

\textit{Nulliparity}
Women who are pregnant for the first time are more likely to develop PE (RR2.35, 95\% CI 1.80-3.06) compared to women who have previously had an uncomplicated pregnancy.\textsuperscript{9} Factors such as paternity and short term pre-pregnancy cohabitation increase a woman’s risk of PE. Prolonged exposure to paternal antigens appears to have a protective effect against PE for women; the theory of immunological priming of the maternal body is thus suggested.\textsuperscript{3,10-12} Although recurrent miscarriages (3 or more miscarriages) do not reduce
the risk of PE, the risk of PE is reduced (P=0.038) in women with a history of induced abortion compared to women who have never been pregnant.\textsuperscript{13,14}

\textit{Obesity.}

Pre-pregnancy weight is considered as an important risk factor for PE. Risk of PE is increased by 50\% in women with a body mass index (BMI) higher than the normal range of 19 to 25,\textsuperscript{9} and increases incrementally with each additional unit of BMI.\textsuperscript{3,9} When BMI is over 35 kg/m\textsuperscript{2}, the risk of developing PE is over 4 times compared to women with BMI under 27 kg/m\textsuperscript{2}.\textsuperscript{9,15} Instead, underweight women (BMI < 20 kg/m\textsuperscript{2}) have lower risk, odds ratio of 0.76, (99\% CI 0.62-0.92), of developing PE compared to women with normal BMI.\textsuperscript{9,16} With increasing rates of overweight and obesity amongst women who are of reproductive age, this becomes a very concerning modifiable risk factor.\textsuperscript{3}

1.1.3 \textbf{Pathogenesis}

Preeclampsia-eclampsia has been recognized for thousands of years, however, the exact cause or etiology of PE is still unknown.\textsuperscript{17} PE is a complex pregnancy disorder that occurs almost solely in humans.\textsuperscript{18} It is a multi-system disorder which is thought to be a two-stage disorder that starts with inadequate placentation.\textsuperscript{1,19} During early stages of a normal pregnancy, extravillous trophoblasts develop into cytotrophoblast cell columns.\textsuperscript{20} The cytotrophoblasts from the cell columns invade deep into maternal decidua and myometrium. In this process, cytotrophoblasts transform into elongated interstitial trophoblasts which invade between the maternal stromal cells and eventually resides around the uterine spiral arteries.\textsuperscript{20,21} Some cytotrophoblasts from the cell columns also invade the distal opening of the spiral arteries and migrate along the lumen of the vessels. These cytotrophoblasts adapt the adhesion molecules similar to that of endothelial cells.
and give rise to endovascular trophoblasts.\textsuperscript{20,22} Interstitial trophoblasts destroy the smooth muscle cell organization in the spiral arteries through fibrinoid necrosis of the media layer of the vessel\textsuperscript{20} which is followed by the replacement of maternal endothelia by the endovascular trophoblasts.\textsuperscript{18,20} Under normal circumstances, this complex remodeling process is mostly completed by 20-22 weeks gestation.\textsuperscript{23} In preeclamptic pregnancies, however, modifications to the spiral arteries are minimal and only occur in the decidua layer of the uterine wall.\textsuperscript{23} Such shallow invasion of the interstitial trophoblasts fail to promote smooth muscle disorganization in the spiral arteries. With the smooth muscle layer of the spiral arteries still intact or partially intact, the diameter of these arteries would remain relatively unchanged and responsive to vasoconstrictors.\textsuperscript{1,12,24}

As the nutritional and oxygen demands of the rapidly growing fetus increases after 20 weeks of gestation, the inadequate uteroplacental perfusion can no longer sustain the growth of the placenta and fetus, which then leads to increased placental ischemia and oxidative stress.\textsuperscript{12,25} In response to ischemic stress, the placenta releases factors such as free radicals, angiogenic factors, immune and inflammation activators into the maternal circulation. This then leads to the second stage of the disorder: maternal endothelial dysfunction, which is characterized as alteration of endothelium hemodynamic response, endothelial activation, increased in platelet activation and cell adhesion molecules.\textsuperscript{1,12,18,24,25} Factors released by the placenta can have a profound effect on multiple organs and systems in the maternal body which then present as typical maternal syndrome of PE.\textsuperscript{1}
Release of antiangiogenic factors

The release of large quantities of soluble fms-like tyrosine kinase (sFlt-1) by ischemic placenta plays an important role in the progression of PE. The released sFlt-1 is a vascular endothelial growth factor (VEGF) receptor that lacks the transmembrane and cytoplasmic domain. VEGF is an angiogenic growth factor that has high particularity for endothelial cells and VEGF is also a critical factor in vasculogenesis and angiogenesis.\textsuperscript{26} VEGF receptor (VEGFR) family include VEGFR-1 (fms-like tyrosine kinase-1), VEGFR-2 (fetal liver kinase-1) and VEGFR-3.\textsuperscript{26} VEGFR-1 and VEGFR-2 receptors are largely expressed in endothelial cells, including glomerular endothelial cells.\textsuperscript{26,27} The binding of VEGF to VEGFR-1 is associated with inducing cell migration whereas the activation of VEGFR-2 induces cell proliferation.\textsuperscript{26} VEGF, particularly VEGF-A which is expressed by the podocytes, has shown to be essential for signalling between podocytes and glomerular endothelial cells for proliferation or differentiation and maintaining the filtration barrier in the glomerulus.\textsuperscript{27} Circulating sFlt-1 can bind to VEGF, which deprives the glomerular endothelial cell the factor that is essential for survival.\textsuperscript{27,28} The reduction in VEGF can lead to a distinctive ‘bloodless glomeruli’ appearance in which the endothelial cells swell and occlude the lumen of the vessel, and this unique glomeruli destruction can be commonly found in preeclamptics.\textsuperscript{28}

1.1.4 Clinical Manifestation of Preeclampsia

The clinical manifestations of PE involve multiple organs and systems. The American College of Obstetrics and Gynecology defines preeclampsia-eclampsia as elevation of blood pressure after 20 weeks of gestation with proteinuria or any of the severe symptoms of PE.\textsuperscript{2} Hypertension is defined as either a systolic blood pressure $\geq$140 mmHg, diastolic
blood pressure ≥90 mmHg, or a combination of the two in a woman with a history of normal blood pressure. The clinical diagnostic criteria for proteinuria are protein concentration ≥ 300mg / 24-hour urine collection, protein/creatinine ratio ≥ 0.3 mg/dL or repeated dipstick reading of 1+ in cases where quantitative methods of measurement are not available. Severe symptoms of PE include thrombocytopenia (platelet count <100,000/uL), renal insufficiency (serum creatinine > 97.26 µmol/L or a doubling of serum creatinine without the implication of other renal disease), impaired liver function (increased liver transaminases concentration in the blood), pulmonary edema, and/or cerebral/visual disturbances.

The maternal endothelial dysfunction underlying PE can exacerbate and increase in liver enzymes and platelet aggregation which then lead to the development of hemolysis, elevated liver function enzymes and low platelet (HELLP) syndrome. If PE is not diagnosed and managed appropriately, eclampsia may develop, in which the mother experiences severe headaches, visual blurriness, convulsions, and even maternal and fetal mortality.

1.1.5 Long-Term Mortality and Morbidity after Preeclampsia

Pregnancy is essentially a cardiovascular stress test. Maladaptation of maternal systems during this period can have serious consequences later in life. Women with a history of PE are at increased risks of developing hypertension (RR3.70, 95% CI 2.7-5.05), ischemic heart disease (RR2.16, 95% CI 1.86-2.52), stroke (RR 1.81, 95% CI 1.45-2.27), venous thromboembolism (RR1.19, 95% CI 1.37-2.33), and all-cause of mortality (RR1.49, 95% CI 1.05-2.14). Furthermore, women with history of PE with preterm delivery have 5- and
8-fold relative hazard rate of dying from stroke and cardiovascular causes, respectively, as compared to women with no such history.\textsuperscript{33} The hazard risk of developing late-onset type 2 diabetes mellitus is 2.1 in women with a history of PE.\textsuperscript{34,35}

1.2 Renal Function

1.2.1 General Renal Function in Health and Disease

One of the most important functions of the kidneys is to help maintain the homeostatic state, especially the consistency of plasma composition.\textsuperscript{36} Deviation from normal kidney functions can result in changes in blood pH, volume, coagulation, and abnormal waste removal.\textsuperscript{36} The functional unit of a normal kidney is the nephron. It consists of 5 different regions: glomerulus, proximal convoluted tubules, loop of Henle, distal convoluted tubules and collection duct. Each nephron begins with the renal corpuscle which contains a network of fenestrated capillaries called glomerulus.\textsuperscript{37} After filtrates enter the capsular space, it then enters the adjacent proximal convoluted tubules. Important nutrients such as glucose, fatty acids, and organic nutrients are reabsorbed by the simple cuboidal epithelium. Inorganic nutrients such as potassium, calcium, and phosphate are also actively reabsorbed.\textsuperscript{37} The inability of the proximal tubule to reabsorb nutrients in the filtrate leads to excessive urinary excretion of phosphate, amino acids, glucose, and other ions. It can be seen clinically as chronic renal deficiency, and the symptoms include proteinuria and electrolyte imbalance. The accumulation or precipitation of calcium from inappropriate excretion can also lead to the formation of kidney stones.\textsuperscript{38}
The kidney involvement in regulating long-term arterial pressure is well known.\textsuperscript{39–42} Resting blood pressure is influenced by vascular volume which in turn depends on extracellular fluid volume.\textsuperscript{40} Sodium balance is an important determinant of extracellular fluid volume as well as blood serum osmolality.\textsuperscript{36,40} As the systemic blood pressure increases, the renal arterial pressure increases. The kidneys respond to this pressure change by increasing sodium and water excretion which leads to the reduction in extracellular fluid volume and this mechanism is referred to as pressure natriuresis.\textsuperscript{39–41} This response is mediated mainly through the renin-angiotensin system. The reduction in expression of one of the hormones, angiotensin II, leads to decrease in sodium reabsorption in the proximal tubules and ultimately sodium excretion and decrease in blood pressure.\textsuperscript{40,41} Inability to respond by the kidneys can lead to changes in blood pressure, ion imbalance, and extracellular fluid volume.\textsuperscript{40}

\subsection*{1.2.2 Types of Renal Dysfunction}
Damage to the kidneys can be either acute or chronic. According to the Kidney Disease: Improving Global Outcomes 2012 guideline, acute kidney injury/impairment (AKI) is defined as increases in serum creatinine by more than 0.3mg/dL within 48 hours, or increases in serum creatinine to more than 1.5 times the baseline, or urine volume of less than 0.5mL/kg/h for 6 hours.\textsuperscript{43} Chronic kidney disease is defined as abnormalities of kidney structure or function for more than 3 months and in combination of one or more markers of kidney damage.\textsuperscript{44}

Risk factors for both acute and chronic kidney diseases include advanced age, diabetes mellitus, obesity, and cardiovascular diseases.\textsuperscript{43,44} AKI can be caused by different insults
including: medications or toxins, exposure to infections, cardiac surgery, radiocontrast agents or other morbidities. The major symptom of AKI is fluid overload. Thus, the most common treatment when individuals are at risk or suspected of AKI is diuretics. Chronic kidney diseases can have very different etiologies. Since different renal regions have different functions, evaluation of different markers of renal damage in combination with other laboratory testing are used to guide diagnosis of kidney diseases. For instance, protein in urine suggests a possible increase of pore sizes in the glomerulus (glomerular proteinuria), inadequate reabsorption of proteins in the proximal tubules (tubular proteinuria), or increase in plasma protein level (overproduction proteinuria). Proteinuria can play an important role in the development or worsening of chronic kidney disease. It has been shown that proteinuria itself can cause progressive renal dysfunction by increasing inflammation, and the reduction of proteinuria has shown a decline in progression in end-stage renal disease.

1.2.3 Renal function indicators
Glomerular filtration rate (GFR) is defined as a measure of the volume of fluid filtered from the renal glomerulus into the Bowman’s capsule per unit time, and it has been widely recognized as the best indicator of glomerular function. Since it is improbable to measure individual glomerulus filtration all at once, indirect methods of measuring GFR were developed. Inulin clearance, which requires continuous exogenous inulin infusion and urine collection, has been generally accepted as the gold standard of indirectly measuring GFR. However, it is a cumbersome and time-consuming method which is not commonly used as a clinical diagnostic tool. Since testing for endogenous markers is less invasive and more practical, a variety of biomarkers are currently used as an alternative to estimate
GFR. Creatinine, a by-product of muscle metabolism, has been widely used clinically as a biomarker for estimating GFR (eGFR). Since populations with differences in age, sex and race would have different muscle mass and rates of muscle metabolism, creatinine-based eGFR equation needs to take the listed factors into consideration.

1.2.4 Cystatin C

Serum cystatin C, an endogenous cysteine proteinase inhibitor, is thought to be produced by all known nucleated cells in the body at a constant rate. As cystatin C has a molecular weight of 13.3kDa, it can be freely filtered by the glomerulus and mostly reabsorbed by the proximal renal tubules. Cystatin C production has been mainly described in the literature to be independent of factors such as age and gender and thus has been proposed to be a better biomarker for detecting minor reductions in GFR than serum creatinine. Due to the smaller contribution of muscles in cystatin C production, serum cystatin C concentration have a weaker association with muscle mass than serum creatinine. Thus it has been suggested that cystatin C may be a more reliable endogenous biomarker in estimating GFR among populations that have variations in diet or conditions that could affect muscle mass. Cystatin C-based eGFR also improves the calculation of risks of death from any cause, cardiovascular causes, and renal disease and it can better detect adverse outcome that may not be apparent when eGFR is calculated with creatinine-based equations. Cystatin C has proven to be a promising predictor of preclinical chronic kidney disease in the elderly and diabetic populations. Furthermore, it has been proposed that individuals, without clinically diagnosed kidney disease, who have an eGFR greater than 60ml per minute per 1.73m² and cystatin C concentration higher or equal to 1.0 mg per liter should be classified as having preclinical kidney disease.
1.2.5 Phosphate Metabolism

Total body phosphorous content accounts for approximately 1% of the body weight. The majority of phosphorous is stored in bones and only 1% of total phosphorus is extracellular phosphorus, only 15% of which can be readily measured in the blood.\(^{65}\) Around 25% of plasma phosphate is protein bound, where the rest can be filtered by the glomerulus, referred to as ultrafilterable phosphate.\(^{65,66}\) Extracellular phosphorus fluctuates during the course of a day and depends on intestinal absorption, renal excretion, and bone turnover.\(^{67}\) The different food groups contain at least some level of bioavailable phosphate, hence dietary deficiencies of phosphate are rare.\(^{65,66}\) Approximately 65% of phosphate from food intake is absorbed by the small intestine.\(^{66}\) Intestinal absorption is mediated by IIb type sodium phosphate cotransporter in the lumen of the small intestine.\(^{65,67}\)

Phosphate excretion depends heavily on kidney function, as almost 70% of ingested phosphate is excreted in the urine.\(^{65,66}\) At the level of the glomerulus, 90 to 95% of the extracellular phosphate is ultrafilterable phosphate.\(^{66}\) The kidney filters approximately 7 g of phosphorus daily and 80% to 90% is then reabsorbed by the renal tubules. Of the reabsorbed phosphate, over 60% to 70% is done by IIa and IIc type sodium phosphate cotransporters in the brush border membrane of the proximal renal tubules.\(^{66,68,69}\) With the remaining filtered phosphate excreted in urine, phosphate balance is thus maintained (Figure 1-1).\(^{66,68}\) The exchange of phosphate between extracellular fluid, intracellular reserve, and bone can be influenced by insulin and respiratory alkalosis. Phosphate uptake by the cell is increased when there is an increase of intracellular pH.\(^{68}\) Multiple feedback
loops are involved in regulating serum phosphate balance, however, the specific sensor of phosphate in the body is still unknown.\textsuperscript{68}

Abnormal calcium and phosphate metabolism and bone remodeling due to renal insufficiency have been associated with vascular calcification and it continues to be the leading cause of comorbidity in patients with chronic kidney disease.\textsuperscript{70,71} This type of vascular calcification differs from atherosclerotic lesions in that calcification occurs in the media, as opposed to intima of the blood vessels.\textsuperscript{72,73} Dialysis patients given noncalcium-containing phosphate binder have shown reduced progression of vascular calcification compared to patients given calcium-containing phosphate binders. Though the exact mechanism is unclear, the improvement in these patients supports the association of mineral metabolism imbalance and medial vascular calcification.\textsuperscript{73} High concentration of phosphate, high glucose and oxidized lipids can induce vascular smooth muscle cell transformation into chondrocyte or osteoblast-like cells.\textsuperscript{73,74} Phosphate at high levels in the circulation binds with calcium forming calcium phosphate apatite crystals, which precipitate and lead to vascular calcification at the smooth muscle layers.\textsuperscript{70} Extensive vascular calcification particularly in the elastic vessels such as the aorta and the common carotid arteries is associated with arterial stiffening and it is independent of age and blood pressure.\textsuperscript{75}
Figure 1-1 Schematic diagram of phosphate metabolism from dietary phosphate intake to phosphate excretion. Note: Pi = phosphate, NaPi-IIa, IIc = Sodium phosphate cotransporter type IIa, and type IIc.
1.2.6 Parathyroid Hormone

Parathyroid hormone (PTH) produced by the parathyroid gland is primarily considered to be a calcitropic hormone, however, it is also consider to be one of the major humoral factors that regulates phosphate homeostasis.\textsuperscript{76,77} The synthesis and release of PTH are governed by calcium concentration in blood via calcium sensing receptors in the parathyroid gland.\textsuperscript{68} However, there is also evidence that phosphate stabilizes posttranscriptional PTH mRNA, hence increases PTH production.\textsuperscript{77} The intact and bioactive form of PTH has a half-life of less than 4 minutes in circulation before it is cleaved by the liver or the kidneys.\textsuperscript{78} The liver degrades the intact PTH into small fragments before they are released back into the circulation. The carboxyl-terminal fragments have a much longer half-life in circulation before it is cleared by the glomerulus.\textsuperscript{78}

Alongside with regulating calcium concentration in blood and bones, PTH also plays an essential role in regulating phosphate balance (Figure 1-2). In response to decreased circulating calcium, intact PTH can stimulate the release of calcium and phosphate from bones through osteoclast resorption. It can also increase calcium and decrease phosphate reabsorption in the renal proximal tubules.\textsuperscript{78} The PTH-mediated phosphate excretion is accomplished through increase internalization of IIa and IIc type sodium phosphate cotransporters in the proximal tubules, thereby increasing excretion of phosphate and decreasing phosphate levels in the body.\textsuperscript{68,79} Following the increase of serum calcium concentration, PTH concentration in circulation decreases (Figure 1-2).\textsuperscript{78}
PTH can also indirectly increase phosphate by stimulating the synthesis and secretion of calcitriol.\textsuperscript{78} Calcitriol or 1,25(OH)\textsubscript{2}D\textsubscript{3} can restore low serum phosphate concentrations by increasing the absorption of phosphate in the intestine through increasing expression of \textit{IIb} type sodium phosphate cotransporter.\textsuperscript{66,68,80} In turn, calcitriol synthesis can be reduced by elevation of phosphate.\textsuperscript{68} Individuals with impaired renal function show elevated serum phosphate concentrations (normal range of 0.74 to 1.54mmol/L).\textsuperscript{71} In chronic kidney disease patients, the inability to remove excess phosphate in circulation leads to hyperphosphatemia and the chronic elevation of PTH subsequently leads to secondary hyperparathyroidism.\textsuperscript{81} The following figure demonstrates the dynamic and complex interactions of PTH and other organs in the body (Figure 1-2).
Figure 1-2 Negative feedback loop of PTH metabolism. In respond to calcium concentration decrease in circulation, the secretion of PTH increases calcium concentration. The increase in calcium inhibits PTH release. Note: \( \text{Ca}^{2+} = \text{Calcium}, \text{Pi} = \text{phosphate.} \)
1.2.7 Fibroblast Growth Factor 23

Fibroblast Growth Factor 23 (FGF23) is a peptide hormone produced by osteocytes and osteoblasts. It is one of the major hormones in regulating phosphate homeostasis, and it is a potent phosphaturic agent.\textsuperscript{68,77,82} The full-length intact FGF23 is the biologically active form, which is secreted from bone and can be detected in circulation.\textsuperscript{68,83} Activity of FGF23 is regulated by proteolytic cleavage at \textsuperscript{179}Arg and \textsuperscript{180}Ser to render it inactive, however, the specific enzyme which cleaves FGF23 to its inactive form is still unknown.\textsuperscript{66,68,84} Some human hereditary diseases have been associated with FGF23 mutations. Certain \textit{FGF23} gene mutations can cause the peptide to be resistant at the cleavage site, which then leads to phosphate wasting or hypophosphatemia. In contrast, some mutations in \textit{FGF23} gene can cause the instability of the protein and result in hyperphosphatemia.\textsuperscript{82,85}

Biologically active FGF23 binds to the Klotho-FGF receptor complex to exert its function.\textsuperscript{68,86} Klotho, a single-pass transmembrane protein, is predominantly found in the kidney. It binds to multiple FGF receptors to form a Klotho-FGF receptor complex.\textsuperscript{86} FGF23 responds to increased phosphate burden by downregulating sodium dependent phosphorus transporter type IIa and IIc in the renal proximal tubules. This translates to a decrease in phosphate reabsorption that is independent of PTH.\textsuperscript{68,82,87} FGF23 also suppresses the synthesis of calcitriol by inhibiting the expression of 1α(OH)ase (an enzyme found in the proximal tubule that catalyze calcifediol to the bioactive form calcitriol) and stimulating the expression of 24(OH)ase (an mitochondrial enzyme that degrades calcitriol into calcitroic acid).\textsuperscript{88,89} Calcitriol in turn increases the production of FGF23 in the osteocytes by stimulating the promotor region of \textit{Fgf23}.\textsuperscript{88} In an animal study with bone
protein phosphate regulating endopeptidase, X-linked (PHEX) and dentin matrix acidic phosphoprotein 1 (DMP1) deficient mice, PHEX and DMP1 were shown to control the FGF23 production in osteocytes in that inactivation of either protein led to intrinsic elevation of FGF23.88

When healthy volunteers were subjected to low phosphate/phosphate binder diet followed by phosphate replenishment experiment within a five day study period, serum FGF23 concentration had no detectable changes. This suggests that FGF23 is not the hormonal factor that regulates rapid phosphate excretion within short period of time.71 Instead, it is suggested to be a long term regulator of phosphate elevation. Serum phosphate concentrations in chronic kidney disease (CKD) patients are significantly elevated due to impaired renal excretion of phosphate.71 Serum FGF23 levels are also significantly elevated in these individuals.71 It is postulated that the synthesis of FGF23 is induced by long-term retention of phosphate as renal function progressively declines.67 In CKD patients, elevated FGF23 in circulation fails to decrease phosphorus reabsorption and leads to calcium phosphate deposition in the peripheral vasculature; most common comorbidity of CKD.90 It is also shown that plasma FGF23 level increases before changes in other traditional biomarkers of phosphorus imbalance (e.g. increase in PTH level). This suggests that FGF23 is an ideal ‘early’ biomarker for assessing phosphorus metabolism and renal function.91

1.3 Preeclampsia and Renal Dysfunction

PE and renal disease share common risk factors including obesity, hypertension, diabetes and endothelial dysfunction.92,93 Even though signs and symptoms of PE usually resolve
early postpartum, women with a history of PE have a higher risk for developing renal dysfunction later in life.\textsuperscript{94} Furthermore, women with a history of mild and severe PE have 4-fold and 8-fold increased risk, respectively, of postpartum microalbuminuria compared to women with uncomplicated pregnancies.\textsuperscript{95} The risk of developing kidney disease that requires kidney biopsy is also increased in the women with a history of PE population, with the highest risk during the first five years postpartum.\textsuperscript{96} In terms of severe renal outcome later in life, women who have had a history of PE are at four to five times higher risk of end-stage kidney disease (ESKD).\textsuperscript{92} It has been suspected that the increased renal disease risk may be a result of PE directly causing permanent renal damage during pregnancy or indirectly initiating endothelial dysfunction which slowly leads to renal dysfunction later in life.\textsuperscript{97} However, it is still unclear whether PE is a presentation of pre-existing subclinical kidney dysfunction or whether it is the origin of future kidney diseases.\textsuperscript{97,98}

It has been shown in the literature that the traditional renal function assessment tools such as serum creatinine levels and eGFR do not significantly differ between women who did or did not have a pregnancy complicated by PE seven years postpartum.\textsuperscript{99} However, when specific characteristics of renal function were investigated, the differences between women with a history of PE and healthy parous controls became apparent.\textsuperscript{100} A study investigated the difference in renal hemodynamic values such as effective renal plasma flow (ERPF) and GFR between women with a history of PE and healthy parous controls at least four months postpartum.\textsuperscript{101} It was shown that women with a history of PE had a significantly lower (P<0.05) ERPF compared to controls, 482 ± 88 and 553 ± 67 mL/min/1.73m\textsuperscript{2}, respectively. However, there was no difference between women with a history of PE and
controls in GFR. When renal vascular resistance was calculated in these two groups, women with a history of PE had significantly higher values (P<0.001) than controls, 9981 ± 2468 and 7490 ± 868 dyne-seconds/cm^5, respectively. A more recent study measuring the effect of PE 23 years postpartum \(^{102}\) found that women with a history of PE had significantly lower (P=0.003) ERPF compared to controls, 399 ± 61 and 463 ± 83 mL/min/1.73m^2, respectively. Also, renal vascular resistance remained high (P<0.001) compared to parous controls, 122 ± 28 and 95 ± 20 (100 dyne-seconds/cm^5), respectively. There was no difference between hypertensive pre women with a history of PE and normotensive women with a history of PE in terms of ERPF or renal vascular resistance. Another study examined kidney biopsies in women with a history of PEs 3 months to 4 years postpartum reported that the glomerular lesions occurring during pregnancy resolved within 6 months.\(^{100}\) Grade I or II arteriolar lesions remained unchanged or progressively disappeared. Grade III arteriolar lesions which often presented as edema of the media however, were directly associated with high or increased blood pressure.\(^{100,103}\)

*In vitro* studies have shown that exposing proximal tubular cell culture to plasma protein can lead to the synthesis of endothlin-1 which is a progressive renal injury mediator. Free fatty acid-bound albumin has also shown to cause proinflammatory activation or injury in proximal tubular cell cultures.\(^{46}\) Since women with a history of PE have higher risk of proteinuria and microalbuminuria, it is then of no surprise that women with history of PE are more likely to suffer from progressive renal injury. Although sFLT-1 concentration in circulation rapidly decreases within the first week after delivery, women with history of PE still have an increased level of sFLT-1 a year postpartum.\(^{104}\) As mentioned above,
sFLT-1 can bind to VEGF which is essential to glomerular vascular health and development. The exact complex mechanism from PE to kidney disease is still unclear, however, several possible pathways have been proposed, one of which suggested that the persistently high level of sFLT1 can lead to glomerulus dysfunction and altered renal hemodynamic. Subsequently, microalbuminuria or other forms of proteinuria may develop due to the damaged glomerulus. Combining with residual or worsened endothelial dysfunction from the pregnancy complication, women with a history of PE would thus have a higher risk of developing CKD later in life.¹⁰⁰
Chapter 2

Materials and Methods

2.1 Assessing renal function and phosphate excretion in women with history of preeclampsia

This study was approved by the Queen’s University Health Sciences Research Ethics Board (OBGY-270-15). Written informed consent was obtained from all participants.

2.1.1 Participant selection

Women with a history of singleton PE pregnancies were identified from the Maternal Health Clinic at Kingston General Hospital through chart review. Women with a history of singleton uneventful pregnancies who were under the care of obstetrician Dr. Susan Chamberlain at Kingston General Hospital during pregnancy were also identified through chart review. Individuals who were at least 6 months postpartum were considered. Women with a history of prepregnancy cardiovascular, renal, or endocrine diseases were excluded from the study.

2.1.2 Participant recruitment

Women with history of PE satisfied the initial screening were contacted through letters of invitation. Letters of invitation included detail information on the purpose of the study and how the study would be conducted. Upon telephone follow-up, participant selection was further narrowed down to exclude women who were currently smoking, currently pregnant or breastfeeding. Maternal mineral balance can be altered during lactation\textsuperscript{105}, however, no longitudinal study has shown a definitive time where phosphate metabolism returns to the prepregnancy level. Thus, based on expert opinion, the timeline of 2 months (8 weeks) of
post-weaning was used as a criterion for participant selection. Thus, women who stopped breastfeeding for less than 2 months were also excluded. Individuals who matched all criteria and agreed to participate were recruited for the study. A detailed description of the study was explained to the participant and two copies of the consent form were signed by the participant and research coordinator. One copy was given to the participant and another copy was kept for record purposes. The PEs were matched with controls by age (± 5 years), parity, and similar time postpartum (± 5 years).

2.1.3 Participant demographic data
A total of 10 PE and 8 matched controls were recruited. However, 2 PE as well as 1 matched control samples were not included in the analysis due to improper sample collection and 1 PE had no match controls. Demographic data such as current age, age at index pregnancy, time postpartum(in months), height, prepregnancy and current weight, prepregnancy and current BMI, waist and hip circumference, current systolic and diastolic blood pressure were collected during the study through a questionnaire (appendix A) or through the hospital record. Participants were asked to rate the average intensity of their weekly activity based on descriptions provided in the Borg Scale of Perceived Exertion chart (Appendix B).

2.1.4 Demographic statistical analysis
Since demographic data were only collected at a single time point, unpaired t test was performed for statistical analysis. All data sets were tested for normal distribution using the D’Agostino Omnibus test. For data sets that failed the normality test, nonparametric
Mann-Whiney test was performed. For data sets that follow the normal Gaussian distribution, unpaired t test with Welch correction was performed.

2.1.5 Blood collection

Each participant’s venous blood samples were collected through a saline lock established in the anti-cubital vein by a registered nurse. Serum and plasma samples were collected at three different time points. A discard vacutainer was used to remove air or saline solution before each set of sample collection at each time point. Serum samples were obtained by using two 3mL serum-separator vacutainers that contain silicone, micronized silica particles, and serum separator gels (BD Vacutainer®, SST™ Gel Separator Tube). One 6mL plasma sample was obtained by drawing blood into vacutainers containing K$_2$EDTA (BD Vacutainer®, K$_2$EDTA). Serum samples collected with separator gel tubes were drawn first and followed by plasma samples collected with K$_2$EDTA tubes to avoid cross-contamination. At least 3mL of saline was flushed into the tubing at the end of each set of sample collection to prevent clotting in the saline lock components. Serum samples were allowed to clot for 30 minutes at room temperature. Then the samples were centrifuged (Sigma 2-6 Compact Centrifuge, Montreal biotech inc., Canada) at 2000xg for 15 minutes and then aliquoted into minimum of eight 2 mL micro tubes (Sarstedt, Germany). Plasma samples were spun down at room temperature for 20 minutes at 1800xg immediately after collection. Samples were then aliquoted in minimum of five 2mL micro tubes (Sarstedt, Germany). Samples were initially stored in -20°C freezer until the end of study visit period at which point they were moved to -80°C freezer for long term storage until analysis.
2.1.6 Urine Collection

Participants were instructed to collect the urine samples with a sterile urine collection cup for each time point. Urine samples were immediately aliquoted into a minimum of eight 2 mL micro tubes (Sarstedt, Germany) and stored in -20°C freezer until the end of the study visit period. Samples were later transferred and stored at -80°C freezer for long term storage until analysis.

2.1.7 Phosphate loading

3.9 mL of liquid sodium phosphate oral solution (Phoslax, Odan Laboratories) were diluted with 125mL of demineralized water (Aquafina, USA) in a disposable plastic cup. The sodium phosphate solution was equivalent to 500mg of sodium phosphate, which was adapted from a previous study done on a difference population.107 Participants were instructed to ingest the 500mg of sodium phosphate solution within 5 minutes.

2.1.8 Baseline, 1-hour and 2-hour sample collections

All study visits were held in a clinical research room at the Kingston General Hospital. Each participant was instructed to come in following an overnight fast (12-hour fast to the start of the study visit) and hydrated with only water. Consent forms were signed. Bottles of demineralized water were provided throughout the study period.

Baseline: the participant was instructed to collect the baseline urine sample with a sterile urine collection cup. Upon return, 2 tubes of baseline serum were collected first with serum-separator vacutainers and followed by 1 tube of baseline plasma collected with K₂EDTA described above. All blood samples were collected via the saline lock. Then, the
participants consumed a cup of 500mg sodium phosphate solution prepared as described above within 5 minutes.

1-hour and 2-hour: 60 minutes and 120 minutes after complete consumption of the 500mg sodium phosphate solution, the participant was instructed to collect urine sample with sterile urine collection cups. A set of 1- and 2-hour blood samples were obtained via the saline lock. 2 tubes of serum were collected first with serum-separator vacutainers and followed by 1 tube of plasma collected with K₂EDTA at each time point. The saline lock was then removed after the second hour sample collection and this concluded the study period (Figure 2-1).
Figure 2-1 Study design of project 1. ▲ Represent blood and urine sample collection time points.
2.1.9 Colorimetric assay

All assays were done using the serum and urine aliquots collected. Serum and urine samples used in the following assays had been subjected to a maximum of two freeze/thaw cycles. All samples were assayed in duplicates. Samples were kept on ice until plating and then labeled to return to -80°C freezer. Optical density (OD) of each sample well was determined by automated microplate reader (SpectraMax Plus Microplate Reader, Molecular Devices, USA) and corresponding concentration of each sample was calculated by automated microplate reader software (SoftMax® Pro version 4.3), unless otherwise specified.

**Phosphate concentration analysis**

The concentration of phosphate in both serum and urine were determined using the assay protocol adapted from Heresztyn and Nicholson (2001). 10 phosphate standards were prepared from 100µM stock potassium phosphate solution and these standards were 0.1, 0.2, 0.3, 0.4, 0.5, 1, 2, 3, 4, and 5nmols. 3 reagents were needed to make the working reagent: 7.5% ammonium molybdate solution (reagent 1), malachite green chloride solution (reagent 2), and 11% tween-20 solution (reagent 3). Standards and reagents were prepared by a technician from the laboratory of Dr. Adams/Dr. Holden in the Department of Biomedical and Molecular Sciences. 1.7mL of reagent 1, 2.5mL of reagent 2 and 20µL of reagent 3 were combined to make the working reagent, which was prepared fresh at the beginning of each assay. According to the protocol provided, serum samples were diluted to 1:10 and urine samples were diluted to 1:100 with double distilled water. 50µL of standards and 10µL samples in duplicate were loaded onto a 96-well plate. 150µL of
double distilled water was added to the standard wells and 190µL of double distilled water was added to the sample wells to bring the total volume of all wells to 200µL. 30µL of the prepared working reagent was quickly added to each well. The plate was incubated on the plate shaker (Titer Plate Shaker, Barnstead/Lab-Line, USA) speed set at 5 at room temperature (22°C) for 10 minutes. The plate was then quickly read at 630nm by the automated microplate reader.

*Creatinine concentration analysis*

The concentration of creatinine in serum and urine samples was determined using the Jaffe method which utilizes picrate to form a complex with creatinine. (QuantiChrom™ creatinine assay, BioAssay Systems, USA). For serum sample analysis, 5µL of 50mg/dL standard stock solution provided by the kit was diluted with 120µL of double distilled water to produce 2mg/dL standard. Serum samples were analysed without dilution. 30µL of double distilled water (blank), diluted standard, and serum samples were transferred to wells of a 96-well plate. To prepare for the working reagent, Reagent A and B provided by the kit was mixed in 1:1 ratio. 200µL of the final working reagent was added to each well. The plate was quickly shaken in the automated microplate reader before it was read at 510nm wavelength. For urine sample analysis, 5µL of double distilled water, 50mg/dL standard stock solution and urine samples were added into wells of a 96-well plate. Working reagent of each well was composed of 50µL of Reagent A, 50µL of Reagent B and 100µL of double distilled water. The plate was quickly shaken and read at 510 nm wavelength by the automated microplate reader after the prepared working reagent was added to each well. All blank, standards and samples were analyzed in duplicate. All serum
and urine plates were read twice with the microplate reader software to determine the OD of each samples. The samples were first read immediately after adding the working reagent and then it was read again 5 minutes later. Then, the average OD of duplicates of both standards and samples were used to calculate creatinine concentration with the equation shown as \[
\frac{OD_{samples\ 5\ min} - OD_{samples\ 0\ min}}{OD_{standards\ 5\ min} - OD_{standards\ 0\ min}} \times \text{Standards concentration (mg/dL)}.
\]

**Calcium concentration analysis**

Total calcium concentration in urine and serum was measured using the o-Cresolphthalein-calcium reaction method (Cayman Chemical, Michigan, USA). 5mL of assay buffer was diluted with 45 mL of double distilled water to be used for standard and sample dilutions. Standards were diluted with prepared assay buffer according to the dilution direction provided by the protocol. Working reagent was prepared by adding calcium detector R1 and R2 provided by the kit in 1:1 ratio. 10\(\mu\)L of standards and samples were added to wells of a 96-well plate. 200\(\mu\)L of prepared working reagent was added to each well. The plate was then shaken on the plate shaker at speed 3 for 30 second. Then the plate was incubated at room temperature for 5 minutes before the plate was read at 580nm wavelength by the automated microplate reader. Serum samples were diluted 1:2 with assay buffer. Urine samples were initially analyzed without dilution. Then, the samples that had higher concentration which were out of range on the initial analysis were analyzed again diluted in 1:2 with assay diluent.
2.1.10 Enzyme-linked immunosorbent assay (ELISA) analysis

All ELISAs were done using the serum or plasma aliquots collected. Serum and plasma samples used in the following ELISA had not been previously thawed. All samples were assayed in duplicates. Samples were kept on ice until after plating. Optical density (OD) of each sample well was determined by automated microplate reader (SpectraMax Plus Microplate Reader, Molecular Devices, USA) and concentrations were calculated by automated microplate reader software (SoftMax® Pro version 4.3), unless otherwise specified.

Cystatin C analysis

Cystatin C concentration in serum was analyzed through the quantitative sandwich enzyme immunoassay technique (Quantikine® Human Cystatin C Immunoassay, R&D systems, USA). A face mask was worn when sample microplate was handled to prevent saliva contamination. Calibrator diluent RD5-24 was diluted 1:5 with double distilled water to serve as the diluent used for standard and sample dilutions. The standard provided by the kit was reconstituted with 1 mL of double distilled water to produce a 200ng/mL standard stock solution. The high standard was prepared by pipetting 300µL of standard stock solution into 300µL of diluted calibrator diluent. Serial dilution of the high standard produced the 50ng/mL, 25ng/mL, 12.5ng/mL, 6.25ng/mL and 3.13ng/mL standards. The diluted calibrator diluent served as the 0 ng/mL standard (blank). 10 µL of each serum sample was diluted with 390µL of prepared calibrator diluent to make 40-fold dilution. 100 µL of assay diluent provided by the kit was added to each well of human cystatin C monoclonal antibody coated plate before 50µL of each standard and sample were added.
After the plate was incubated at 4°C for 3 hours, the plate was then subjected to 4 wash cycles by automated plate washer (ELx50 Auto Strip Washer, Bio-Tek Instruments, USA) with diluted wash buffer. 200 µL of cold cystatin C conjugate provided by the kit was then added to each well before the plate was incubated at 4°C. The plate was washed again following the same protocol and 200 µL of substrate solution was added to each well. To protect from light exposure, the plate was wrapped in aluminum foil to incubate at room temperature (22°C) for 30 minutes. 50µL of stop solution provided by the kit was added to each well and to ensure thorough mixing, each well was pipetted up and down at least once. The plate was then read at 450nm and 570nm wavelength and concentration of each well was calculated by the plate reader by subtracting 570nm reading from 450nm reading to account for imperfections in the plate.

**Parathyroid hormone (PTH) analysis**

Plasma concentration of PTH was analyzed using ELISA kit (Human bioactive PTH 1-84, immutopics, USA). Bioactive PTH was detected using antibodies that recognized both the carboxyl and the amino terminal of the intact PTH. All standards and controls were reconstituted with double distilled water. Working antibody solution was prepared by mixing equal volume of biotinylated human PTH antibody and HRP conjugated human PTH antibody. Plasma samples were analyzed with no dilution. 50µL of standard, control, and sample were loaded onto streptavidin coated plate provided by the kit. 50µL of prepared working antibody solution was then added to each well before the plate was covered with aluminum foil for 3 hour incubation at room temperature on the plate shaker (Titer Plate Shaker, Barnstead/Lab-Line, USA) speed set at 5. The plate was then washed
5 times with diluted wash solution by the automated microtiter plate washer. 100µL of ELSIA HRP substrate solution was pipetted into each well before it was re-covered for another 30 minutes of incubation at room temperature on the titer plate shaker. The plate was then read at 620nm wavelength by the plate reader using the 0 RU/mL standard as blank. 50 µL of stop solution was immediately added to all the wells and shaken for 1 minute. The plate was read at 450nm wavelength against reagent blank (100µL of HRP substrate and 50 µL of stop solution). All samples presented in this study had PTH concentration within standard curve generate with 450nm wavelength read.

Fibroblast growth factor 23 (FGF23) analysis

Plasma concentration of FGF23 was determined using an ELISA kit (Human FGF-23 (c-term), immutopics, USA). Intact FGF23 hormone and large FGF23 carboxyl-terminal fragment were detected by two site ELISA which contains two types of antibodies that capture and detect the hormone. Standards used were prepared by reconstituting each standard vial with double distilled water. Two controls were also provided by the kit and they were reconstituted with double distilled water as well. Working antibody solution was prepared by mixing equal volume of biotinylated human FGF23 antibody and horseradish peroxidase (HRP) conjugated human FGF23 antibody. Plasma samples were analyzed in duplicate with no dilution. 100µL of prepared standards, controls and samples were loaded onto streptavidin coated plate provided before adding 50µL of prepared working antibody solution. The plate was then covered with aluminum foil to protect from light and placed on the plate shaker speed set at 5 for 3 hours. The plate was then subjected to 5 wash cycles with diluted wash solution in the automated microtiter plate washer. 150µL of HRP
substrate was added to each well followed by covering the plate with aluminum foil before it was incubated at room temperature for 30 minutes on the plate shaker speed set at 5. The plate was read at 620nm wavelength by the plate reader against 0 RU/mL standard as the blank. 50µL of stop solution was added immediately and plate was shaken for 1 minute before it was read again at 450nm wavelength against reagent blank (150µL of substrate and 50µL stop solution). All the samples presented in this study had FGF23 concentration within standard curve generated with 450nm wavelength read.

2.1.11 Biomarker statistical analysis

For statistical analysis of the biomarkers, 2 way Analysis of Variance (ANOVA) and Bonferroni’s multiple comparison test were performed to compare the difference in mean concentration of biomarkers of interest between the two groups at each time point as well as comparing between each time points within each group. Results for each biomarker of interest were graphed separately as well as together. Individual graphs were better for representing differences within each group whereas combined graphs can better demonstrate differences between the groups. Sample concentrations were plotted against each time points. Statistical significance is indicated on the graphs. To better separate data plots from two groups, data from PE group are depicted with open circles with solid lines whereas controls are shown in solid circles with dotted lines. Some graphs also include the mean ± SD of each time point and they were shown in grey triangles. The area under the curve (AUC) was also analyzed for each individual to calculate concentration over time. Then the significance of AUC from each group was compared using unpaired t test. Similar to statistical calculation of the demographic data, data that failed the normality test were tested with Mann-Whiney test whereas data that followed the normal Gaussian distribution,
unpaired t-test with Welch correction was performed. The mean and standard error of the mean (SEM) of AUC were graphed as well.

### 2.2 Assessing plasma cystatin C in the first trimester of pregnancy

This study was approved by the Queen’s University Health Sciences Research Ethics Board (OBGY-271-15).

#### 2.2.1 Sample collection

Maternal plasma samples were collected between 12 to 20 weeks of gestation before the diagnosis of PE. Samples were collected by the Ottawa and Kingston (OaK) birth cohort team between the years 2002 to 2009. Plasma samples were collected in EDTA tubes and centrifuged (make and model not available) at 4°C for 10 minutes at 3000xg. All samples were stored at a minimum of -80°C freezer in the Ottawa site. For the details of sample collection, refer to the method sections of OaK birth cohort publications. Nineteen selected plasma samples from women who eventually developed PE (cases) and 19 matched samples from women who had an uncomplicated normotensive pregnancy (controls) were transferred from Ottawa to Kingston on dry ice. Samples were stored in -80°C freezer until analysis. An electronic file of the clinical information associated with the samples was also transferred to researchers in Kingston for analysis.

#### 2.2.2 Sample selection

A list of inclusion and exclusion criteria was used to select appropriate case and control samples for testing. Only singleton pregnancy samples were included in this study. Samples from participants with no history of clinically diagnosed cardiovascular diseases, renal disease, thyroid/parathyroid disorders, diabetes mellitus (type I or type II), smoking
or have ceased smoking for more than 2 years were selected. Cases and controls were matched based on different maternal characteristics. Maternal age (± 5 years), the number of parturitions (± 2) and year of sample collection (± 1 year) were matched between cases and controls. Cases and controls were also matched based on the BMI categories (<20, 20 to 25, 25 to 30, > 30 kg/m²). The number of freeze/thaw cycle which the samples were subjected to was not recorded and some samples were used previously in other studies.

2.2.3 Cystatin C analysis

Plasma samples were thawed quickly in 37°C warm water bath prior to analysis. The samples were then mixed thoroughly before they were spun in the centrifuge (Sigma 1-14 Microfuge, Sigma laborzentrifugen, Germany) at 5000xg for approximately 1 minute. Plasma samples were then stored at 4°C on ice bath for a minimum of 20 minutes to allow the aerosolized material to set before use. Cystatin C was analysed as described in the above section (2.1.10).
Chapter 3

Results

3.1 Project 1: Assessing renal function and phosphate excretion in women with history of preeclampsia

All participants for this study were recruited by the author between June 2015 and April 2016.

3.1.1 Demographic results

The mean (± standard deviation, SD) pre-pregnancy weight of the women with a history of PE group (72.62 ± 22.73 Kg) was significantly higher (P = 0.048) than the matched control group (59.51 ± 14.20 Kg). Also, the mean pre-pregnancy BMI of women with a history of PE was significantly higher (P=0.026) than controls, 27.76 ± 7.96 and 21.96 ± 2.41kg/m², respectively. The details of the demographic data are shown in Table 3-1.

Obstetrical history, family medical history and breastfeeding history collected from both the questionnaire and hospital chart review were shown below (Table 3-2). Information regarding physical activity frequency and intensity before and after the index pregnancy were also collected. Average times of activity were estimated and the Borg Scale of Perceived Exertion was used to estimate intensity. Frequency per week was estimated and greater than 3.5 times a week was considered to be active more than half of the week. Participants were asked to rate their level of exertion on average after each exercise regimen using the Borg Scale. No to low exertion was rated between 6 and 10, moderate exertion rated between 11 and 14, and high level of exertion rated between 15 and 20.
Table 3-1. The mean (±SD) of participant demographic data

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<th>PEs (n=7)</th>
<th>Controls (n=7)</th>
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<tr>
<td></td>
<td>Mean ± SD</td>
<td>Mean ± SD</td>
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<tr>
<td>Age (years-old)</td>
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<td>Time postpartum (Months)</td>
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<td>Height (m)</td>
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<td>1.6 ± 0.1</td>
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<tr>
<td>Pre-pregnancy weight (Kg)</td>
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<td>59.5 ± 14.2</td>
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<tr>
<td>Pre-pregnancy BMI (Kg/m²)</td>
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<tr>
<td>Current weight (Kg)</td>
<td>74.8 ± 23.6</td>
<td>66.4 ± 16.4</td>
<td>0.45</td>
</tr>
<tr>
<td>Current BMI (Kg/m²)</td>
<td>28.6 ± 8.3</td>
<td>24.6 ± 3.9</td>
<td>0.26</td>
</tr>
<tr>
<td>Waist Circumference (cm)</td>
<td>84.8 ± 15.8</td>
<td>77.7 ± 10.6</td>
<td>0.35</td>
</tr>
<tr>
<td>Hip Circumference (cm)</td>
<td>102.2 ± 19.4</td>
<td>97.4 ± 10.9</td>
<td>0.97</td>
</tr>
<tr>
<td>Average systolic blood pressure (mmHg)</td>
<td>115.3 ± 10.2</td>
<td>106.7 ± 6.5</td>
<td>0.09</td>
</tr>
<tr>
<td>Average diastolic blood pressure (mmHg)</td>
<td>72.1 ± 6.9</td>
<td>71.1 ± 6.7</td>
<td>0.78</td>
</tr>
</tbody>
</table>

Unpaired t test with Mann-Whiney test, *P<0.05. Note: PE = previous preeclamptics, BMI = body mass index
<table>
<thead>
<tr>
<th>Medical History</th>
<th>PEs (n=7)</th>
<th>Controls (n=7)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Parity</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Primiparity, n (%)</td>
<td>5 (71.4%)</td>
<td>5 (71.4%)</td>
</tr>
<tr>
<td>Multiparity, n (%)</td>
<td>2 (28.6%)</td>
<td>2 (28.6%)</td>
</tr>
<tr>
<td><strong>Term/Preterm</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Term, n (%)</td>
<td>4 (57.14%)</td>
<td>7 (100%)</td>
</tr>
<tr>
<td>Late Preterm &gt;34 weeks, &lt;37 weeks, n (%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Early Preterm &lt;34 weeks, n (%)</td>
<td>3 (42.86%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td><strong>Maternal 1st degree pregnancy history</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Complication, n (%)</td>
<td>4 (57.14%)</td>
<td>1 (14.29%)</td>
</tr>
<tr>
<td>No complication, n (%)</td>
<td>3 (42.86%)</td>
<td>6 (85.71%)</td>
</tr>
<tr>
<td><strong>Family History of CVD (1st Degree)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes, n (%)</td>
<td>1 (14.29%)</td>
<td>1 (14.29%)</td>
</tr>
<tr>
<td>No, n (%)</td>
<td>6 (85.71%)</td>
<td>6 (85.71%)</td>
</tr>
<tr>
<td><strong>Breastfeeding duration (months)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Never</td>
<td>2 (28.6%)</td>
<td>2 (28.6%)</td>
</tr>
<tr>
<td>Less than or equal to 6 months</td>
<td>1 (14.29%)</td>
<td>4 (57.14%)</td>
</tr>
<tr>
<td>More than 6 months</td>
<td>4 (57.14%)</td>
<td>1 (14.29%)</td>
</tr>
<tr>
<td><strong>Currently on Hormonal Contraception</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes, n (%)</td>
<td>4 (57.14%)</td>
<td>2 (28.57%)</td>
</tr>
<tr>
<td>No, n (%)</td>
<td>3 (42.86%)</td>
<td>5 (71.43%)</td>
</tr>
</tbody>
</table>
Pre-pregnancy physically active

| Active, n (%) | 5 (71.4%) | 6 (85.71%) |
| Non-active, n (%) | 2 (28.6%) | 1 (14.29%) |

Average times active per week in active individuals

| < 3.5 times, n (%) | 2 (40%) | 3 (50%) |
| > 3.5 times, n (%) | 3 (60%) | 3 (50%) |

BORG activity Scale in active individuals

| Light (≤ 10), n (%) | 1 (20%) | 2 (33.33%) |
| Moderate (11 ≤ x ≤ 14), n (%) | 1 (20%) | 4 (66.67%) |
| Hard or extremely hard (≥15), n (%) | 3 (60%) | 0 (0%) |

Currently physically active

| Yes, n (%) | 6 (85.71%) | 6 (85.71%) |
| No, n (%) | 1 (14.29%) | 1 (14.29%) |

Average times active per week

| < 3.5 times, n (%) | 6 (100%) | 3 (50%) |
| > 3.5 times, n (%) | 0 (0%) | 3 (50%) |

BORG activity Scale

| Light (≤ 10), n (%) | 3 (50%) | 2 (33.33%) |
| Moderate (11 ≤ x ≤ 14), n (%) | 0 (0%) | 4 (66.67%) |
| Hard or extremely hard (≥15), n (%) | 3 (50%) | 0 (0%) |

Note. CVD = Cardiovascular disease. PE = previous preeclamptics
3.1.2 Serum Phosphate

Phosphate concentration was measured in mmol/L. The mean (± SD) of phosphate concentration at each time point was graphed with a lighter color in the individual group graphs. Serum phosphate concentration of the women with a history of PE group was significantly higher at 1-hour compared to baseline (P<0.01), also, concentration at 2-hour was significantly higher than baseline (P<0.001) (Figure 3-1A). No such difference was observed in the control group (Figure 3-1B). There was also no difference between the two groups at each time point (Figure 3-1C). There was no statistical significant difference in AUC between the two groups (Figure 3-1D).

3.1.3 Urine Phosphate

Urine phosphate concentration was measured in mmol/L. The mean (± SD) of phosphate concentration at each time point was graphed with a lighter color in the individual group graphs. There was no significant difference in urine phosphate within individual group or between the groups. Similarly, there was no difference in AUC between the groups (Figure 3-2).

3.1.4 Serum creatinine

Serum creatinine concentration was measured in micromole per liter (µmol/L). The mean (± SD) of creatinine concentration at each time point were shown in a lighter color in the individual group graphs. There was no difference observed between the groups or time points (Figure 3-3).
3.1.5 Urine creatinine

Creatinine concentration in the urine was measured in mmol/L. The mean (± SD) of creatinine concentration were depicted in a lighter colour in the individual group graphs. In both the women with a history of PE and matched control groups, the mean urine creatinine concentration at 1-hour and at 2-hour were significantly lower than baseline, P<0.05 and P<0.01, respectively (Figure 3-4A, B). There was no difference in urine creatinine concentration between the groups at each time point (Figure 3-4C).

3.1.6 Serum calcium

Serum calcium concentration was measured in mmol/L. There was no difference observed between the groups or throughout each time points. Similarly, there was no difference found in AUC between the two groups (Figure 3-5).

3.1.7 Urine calcium

Urine calcium concentration was measured in mmol/L. The y-axis of each graph were displayed in two segments to better demonstrate the individual concentration of urine calcium. The mean (± SD) of calcium concentration were depicted in a lighter colour in the individual groups. The controls had significantly lower urine calcium at 2-hour than baseline (P<0.05) (Figure 3-6B). There was no difference observed between the two groups, similarly, no difference was observed comparing AUC (Figure 3-6D).

3.1.8 Urine phosphate and urine creatinine ratio (U_{pi}:U_{crea})

Urine phosphate concentration was compared to urine creatinine for better comparison between participants. Urine phosphate and urine creatinine were measured in mmol/L. The ratio was calculated by dividing urine phosphate by urine creatinine. The U_{pi}:U_{crea} in women with a history of PE group at 2-hour was significantly higher than baseline
(P<0.0001) and at 1-hour (P<0.001) (Figure 3-7A). As for the matched controls, the U_{pi}:U_{crea} at 1-hour and at 2-hour were significantly higher than baseline (P<0.01) and (P<0.0001), respectively (Figure 3-7B). U_{pi}:U_{crea} of the control group was also significantly higher at 2-hour compared to 1-hour (P<0.01) (Figure 3-7B). However, there was no statistical significance between the two groups across the time points. The AUC of U_{pi}:U_{crea} of each group was also calculated, no significance was found between the groups (Figure 3-7D).

3.1.9 Absolute change of U_{pi}:U_{crea}

The absolute change of U_{pi}:U_{crea} was calculated by subtracting U_{pi}:U_{crea} at 1-and 2-hour from baseline. There was no significant difference observed between 1-and 2-hour within individual group or between the groups. The AUC of absolute change of each group was also calculated and there was no significant difference between the groups (Figure 3-8).

3.1.10 Percentage change of U_{pi}:U_{crea}

The percentage change of U_{pi}:U_{crea} was calculated by dividing absolute change of U_{pi}:U_{crea} at 1 and 2-hour from baseline. The percentage change at 2-hour of control group was significantly higher than at 1-hour (P<0.05) (Figure 3-9B). However, no such difference was observed in the women with a history of PE group (Figure 3-9A). There was no statistical difference between two groups throughout time points (Figure 3-9C) and similarly, there was no difference in AUC of percentage change of each group (Figure 3-9D).
3.1.11 Fractional excretion of phosphate (FE\textsubscript{Pi})

Fractional excretion of phosphate was calculated with urine and serum phosphate as well as urine and serum creatinine. The formula used to calculate the fractional excretion could be depicted as such: FE\textsubscript{Pi} (%) = \frac{PO_{4(S)} \times Cr(U)}{PO_{4(U)} \times Cr(S)} \times 100\%. All phosphate and creatinine concentration were measured in mmol/L. FE\textsubscript{Pi} in the women with a history of PE group was significantly higher at 2-hour compared to baseline and 1-hour, P<0.001 and P<0.01, respectively (Figure 3-10A). Similarly, the mean of FE\textsubscript{Pi} in the matched control group was also significantly higher at 2-hour compared to baseline and 1-hour, P<0.0001 and P<0.01, respectively. Also, the controls at 1-hour were significantly higher than baseline as well (P<0.01) (Figure 3-10B). However, there was no significant difference between the 2 groups across time points (Figure 3-10C). The AUC of each individuals were calculated and no difference was found between the two groups (Figure 3-10D).

3.1.12 Urine calcium and urine creatinine ratio U\textsubscript{cal}:U\textsubscript{crea}

Urine calcium concentration was compared to urine creatinine concentration so that the ratio can be compared across participants. Both calcium and creatinine were measured in the mmol/L. The ratio was calculated by dividing urine calcium concentration from urine creatinine concentration. There was no difference observed within each group (Figure 3-11A, B), however, there was a statistically significant difference between the 2 groups at baseline and 1-hour (P<0.05) (Figure 3-11C). The AUC of each individual was also calculated and no difference was found between the two groups (Figure 3-11D).
3.1.13 Absolute change of U_{cal}:U_{crea}

Absolute change of U_{cal}:U_{crea} was calculated by subtracting the U_{cal}:U_{crea} ratio at 1 and 2-hour from baseline. There was no such difference observed in either women with a history of PE or control group (Figure 3-12A, B). There was no significance between the 2 groups observed (Figure 3-12C). The individual AUC of each participant was calculated and there was no difference in AUC when compared between the two groups (Figure 3-12D).

3.1.14 Percentage change of U_{cal}:U_{crea}

The percentage change of U_{cal}:U_{crea} was calculated by dividing absolute change of U_{cal}:U_{crea} at 1 and 2-hour from baseline. There was no significant difference within each group or between the two groups. The AUC of percentage change of each group was also calculated. There was no difference between the two groups (Figure 3-13).

3.1.15 Serum cystatin C

Serum cystatin C protein concentration was analyzed with ELISA kits and measured in milligram per liter (mg/L). There was no statistical significance within each group across the time points or between the two groups (Figure 3-14).

3.1.16 Estimated GFR

The Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) – serum creatinine and cystatin C (CKD-EPI_{Scr-cys}) equation was used to calculate estimated GFR because it was shown to be the best equation for young individuals, female, and populations expected to have higher GFR, which was the population of interest in this study.\textsuperscript{110} The following table depicted the details of the CKD-EPI_{Scr-cys} equations (Table 3-3) and depending on the concentration of creatinine and cystatin C of each individual, different factors were used for calculation.\textsuperscript{111} Only one women with a history of PE participant was black, thus the
factor of 1.08 was only applied to that particular participant. The serum creatinine concentrations of all participants were under 0.7 mg/dL, thus only the top two equations were applicable to eGFR calculation in this study. The eGFR was calculated in mL/min/1.73m². There was no statistical significance within individuals groups or between the two groups (Figure 3-15).

3.1.17 Plasma PTH

Intact plasma PTH was measured in picogram per milliliter (pg/mL). The mean (± SD) of PTH concentration at each time point was graphed with a lighter color in the individual group graphs. One control participant had abnormally high level of PTH throughout the time points. A robust regression and outlier removal method was employed to analyze all the control participant PTH concentrations to identify the outliers, which then identified one control participant PTH concentration at baseline was an outlier. Since the analysis would require the full set of data from all 3 time points, all PTH concentrations from this participant were removed from analysis. In order to maintain the comparability between the two groups, the matching women with a history of PE participant was also removed from statistical analysis. After excluding the outlier and matched women with a history of PE, controls had significantly higher intact PTH concentration compared to baseline (P<0.05) (Figure 3-16B). Controls were also significantly higher at 1-hour than women with a history of PE (P<0.001) (Figure 3-16C).

3.1.18 Plasma FGF23

Plasma FGF23 was measured in relative unit per milliliter (RU/mL). The mean (± SD) of FGF23 concentration at each time point was graphed with a lighter color in the individual
group graphs. One control participant and one women with a history of PE participant, not matched with one another, had abnormally high level of FGF23 throughout the time points. A robust regression and outlier removal method was employed to analyze both control and women with a history of PE participant FGF23 concentrations to identify the outliers, which then identified this control and women with a history of PE participant FGF23 concentration to be outliers throughout all three time points. All FGF23 concentration from these two participants were removed from analysis. In order to maintain the comparability between the two groups, the matching women with a history of PE and control participant for each of the excluded participants were also removed from statistical analysis. After excluding the four participants, there was no statistical difference found within each group or between the two groups (Figure 3-17).

3.2 Assessing plasma cystatin C in the first trimester of pregnancy
All participant were recruited by the OaK birth cohort between the year of 2002 and 2009.

3.2.1 Demographic data
A total of 19 PE and 19 matched controls were provided by the OaK birth cohort study. Demographic information were provided by the birth cohort in an excel file. Demographic factors provided included age, height, pre-pregnancy weight, and pre-pregnancy BMI. Information such as gestational age at delivery and baby’s weight were also provided. Since demographic data were not collected continuously, unpaired t test was used to perform the statistical analysis and followed the same principle described in project 1 (see 3.1.1). The mean (±SD) gestational age at delivery of the PE group (37.46 ±2.322 weeks) was significantly lower (P<0.01) than the matched control group (39.37±1.321 weeks). Unsurprisingly, the mean (±SD) of baby’s weight of PE group (2924±796.6g) was
significantly lower (P<0.05) than the matched controls group (3480±492.4g). The details of the demographic data were depicted in the following table (Table 3-4).

3.2.2 Plasma cystatin C
Unpaired t-test was used to analyze cystatin C concentration between the PE and control groups. Since the two groups followed normal distribution which was tested with D’Agostino Omnibus test, unpaired t-test with Welch correction was performed. The mean (±SD), depicted in black in Figure 3-18, cystatin C concentration of the PE group was 0.5198 (±0.08063) mg/L, whereas the cystatin C concentration of controls was 0.4912 (±0.06588) mg/L. Women who were destined to develop PE were depicted in open circles and controls were depicted in solid black circles. The mean ± SD of each group was shown in black bars. There was no significant difference between the two groups in cystatin C concentration in the first trimester (Figure 3-18).
### Table 3-3 Female CKD-EPI Scys-crea equations for estimating GFR, shown for serum creatinine level and serum cystatin C.

<table>
<thead>
<tr>
<th>Serum Creatinine (mg/dL)</th>
<th>Serum Cystatin C (mg/L)</th>
<th>Equation for estimating GFR</th>
</tr>
</thead>
<tbody>
<tr>
<td>≤ 0.7</td>
<td>≤ 0.8</td>
<td>$130 \times \left(\frac{Scr}{0.7}\right)^{-0.248} \times \left(\frac{Scys}{0.8}\right)^{-0.375} \times 0.995^{Age}$ $\times [1.08 \text{ if black}]$</td>
</tr>
<tr>
<td>≤ 0.7</td>
<td>&gt; 0.8</td>
<td>$130 \times \left(\frac{Scr}{0.7}\right)^{-0.248} \times \left(\frac{Scys}{0.8}\right)^{-0.711} \times 0.995^{Age}$ $\times [1.08 \text{ if black}]$</td>
</tr>
<tr>
<td>&gt; 0.7</td>
<td>≤ 0.8</td>
<td>$130 \times \left(\frac{Scr}{0.7}\right)^{-0.601} \times \left(\frac{Scys}{0.8}\right)^{-0.375} \times 0.995^{Age}$ $\times [1.08 \text{ if black}]$</td>
</tr>
<tr>
<td>&gt; 0.7</td>
<td>&gt; 0.8</td>
<td>$130 \times \left(\frac{Scr}{0.7}\right)^{-0.601} \times \left(\frac{Scys}{0.8}\right)^{-0.711} \times 0.995^{Age}$ $\times [1.08 \text{ if black}]$</td>
</tr>
</tbody>
</table>

Note: Scr = serum creatinine, Scys = serum cystatin C
Table 3-4 The mean (±SD) of maternal and infant demographic data

<table>
<thead>
<tr>
<th>Demographic factors</th>
<th>PE (n=19)</th>
<th>Control (n=19)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Maternal characteristic</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>30.6 ± 5.09</td>
<td>30.8 ± 4.5</td>
<td>0.87</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>161.8 ± 5.3</td>
<td>164.9 ± 6.3</td>
<td>0.11</td>
</tr>
<tr>
<td>Pre-pregnancy Weight (kg)</td>
<td>72.3 ± 15.2</td>
<td>77.00 ± 16.0</td>
<td>0.36</td>
</tr>
<tr>
<td>Pre-pregnancy BMI (kg/m²)</td>
<td>27.6 ± 5.26</td>
<td>28.5 ± 6.9</td>
<td>0.64</td>
</tr>
<tr>
<td><strong>Infant characteristic</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gestational age at birth (weeks)</td>
<td>37.5 ± 2.3</td>
<td>39.3 ± 1.3</td>
<td>0.007**</td>
</tr>
<tr>
<td>Weight (g)</td>
<td>2924 ± 796.6</td>
<td>3480 ± 492.4</td>
<td>0.012*</td>
</tr>
</tbody>
</table>

Note: BMI=body mass index, PE=pre-eclamptic *: P<0.05, **: P<0.01
Figure 3-1 Serum phosphate concentration. The serum phosphate concentration of women with a history of PE (A) and controls (B) were graphed separately as well as together (C). The mean Spi of women with a history of PE at 1- and 2-hour was significantly higher than baseline. The AUC of Spi was compared between the two groups (D). ○—○: women with a history of PE; ●--●: controls; grey▲ and error bars: mean ± SD. Note: Spi = serum phosphate. **:P<0.01, ***:P<0.001.
**Figure 3-2 Urine phosphate concentration.** The urine phosphate concentration of women with a history of PE (A) and controls (B) were graphed separately as well as together (C). The individual AUC and mean (±SEM) AUC of urine phosphate were plotted in (D). ○—○: women with a history of PE; ●—●: controls; grey▲ and error bars: mean ± SD. Note: Upi = urine phosphate.
Figure 3-3 Serum creatinine concentration. Serum creatinine concentration in µmol/L was plotted against each time point for the women with a history of PE (A) and matched control group (B). The two groups were also plotted together shown in (C). ○─○: women with a history of PE; ●--●: controls; grey▲ and error bars: mean ± SD. Note: Screa = Serum creatinine.
Figure 3-4 Urine creatinine concentration. Urine creatinine concentration in mmol/L was plotted against each time point for the women with a history of PE (A) and matched control group (B). The mean urine creatinine concentration of both women with a history of PE and the mean of controls were significantly lower at 1 and 2-hour than baseline. There was no significant difference in urine creatinine concentration between the groups (C). ○—○: women with a history of PE; ●--●: controls; grey▲ and error bars: mean ± SD. Note: Ucrea = urine creatinine. *: P<0.05, **: P<0.01.
Figure 3-5 Serum calcium concentration. The serum calcium concentration of women with a history of PE (A) and matched controls (B) were plotted separately and together (C). The individual AUC and mean (±SEM) AUC of serum calcium were plotted in (D). ○—○: women with a history of PE; ●--●: controls. Note: Scal = Serum calcium
Figure 3-6 Urine calcium concentration. The urine calcium concentration of women with a history of PE (A) and matched controls (B) were plotted separately and together (C). The mean urine calcium concentration of control group at 2-hour was significantly lower than baseline. The individual AUC and mean (±SEM) AUC of urine calcium were plotted in (D). ○—○: women with a history of PE; •--•: controls; grey▲ and error bars: mean ± SD. Note: Ucal = urine calcium. *: P<0.05
Figure 3-7 Urine phosphate urine creatinine ratio. The $U_{\text{Pi}}:U_{\text{Crea}}$ of women with a history of PE (A) and matched controls (B) were plotted separately and together (C). The mean ratio at 2-hour of both women with a history of PE and controls were significantly higher than respective baseline and 1-hour. The mean ratio of control group was also significantly higher at 1-hour than baseline. The individual AUC and mean (±SEM) AUC of $U_{\text{Pi}}:U_{\text{Crea}}$ were plotted in (D). ○--○: women with a history of PE; ●--●: controls. Note: $U_{\text{Pi}}:U_{\text{Crea}} = $ urine phosphate urine creatinine ratio. **: P<0.01, ***:P<0.001, ****:P<0.0001.
Figure 3-8 Absolute change of urine phosphate and urine creatinine ratio. The absolute change of $U_{\text{pi}}:U_{\text{crea}}$ of women with a history of PE (A) and matched controls (B) were plotted separately and together (C). The mean absolute change of both women with a history of PE and controls at 2-hour were significantly higher than respective 1-hour. The mean absolute change of $U_{\text{pi}}:U_{\text{crea}}$ of control was significantly higher at 1-hour than controls. The individual AUC and mean (±SEM) AUC of $U_{\text{pi}}:U_{\text{crea}}$ were plotted in (D). ○─○: women with a history of PE; ●→●: controls. Note: absolute $\Delta U_{\text{pi}}:U_{\text{crea}} = $ absolute change in urine phosphate urine creatinine ratio.*: P<0.05, **: P<0.01.
Figure 3-9 Percentage change of urine phosphate urine creatinine ratio. The percentage change of \( U_{\text{pi}}:U_{\text{crea}} \) of women with a history of PE (A) and matched controls (B) were plotted separately and together (C). The mean percentage change at 2-hour of controls was significantly higher than 1-hour. The individual AUC and mean (±SEM) AUC of \( U_{\text{pi}}:U_{\text{crea}} \) were plotted in (D). ○-○: women with a history of PE; ●--●: controls. Note: \( \% \Delta U_{\text{pi}}:U_{\text{crea}} \) = percentage change of urine phosphate urine creatinine ratio. *: \( P<0.05 \)
Figure 3-10 Fractional excretion of phosphate. The fractional excretion of phosphate of women with a history of PE (A) and matched controls (B) were plotted separately and together (C). The mean fractional excretion of both women with a history of PE and controls at 2-hour were significantly higher than respective baseline and 1-hour. The mean fractional excretion at 1-hour was significantly higher than baseline. The mean (±SEM) AUC of fractional excretion were plotted in (D). ○—○: women with a history of PE; ●—●: controls. Note: FE(pi) % = fractional excretion of phosphate. **: P<0.01, ***: P<0.001, ****: P<0.0001
**Figure 3-11 Urine calcium and urine creatinine ratio.** The \( \text{U}_{\text{calc}}:\text{U}_{\text{crea}} \) of women with a history of PE (A) and matched controls (B) were plotted separately and together (C). The mean \( \text{U}_{\text{calc}}:\text{U}_{\text{crea}} \) of control group at baseline and 1-hour were significantly higher than women with a history of PE. The individual AUC and mean (±SEM) AUC of \( \text{U}_{\text{calc}}:\text{U}_{\text{crea}} \) were plotted in (D). ○—○: women with a history of PE; ●—●: controls. Note: \( \text{U}_{\text{calc}}:\text{U}_{\text{crea}} \) = urine calcium urine creatinine ratio. *:*P<0.05.
Figure 3-12 Absolute change of urine calcium and urine creatinine. The absolute change $U_{\text{cal}}:U_{\text{crea}}$ of women with a history of PE (A) and matched controls (B) were plotted separately and together (C). The individual AUC and mean (±SEM) AUC of absolute change of $U_{\text{cal}}:U_{\text{crea}}$ were plotted in (D). ○—○: women with a history of PE; ●--●: controls. Note: Absolute Δ of $U_{\text{cal}}:U_{\text{crea}}$ = absolute change of urine calcium urine creatinine ratio.
Figure 3-13 Percentage change of urine calcium urine creatinine. The percentage change $U_{\text{cal}}:U_{\text{crea}}$ of women with a history of PE (A) and matched controls (B) were plotted separately and together (C). The individual AUC and mean (±SEM) AUC of percentage change of $U_{\text{cal}}:U_{\text{crea}}$ were plotted in (D). ○—○: women with a history of PE; ●—●: controls.

Note: Percentage $\Delta$ of $U_{\text{cal}}:U_{\text{crea}}$ = percentage change of urine calcium urine creatinine ratio.
Figure 3-14 Cystatin C concentration. The cystatin C concentration of women with a history of PE (A) and matched controls (B) were plotted separately and together (C). ○•○: women with a history of PE; ●--●: controls.
Figure 3-15 The CKD-EPI\textsubscript{Scys-crea} calculated estimated glomerular filtration rate. The eGFR of women with a history of PE (A) and matched controls (B) were plotted separately and together (C). ○—○: women with a history of PE; ●--●: controls.
Figure 3-16 Intact parathyroid hormone. The iPTH concentration of women with a history of PE (A) and matched controls (B) were plotted separately and together (C). The mean iPTH concentration of control at 1-hour was significantly higher than baseline. The mean iPTH concentration of control was significantly higher than women with a history of PE at 1-hour. ○—○: women with a history of PE; ●--●: controls; grey▲ and error bars: mean ± SD. Note PTH = parathyroid hormone *: $P<0.05$, **: $P<0.001$. 
Figure 3-17 Fibroblast growth factor 23. The FGF23 concentration of women with a history of PE (A) and matched controls (B) were plotted separately and together (C). ○—○: women with a history of PE; ●—●: controls; grey ▲ and error bars: mean ± SD. Note FGF23 = fibroblast growth factor 23.
Figure 3-18 Cystatin C concentration. Cystatin C concentration of PE and control groups. The mean ± SD of each group was shown in black error bars. ○: women destined to develop PE; ●: controls.
Chapter 4
Discussion

Project 1: Assessing renal function and phosphate excretion in women with history of preeclampsia

Present study provided novel insights into phosphate metabolism of early postpartum women who did or did not have a history of PE. In this study, it was discovered that women with a history of PE had significantly elevated serum phosphate concentration after a 500mg phosphate dose. A significant elevation in serum phosphate was only expected in higher doses of phosphate, which was demonstrated in several other phosphate loading studies.\(^{71,82,112}\) This elevation in serum phosphate could be due to the delay in urine phosphate excretion. As shown in several calculations in this study, women with a history of PE had a lower phosphate excretion rate in the first hour after phosphate ingestion compared to controls. Though there was no difference in total phosphate excretion over the two hour period, women with a history of PE had longer exposure to elevated serum phosphate compared to controls. Prolong exposure to serum phosphate elevation can have serious consequences to the cardiovascular system. Two of the major regulating factors involved in phosphate excretion were investigated, women with a history of PE had no detectable fluctuation in either of the regulating hormones. This finding was unexpected since elevation in serum phosphate should have stimulated the release of PTH into circulation to reduce phosphate reabsorption and subsequently increase urine phosphate excretion. The absence in hormone response to serum phosphate elevation can also explain the delay in phosphate excretion seen in women with a history of PE.
Demographic data comparison

Women with a history of PE and matched control women were recruited within 5 years postpartum. It was hypothesized that the majority of the demographic data collected between the two groups would be similar, however, criteria such as pre-pregnancy weight and current average blood pressure were expected to be different. The participants were matched based on age, parity and time postpartum, thus there was no difference in these criteria within the participants analyzed. As the majority of the data presented in Table 3-1 were collected through medical records, pre-pregnancy weight was self-reported and pre-pregnancy BMI was then calculated based on the height measured. It was known that women tend to underestimate their weight.\textsuperscript{113,114} In a study done with the Canadian population, women on average under-reported their weight by 2.5kg. In the age group between 25 and 44, the mean difference in self-reported and measured weight was 2.3kg (95%CI 1.9 to 2.6).\textsuperscript{114} Assuming that participants from the present study followed the same phenomenon as the Canadian female population study, the mean prepregnancy weight from both group should be higher but the difference between the two groups should remain the same. It was of no surprise that women with a history of PE had significantly higher prepregnancy weight and BMI compared to controls (Table 3-1). As presented in the risk factor section of the introduction, Section 1.1.2, prepregnancy weight and BMI have shown to be important PE risk factors. Women with prepregnancy BMI of 26 had a 2.1 (95% CI 1.4, 3.4) adjusted risk of PE compared to women with prepregnancy BMI of 21.\textsuperscript{115} The risk of PE increased with prepregnancy BMI and prepregnancy underweight was shown to have a protective effect compared to women of normal BMI.\textsuperscript{115} As for postpartum blood pressure, it has been well documented in the literature that women with a history of PE
have higher risk of hypertension than controls even as early as one year postpartum.\textsuperscript{116–118} A study with 140 women with a history of PE and controls has shown that women with a history of PE had significantly higher blood pressure than control one year postpartum.\textsuperscript{116} After adjusting for age, BMI, and smoking habit, women with a history of PE still have higher risk of being diagnosed with hypertension later in life compared to controls, an OR of 3.98 (95\%CI 2.82 to 5.61).\textsuperscript{117} There was no difference found in average systolic or diastolic blood pressure between the present study groups (Table 3-1). The mean (±SD) of systolic blood pressure of women with a history of PE and matched controls were 115.3 ± 10.19 and 106.7 ± 6.476, respectively. No difference was found in mean (±SD) diastolic blood pressure between women with a history of PE and controls either, 72.10 ± 6.85 and 71.05 ± 6.73, respectively. Given the small sample size, the significance between the two groups may not be apparent. With the demographic data presented, the prepregnancy weight and BMI coincide with other studies done with this population, however, the blood pressure data did not agree with the literature.

The discrepancy between the two groups in hormonal contraception could possibly pose as a confounding variable. Of the 4 women with a history of PE, 2 of which were using levonorgestrel-releasing intrauterine system (Mirena) as a form of hormonal contraception. 2 others were on either monophasic or triphasic oral contraception composed of combinations of synthetic estrogen and progesterone. One control participant was on Mirena and the other was on a type of multiphasic oral contraception with mixture of synthetic estrogen and progesterone. The use of hormonal contraceptives has been associated with changes in the renin-aldosterone system, blood pressure, and renal
hemodynamic. The increase in blood pressure through the activation of renin-aldosterone system by estrogen in hormonal contraceptives has been well documented. Conversely, progesterone lowers blood pressure through natriuresis. In an earlier study, the renal function of women who started, continued or stopped using hormonal contraception between an average of 4.2 years follow up period were compared to women who had never used hormone contraception. Women who started hormonal contraception between the two assessments had significantly elevated \( P = 0.074 \) urine albumin excretion and reduced \( P<0.001 \) GFR. In a study that investigated the renal response to high sodium diet in women on monophasic oral contraceptives has shown a significant increase \( P<0.05 \) in GFR without significant increase in ERPF. However, such increase in GFR shown in this study could be the result of a 7-day high sodium diet rather than one salt dosage comparable to the present study. The GFR of participants who were on oral contraceptives did not differ from the rest of the respective groups. Since the diet habits and GFR history of these participants were not collected, it is difficult to determine whether the GFR of these women were affected by the oral contraceptives. As shown in a study that examined carbohydrate metabolism, blood glucose, insulin, lipid and total testosterone profile in Mirena users, no difference was found in these metabolic function parameters compared to women with IUD containing no hormones (copper-releasing IUD). Mirena did not appear to affect blood pressure in women after 12 years of usage. Mirena is a locally released progesterone only hormone contraception, low plasma concentration of levonorgestrel is detected in user which explains the minimal systemic effect seen in Mirena users.
The purpose of collecting information on physical activity in this study was to ascertain that both of the study groups have similar physical activity pattern in average. Since moderate/intense physical activity has shown to influence lean body mass, serum creatinine, urinary creatinine and reducing risk of metabolic syndrome\textsuperscript{125,126}, the more active group may have a different metabolic rate of creatinine than the other group. To determine whether such difference was present between the groups, information on the frequency and intensity of physical activity before and after pregnancy were collected (Table 3-2). The two participant groups were relatively active prepregnancy and mostly resumed postpartum. This may represent a biased sampling of the population. Using the BORG intensity scale to estimate each physical activity postpartum, half of PE participants categorized themselves to be in the extremely high intensity range of exercise whereas 67% of controls considered to have moderate intensity exercise in average (Table 3-2). However, the average frequency of physical activity in the PE group was lower compared to prepregnancy whereas controls were consistent before and after pregnancy. Since there was no significant differences or outliers between the two groups in terms of physical activity, the average metabolic rate of creatinine in individuals from each group should be relatively similar.

*Estimated glomerular filtration rate*

Since majority of the creatinine production is done by the skeletal muscle, creatinine production depends on age, sex and lean muscle mass.\textsuperscript{125,127} Since all participants were female and there was no statistical difference in age between participants, creatinine production should not be significantly different in these regards. As for muscle mass, there
was no difference in current weight and BMI, and neither of the groups were particularly athletic. Thus, creatinine concentration in serum should be comparable. Since in steady state, the rate of creatinine production is proportional to metabolic degradation and urinary excretion and with no history of metabolic or kidney diseases, serum creatinine in these individuals were expected to be relatively consistent over time.\textsuperscript{125,127} The adult reference range for serum creatinine concentration in women is 68 to 98 µmol/L.\textsuperscript{128} However, after repeated analysis of the samples, serum creatinine concentrations were all bellow 65 µmol/L in both groups (Figure 3-3). Since both women with a history of PE and control participants had lower values than expected and assuming the low values were proportional to clinical values, the comparison between the two groups should still be sound. As expected, there was no significant changes in serum creatinine throughout the time points in either groups. Because of the unexpectedly low values of serum creatinine across time point, serum creatinine could not be used alone to calculate GFR. Serum creatinine is thought to be freely filtered by glomerulus and secreted by the proximal tubule of the kidneys.\textsuperscript{129} Thus, urine creatinine concentration depends on GFR and rate of proximal tubule secretion. Both women with a history of PE and control groups had significantly lower urinary creatinine at 2-hour compared to respective baselines (Figure 3-4). In another phosphate loading study, no decrease in urinary creatinine excretion over time was observed.\textsuperscript{130} Given that the turnover rate from creatine to creatinine is fairly constant, GFR should remain relatively constant over the study period, and serum creatinine concentrations were consistent over the time period, the reason for this significant decrease in urinary creatinine is unclear.
Cystatin C is thought to be produced by all nucleated cells at a constant rate and freely filtered by the glomerulus and catabolized by the proximal tubules with small amounts of cystatin C excreted in urine.\textsuperscript{61,131} It is generally accepted in the literature that serum cystatin C concentration is independent of age, gender and muscle mass.\textsuperscript{132} However, in some studies, factors such as older age, male gender, height, weight, smoking and inflammation status, and C-reactive protein concentration have been shown to affect serum cystatin C concentration independent of renal function.\textsuperscript{132,133} Nevertheless, it is agreed among these studies that although non-GFR factors were significantly associated with changes in cystatin C, the associations were relatively small.\textsuperscript{61,132} After adjusting for non-GFR determinants, association of factors have higher effect on serum creatinine based GFR calculation than cystatin C.\textsuperscript{61,132} The serum concentration of endogenous biomarkers such as cystatin C can be affect by numerous physiological processes other than glomerular filtration such as tubular reabsorption or secretion and extra-renal elimination.\textsuperscript{61} However, the lack of urine excretion of cystatin C can make clinical analysis on these physiological processes difficult. Measuring urinary creatinine excretion, on the other hand, can possibly provide information in these physiological processes that cystatin C cannot.\textsuperscript{61} Even though cystatin C has its limitations, in comparison, the majority of the literature still consider cystatin C to be a better endogenous biomarker for eGFR than creatinine.\textsuperscript{57,60,134} The normal serum cystatin C concentration range in women is 0.49-0.94mg/dL.\textsuperscript{128} Both groups exhibited normal serum cystatin C concentration (Figure 3-14). Cystatin C is thought to be more sensitive compared to creatinine in detecting early renal impairment whereas traditional methods such as creatinine concentration or creatinine clearance may not be able to detect.\textsuperscript{131} Compared to other eGFR equations such as the more commonly used
Cockcroft-Gault and Modification of Diet in Renal Disease formula\textsuperscript{135}, the CKD-EPI\textsubscript{Scr-cys} was considered to be the more appropriate eGFR equation for the population of interest.\textsuperscript{110} Numerous studies have been done to determine what the normal range of GFR in healthy young women should be, and results from studies involving more than 200 women provided a relatively similar range for different age groups.\textsuperscript{136} Based on a study done with a large number of healthy female participants, women between the age of 20 and 50 should have a GFR between 71 and 141 mL/min/ 1.73 m\textsuperscript{2}.\textsuperscript{136} The CKD-EPI\textsubscript{Scr-cys} equation utilizes both serum creatinine and cystatin C. Factors such as age, race, and gender are also used in the equation to determine eGFR (Table 3-3). Since the majority of the participants, except one black participant, were of Caucasian decedent, the 1.08 factor was only applied to the calculation of eGFR for that particular participant. Given that serum creatinine concentration from both groups are lower than expected, and serum creatinine concentration and CKD-EPI\textsubscript{Scr-cys} have an inverted relationship, the eGFR calculated with CKD-EPI\textsubscript{Scr-cys} may be higher than the actual rate. (Figure 3-15). The mean range of eGFR of both groups calculated with CKD-EPI\textsubscript{Scr-cys} equation was between 129.7 and 133.7 mL/min/ 1.73 m\textsuperscript{2}. The MacIssac equation was shown to be the second best equation to calculate eGFR in a young healthy population.\textsuperscript{110} MacIssac equation is ideal in this situation because it only utilizes cystatin C to calculate eGFR.\textsuperscript{110} The mean eGFR calculated using the MacIssac equation (data not shown) were all within normal range, between 126.5 and 133 mL/min/ 1.73 m\textsuperscript{2}. Both equations demonstrated that the participants from both groups had a relatively consistent GFR throughout the experimental time. Thus, with respect to glomerular filtration, there was no significant difference or deterioration of function in women with a history of PE compared to controls.
After the consumption of phosphate solution, it was expected that the kidneys would excrete the excess phosphate over time while maintaining the relative stability of serum phosphate. AUC of each individual from each group was calculated based on concentration of biomarker of interest, in this case phosphate, over time. Analysis of AUC is commonly used to determine the total drug exposure over time in pharmacokinetics and pharmacodynamics analysis of a drug. Comparing the AUC of each group has shown no difference, similar to that of the comparison between the two groups at each time point. An early study examining the acute effect of oral dosing of 1000mg phosphate in young women had shown a significant increase compared to baseline in serum phosphate at 90 and 120 minutes. Such result was similar to women with history of PE in the present study (Figure 3-1), who were only dosed with 500mg phosphate. However, when young healthy men were dosed with phosphate concentration of 400mg, 800mg, and 1200mg of inorganic phosphate, an increase of serum phosphate in the first 2-hours was only seen after dosing of 800mg and 1200mg. There was no difference in serum phosphate concentration over 2-hours in the control group corresponded to results presented from relatively low phosphate (400mg) loading. The significant serum phosphate elevation from baseline that was seen in the women with a history of PE study group was similar to that of a 800mg to 1 gram phosphate dosing in the literature. The discrepancy between our controls and PE suggests that the PE group required a lower phosphate dosing to increase serum phosphate, while controls may require a higher dosing. Previous studies that investigated urine phosphate excretion after loading showed an increase of urine phosphate excretion over time. In contrast to what was expected, the urine phosphate
concentration in both women with a history of PE and controls remain unchanged over time (Figure 3-2).

Though all participants were advised to keep themselves hydrated for the study, the total amount of water consumption was not controlled for each participant before or during the study. Without controlling for fluid intake, the concentration of urine can vary significantly between individuals. To correct for such variability, an endogenous biomarker was needed to standardize between individuals. The steady serum calcium concentration (Figure 3-5) over the study period demonstrated that there was no dilution in serum concentration from hydration. Thus, serum phosphate was not standardized to serum creatinine. Since the significant decrease in urine creatinine (Figure 3-4) was unlikely due to significant decrease in creatinine production during the present study period but more likely due to hydration which resulted in urine dilution. Due to the absence of urinary excretion of cystatin C, to further examine the difference in urine phosphate excretion between participants, urine phosphate was compared to urine creatinine concentration. As none of the participants has clinically diagnosed kidney diseases of any sort and having no difference in eGFR, urine creatinine was considered adequate biomarkers to be used as reference for phosphate concentration comparison between the two groups. Upon examination of $U_{\text{Pi}}:U_{\text{Crea}}$ and $\text{FEpi}\%$, only the control group showed significant increase ($P<0.01$) from baseline to 1-hour (Figure 3-7 and Figure 3-10, respectively). The difference between the two groups became apparent after comparing the absolute change in $U_{\text{Pi}}:U_{\text{Crea}}$. The absolute change in $U_{\text{Pi}}:U_{\text{Crea}}$ of controls were significantly higher ($P<0.05$) than women with a history of PEs at 1-hour (Figure 3-8).
U_{\text{pi}:\text{U_{crea}}} was calculated, the significant increase from 1-hour to 2-hour was only shown in the control group (Figure 3-9). It was apparent that two of the control participants had percentage change in U_{\text{pi}:\text{U_{crea}}} over 500% at 2-hour. Even though the 2 values were not identified as outlier, the 2 control participants and the matching PE were removed for further analysis (data not shown). After excluding the four participants, both groups exhibited significant increase of percentage change of U_{\text{pi}:\text{U_{crea}}} from 1- to 2-hour. Though the percentage change of U_{\text{pi}:\text{U_{crea}}} was not significantly different between the two groups at 1-hour, the mean difference in percentage change was 80.1% (Figure 3-9). The significant increase in the control group from baseline to 1-hour suggested that the control groups reacted to the phosphate dosing within the first hour and were actively excreting the excess phosphate from circulation and the excretion continued with a significant increase from 1-hour to 2-hour. As for the women with a history of PE group, the significant increase in phosphate excretion was not detected until the second hour. The insignificant difference in all the AUC of U_{\text{pi}:\text{U_{crea}}} between the groups suggested that overall phosphate excretion was similar between the group. The similar overall phosphate excretion combined with the significantly higher excretion at 1-hour of the control group and similar excretion ratio at 2-hour between the groups suggested that the women with a history of PE group had a delay excretion followed by a rapid increase in excretion. This delay in U_{\text{pi}:\text{U_{crea}}} excretion in women with a history of PE group could possibly explain the persistent elevation in serum phosphate in the 2 hour study period. The fast excretion of the control group at 1 and 2-hour could also explain the relative consistency of serum phosphate concentration in this group. Since phosphate dosing in women, particularly the young postpartum population has not been studied, the true underlying cause for this
difference between the groups is unclear. However, inability to eliminate phosphate quickly can have serious long term effects on the cardiovascular system.

*Calcium*

As described previously in (Figure 1-2) phosphate metabolism is closely associated with calcium metabolism due to the shared hormonal regulators. Since the phosphate solution provided only contained sodium phosphate, the response to increase of phosphate intake can have changes in calcium concentration response in both serum and urine as a by-product of hormonal regulation. Since calcium hemostasis, particularly serum calcium, is tightly maintained, serum and urine calcium concentrations were expected to be consistent over the study period.\(^7\)\(^8\) The serum calcium of both women with a history of PE and controls were consistent over time as predicted (Figure 3-5). However, the decrease in urine calcium (Figure 3-6) was likely due to urine dilution from hydration during the study period. Upon further examination, 3 of the control and 1 of the women with a history of PE participants had much higher urine calcium concentration at baseline (> 2.8 mmol/L). To further investigate the difference between women with a history of PE and control groups, urine calcium was also standardized to urine creatinine concentration. The absolute and percentage change of \(U_{\text{cal}}:U_{\text{crea}}\) were not different between the groups (Figure 3-12 and Figure 3-13, respectively) suggesting that there were no significant changes over time from respective baseline. The difference observed between control and women with a history of PE group was likely due to the higher urine calcium at baseline of the control group.
**Hormone regulators**

iPTH was analyzed because it is considered to be a fast acting phosphate regulator. However, PTH is primarily a calcium regulating hormone in which the decrease in serum calcium triggers the release of PTH. An increase in phosphate excretion is achieved by iPTH inhibition of phosphate reabsorption by the kidneys. However, the iPTH has a relatively short half-life in circulation due to the rapid clearance and degradation by the liver and kidneys. When animals are fed with a high phosphate diet, the increase in serum phosphate which was associated with a decrease in circulating calcium concentration was shown to trigger release of PTH. Also, there was evidence from animal studies that showed release of PTH within minutes after a high phosphate diet regardless of serum phosphate and calcium concentration. Thus, it has been proposed that there may be additional signalling in the gastrointestinal tract to trigger the release of PTH. In the analysis of iPTH in the two groups in our study, the control group had a significant increase of iPTH at 1-hour and it was also significantly higher than women with a history of PE at 1-hour (Figure 3-16). The mean difference in elevation of iPTH from baseline to 1-hour of the control and PE groups was 7.78pg/mL and -2.31pg/mL, respectively. Combining all the results from phosphate and calcium concentrations in blood and urine, the control group appeared to exhibit an expected response to an increase in phosphate dose (Table 3-1). The increase in phosphate was associated with decrease in calcium. Such decrease triggered a release of iPTH, which was detected at 1-hour. The increased release of iPTH was possibly able to stabilize serum phosphate concentration and led to an increase in phosphate excretion that was seen in both 1-hour and 2-hour urine collection. Though not significant, a trend of decrease in calcium excretion at 2-hour can be seen in all the Ucalc:Ucrea...
calculations (Figure 3-11, Figure 3-12, and Figure 3-13). On the other hand, there was no significant increase of iPTH or decrease calcium excretion throughout the study period in women with a history of PE and the concentration in iPTH fluctuated less than 2.3pg/mL. It was possible that small amount of iPTH was released minutes after the ingestion of the phosphate load but was quickly degraded before detection at 1-hour. With the significant increase in serum phosphate at 2-hour compared to baseline, the women with a history of PE group still exhibited no elevation of the 2 regulating hormones that were investigated. That being said, the women with a history of PE group was able to excrete phosphate over the study period. Other hormones such as 1,25(OH)\(_2\)D\(_3\) have been shown to regulate phosphate homeostasis. 1,25(OH)\(_2\)D\(_3\) could increase serum phosphate by increasing intestinal absorption and high concentration of serum phosphate suppresses the vitamin D production. 1,25(OH)\(_2\)D\(_3\) has also been shown to suppress the production of PTH and enhance FGF23.\(^{68}\) Without the data on vitamin D status of women with a history of PE, it was difficult to determine the driving force of phosphate excretion in women with a history of PE group.

In terms of plasma FGF23 concentrations of the two groups, the majority of the participants from both groups had no significant differences within or between the groups (Figure 3-17). This was to be expected as FGF23 was shown to be a long term regulator of phosphate balance. Plasma FGF23 concentration remained relatively unchanged throughout phosphate depletion or loading whereas PTH concentration fluctuated significantly with changes in phosphate intake.\(^{71,130,139}\) A previous study has shown that FGF23 elevation preceded the elevation of PTH and hyperphosphatemia in chronic kidney
disease patients.\textsuperscript{91} Elevation of FGF23 can also cause left ventricular hypertrophy and increase rates of mortality in CKD patients.\textsuperscript{140} Clearly, additional testing and multiple sampling should be done in these participants before the confirmation of chronic FGF23 elevation.

\textit{General remark}

Overall, there are some striking differences found in phosphate metabolism between the groups. Since phosphate metabolism is closely associated with kidney function, further studies in this area may be able to detect subtle changes in kidney function that led to the differences observed in this study. Given that both groups were able to excrete phosphate within the 2-hour study period, this study design in general is applicable to this specific population. Perhaps the small differences between the study groups could be the result of a few factors. First, kidney disorders and cardiovascular disease shared the relatively similar risk factors such as obesity and hypertension.\textsuperscript{97} These women were relatively young and healthy individuals with no clinically classified co-morbidities such as diabetes or hypertension. Secondly, since these women were all recruited within five years postpartum, having a young child at home has been reported to keep them very active. Having an active lifestyle has shown to be beneficial to prevent CKD and cardiovascular diseases by improving factors such as vascular endothelial function, insulin sensitivity and oxidative stress. Last but not least, even though women with history of PE have higher risk of CKD or other forms of kidney complication, significant deterioration of kidney function may not occur until much later in life. Thus it was possible that the significant changes in kidney function may not be detectable this early postpartum.
Project 2: Assessing plasma cystatin C in the first trimester of pregnancy

Between 2002 and 2009, over 7000 women in their first trimester provided plasma samples to the OaK birth cohort. Some of these women were later diagnosed with PE. Various exclusion and matching criteria were applied to the selection of 19 samples from women who were destined to develop PE and 19 samples from women who had a healthy and term singleton pregnancy. It was hypothesized that women who were destined to develop PE would exhibit elevation of cystatin C due to early kidney damage in the first trimester.

Demographic data

No difference was found in the maternal characteristics of healthy pregnant women and women who were destined to develop PE later in that pregnancy (Table 3-4). There was no significant pre-pregnancy weight or pre-pregnancy BMI difference between participant groups. PE has been associated with preterm birth, intrauterine growth restriction, in particularly early onset of PE. Not surprisingly, the gestational age and weight of the infants of destined PE were significantly lower than that of the control infants (Table 3-4).

Cystatin C

Assessing cystatin C concentration of first trimester women was done because previous studies had shown significant elevation of cystatin C in third trimester after the diagnosis of PE was made. The increase in cystatin C has been suggested to be a good biomarker for prediction and diagnosis of PE. During normal pregnancy, it has been reported that the filtration of very low molecular mass molecules and water increase over the entire pregnancy was due to increase of renal plasma flow while the filtration of low molecular
mass molecules such as cystatin C were decreased and continued decreasing throughout gestation. The decrease in low molecular mass molecules filtration in late third trimester of normal pregnancy was thought to be caused by endotheliosis where the endothelial cells of the glomerulus swell thus occluding the filtration pores. Glomerular endotheliosis is a common structural alteration present in PE and as the disease progresses, the increase in swelling of the cells can also affect the glomerular filtration rate and subsequently increase serum cystatin C concentration. Assuming that glomerular endotheliosis in pregnancy developed gradually in pregnant women destined to clinically be diagnosed with PE, the aim of this study was to investigate whether the increase in serum cystatin C in destined PE can be identified as early as the first trimester. Such a difference however, was not observed in this study group, the destined PE group and control group had a mean (±SD) of plasma cystatin C of 0.52 (±0.08) mg/L and 0.49(±0.07) mg/L, respectively (Figure 3-18).

Limitations
The two projects presented here focused on young women who had been affected by PE in their previous pregnancy(s) (Project 1) or would be affected by PE later (Project 2) in pregnancy. Both projects included a relatively small sample size. Given the restriction of locations where these participants were recruited, the participants were not as diverse as the general population. Thus, the phenomenon observed in these projects may not be generalizable across populations.

For project 1, there were only seven sets of women with a history of PE and control participants recruited during the study period and except for one black participant, the
majority of participants were Caucasians. It has been shown in the literature that blacks have a higher prevalence of PE, diabetes, early onset hypertension, and hypertension-associated end-stage renal failure.\textsuperscript{145,146} However, the mechanism underlining this racial variation is not fully understood.\textsuperscript{146} The small number of participants does not provide the statistical power to show significant differences in a young and relatively healthy population. Another limitation for the study was that the total volume of urine excretion after phosphate consumption was not collected, thus the total and exact amounts of phosphate and creatinine excreted during the two-hour study period was unknown. Without knowing the total amount of phosphate excreted by the kidneys, the total amount of phosphate that was retained in the body could not be calculated. In future studies with similar methodology, such limitation can be avoided by collecting the entire urine voided rather than just a sample of such.

Due to the design of the study, the selection of participants might have suffered from biases such as participation bias and self-selection bias. For both controls and women with a history of PE participants, it was a great commitment to participate in this study with various requirements and constraints. Thus, there might have been a participation bias\textsuperscript{147} in which potential participants who had the desire to participate but inadvertently excluded from the study because of time conflicts with work or other commitments. Also, participation in the study for both groups was completely voluntary. A self-selection bias\textsuperscript{147} could have also been a problem as individuals who were more motivated and health conscious were more likely to participate compared with the ones that declined.
Another limitation of the study was that samples were collected over eleven months. Though the samples were stored following protocols suggested by the assay kits and the samples were tested together to minimize inter-analysis differences, the variability in sample storage time could have affected the stability of biomarkers of interest. For instance, in a study which tested the stability of serum creatinine after up to 3 months of -20°C storage and up to 10 freeze-thaw cycles, there was no significant decrease in creatinine concentration. However, the stability of the biomarkers after more than 3 months of storage was not investigated. Half of control samples were collected two months before testing and the other half collected 4 months prior, so there was a wide range of storage time differences between participant samples. Whereas women with a history of PE samples were all collected within two months and stored for over eight months before analysis. Since samples with shorter storage time (control samples) did not seem to differ in creatinine concentration compared to women with a history of PE. The wide range and differences in storage time would be unlikely to cause the low creatinine in both groups. However, for future studies, samples collected and analyzed right away by hospital laboratories could avoid or reduce degradation from long term storage.

For project 2, the samples were collected over a long period of time. In order to minimize the effect of long term storage, matched samples were collected within 2 years apart and all samples were collected within 3 years. Not only that the samples provided were again, older samples, but also samples that had been frozen and thawed before. Cystatin C stability has been tested in other studies and has been shown to 15% decrease in value (P<0.05), with the greatest mean difference of 0.16mg/L after 10 freeze/thaw cycles.
The number of freeze thaw cycles was not known for each sample, thus they were not matched in this respect.

**Future directions**

In order to validate the results found in these projects, future studies should include a larger sample size and a more diverse ethnic background. Samples should be analyzed shortly after collection and multiple sample collections at the same time point could also help minimize sample collection error. Due to the short half-life of PTH, a small plasma sample should be collected 5 and 10 minutes immediately after phosphate loading to examine iPTH response to the phosphate load. A longitudinal study of renal function before pregnancy could help better understand whether changes in renal function and handling of minerals are the result of PE or pre-existing underlying physiological conditions which are exacerbated during pregnancy and subsequently worsen over time. Each participant can then serve as their own control and any changes observed after pregnancy can be more clearly shown to be the result of the pregnancy complication. Similarly, it would be interesting to investigate renal handling of phosphate metabolism in women with a history of PE at a later time in life which may provide a better insight on whether the renal alteration during pregnancy persisted long after. For such a study, it would be important to consider and tease out environmental and lifestyle factors which can also contribute to the slow or fast decline in renal function.
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Appendix A

Project 1 Questionnaire

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<thead>
<tr>
<th>Recruitment Code:</th>
<th></th>
</tr>
</thead>
</table>

Assessing Renal and Cardiovascular Functions Following Preeclampsia

Date of study visit:________________________

Time of study visit:________________________

Room Temp:________________________ °C

Last food intake:________________________

Age:________________________

Pregnancy: G____ T____ P____ A____ L____

Ethnicity/Race: Caucasian Black Asian Native American/Inuit Other________________________

Education level: Grade School High School Post-Secondary (completed/not completed)

First day of last menses:________________________

<table>
<thead>
<tr>
<th>FAMILY MEDICAL HISTORY</th>
<th>PERSONAL MEDICAL HISTORY</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hypertension</td>
<td>Hypertension</td>
</tr>
<tr>
<td>Other cardiovascular diseases</td>
<td>Other cardiovascular diseases</td>
</tr>
<tr>
<td>Kidney Disease (CKD, Kidney Failure, etc.)</td>
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</tr>
<tr>
<td>Endocrine disorders</td>
<td>Thyroid Pituitary Other:</td>
</tr>
<tr>
<td>Pregnancy Complications</td>
<td>Gestational Diabetes Preeclampsia Gestational Hypertension Pre-term Birth Serious Infections Other:</td>
</tr>
<tr>
<td>Smoking Habits</td>
<td>Smoking Habits</td>
</tr>
<tr>
<td>Diabetes</td>
<td>Diabetes</td>
</tr>
<tr>
<td>Overweight/Obese</td>
<td></td>
</tr>
</tbody>
</table>
Project 1
Questionnaire (cont.)

**PRE-PREGNANCY HEALTH STATUS**

| Weight (kg) |  |
| Height (cm) |  |
| BMI |  |
| Exposure to Second-hand Smoke | Frequency:  |
| Physical Activity | Week | Hour |
| BORG intensity |  |
| Dietary Supplements | Calcium, Vitamins: Folate Acid Other: |
| Other Medications |  |

**CURRENT HEALTH STATUS**

| Weight (kg) |  |
| Height (cm) |  |
| BMI |  |
| Waist Circumference (cm) |  |
| Hip Circumference (cm) |  |
| Blood Pressure (mm Hg) |  |
| Exposure to Second-hand Smoke | Frequency:  |
| Physical Activity | Week | Hour |

**BREASTFEEDING HISTORY**

| Duration (weeks) |  |
| Intensity |  |
| Reason for Stopping |  |
| Non-breastfeeding Period |  |

| BORG Intensity |  |
| Method of Hormonal Contraception | OC: Depo Provera Hormonal Patches IUD (Mirena) Other: |
| Dietary Supplements | Calcium, Vitamins: Folate Acid Other: |
| Other Medications |  |
# Appendix B

**BORG Scale of Perceived Exertion**

<table>
<thead>
<tr>
<th>Borg Intensity Scale</th>
<th>How It Feels</th>
</tr>
</thead>
<tbody>
<tr>
<td>6 No Exertion</td>
<td>This requires no effort. This is how you feel when sitting with little to no movement.</td>
</tr>
<tr>
<td>7 Extremely Light</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td></td>
</tr>
<tr>
<td>9 Very Light</td>
<td>This should require very light effort. It corresponds to walking slowly for a few minutes.</td>
</tr>
<tr>
<td>10</td>
<td></td>
</tr>
<tr>
<td>11 Light</td>
<td>This should require light effort. Your breathing should speed up slightly and you should be sweating a little. You should be able to talk easily.</td>
</tr>
<tr>
<td>12</td>
<td></td>
</tr>
<tr>
<td>13 Somewhat Hard</td>
<td>This should require moderate effort and speed your heart rate and breathing. You should be sweating more. You should still be able to carry on a conversation.</td>
</tr>
<tr>
<td>14</td>
<td></td>
</tr>
<tr>
<td>15 Hard</td>
<td>This should require moderate to high effort and speed your heart rate and breathing. You should be sweating a lot. You should still be able to talk, but in short bursts.</td>
</tr>
<tr>
<td>16</td>
<td></td>
</tr>
<tr>
<td>17 Very Hard</td>
<td>This is very strenuous. You could continue, but really have to push yourself. It feels very heavy and you are very tired. You can only maintain the pace for a short period of time.</td>
</tr>
<tr>
<td>18</td>
<td></td>
</tr>
<tr>
<td>19 Extremely Hard</td>
<td>This is extremely strenuous. This is the hardest exercise that you have ever experienced.</td>
</tr>
<tr>
<td>20 Maximum Exertion</td>
<td>Total Exhaustion.</td>
</tr>
</tbody>
</table>