THE PATHOGENESIS OF VASCULAR CALCIFICATION IN
CHRONIC KIDNEY DISEASE: CONSEQUENCES AND
TREATMENTS

by

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Abstract

Vascular calcification (VC) is accelerated in patients with chronic kidney disease (CKD), resulting in increased risk of cardiovascular disease and mortality. Although the consequences of VC are associated with elevated pulse wave velocity (PWV) and left ventricular hypertrophy (LVH), the temporal impact on blood pressure changes is unknown. Mineral imbalance in CKD greatly contributes to the development of VC, and elevated serum phosphate is a major risk factor. Magnesium, which plays an important role in bone regulation, has been recently shown to be a modifier of VC, but whether magnesium inhibits calcification in CKD is unknown.

A modified adenine model of CKD was developed in rats, characterized by mineral imbalance and progressive VC. During the development of VC, pulse pressure increased, which was driven by a drop in diastolic blood pressure, rather than systolic hypertension. Continuous pressure recordings in conscious rats using radiotelemetry revealed a significant increase in systolic variability associated with development of VC. Regional VC was associated with regional changes in the hemodynamic profile of the CKD rats. For example, only thoracic aortic calcification was associated with elevated PWV and pulse pressure. In contrast, the presence of abdominal and thoracic calcification differentially affected proximal and distal arterial pressure wave forms. CKD animals exhibited LVH, which was further increased by the presence of VC. In addition, fibroblast growth factor 23, which regulates renal excretion of phosphate, was elevated in CKD animals at every time point and was associated with LVH independently from VC. Development of VC was characterized in an in vitro organ model. Phosphate elevation in vitro caused VC in aortas. In vitro, magnesium supplementation inhibited initiation and
progression of VC. CKD animals given a magnesium diet also demonstrated attenuated development of VC. In patients with stage 3-5 CKD (excluding dialysis), dietary phosphate was associated with the progression of coronary artery calcification even after adjusting for use of phosphate binders, total dietary energy and total dietary protein. Given the serious negative outcomes associated with development of VC, these findings fill key gaps in knowledge regarding the detection, management, prevention and treatment of VC in CKD.
Co-Authorship

The basic research presented in this thesis has been performed and written by Navid Seyed Shobeiri with the following co-authorships and technical assistance (see below). The clinical study in Chapter 4 was a study involving various collaborators in roles as indicated below. Dr. Holden and Dr. Adams, NSS’s supervisors, were involved in obtaining funding, designing studies, performing analysis and interpretation of data and in developing the discussion of all experiments.

Chapter 1:

Chapter 2: Co-authored by Judy J Pang, Michael A Adams and Rachel M Holden

Technical assistance with imaging of von Kossa sections was provided by Dr. Stephen Pang. JJP assisted with preparation of the histology slides. Implantation of radiotelemetric units was performed by Corry Smallegange. Initial presentation of preliminary data and the associated evaluation was contained in one aspect of the Master’s thesis of Navid Shobeiri. However, the interpretations have been changed markedly, experimental data added, much greater analyses performed, and the entire study was re-written for publication. Journal of Hypertension. 31:160-168 2013

Chapter 3: Co-authored by Kristin M McCabe, Michael A Adams and Rachel M Holden
Some of the database was obtained from a set of CKD animals previously used in unrelated studies performed by KMM. Noah Stern provided technical assistance with some of the data acquisition and organization.

Chapter 4: Co-authored by Kristin M McCabe, Cynthia M Pruss, Rachel M Holden and Michael A Adams

Technical assistance was provided by Spencer Barr for the serum and tissue biochemical analyses. Kim Laverty, Alexis Jozefacki and Mason Curtis provided technical assistance in animal handling and daily health checks. KMM provided technical assistance with blood pressure recording. CMP was involved in some of the data analysis. Julie Cruanès under the supervision of NSS assisted with tissue incubation, media changes and mineral quantification assays.


NSS performed FGF23 assay, designed the study, analyzed the results and wrote the Chapter. JSG and RM, were involved in the generation of the database of this cohort of patients, and agreed to share with RMH/MAA research group for this novel analysis. Technical expertise for calcification scores was provided by RLN. WMH provided statistical advice.
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>Ad</td>
<td>Adenine</td>
</tr>
<tr>
<td>ACR</td>
<td>Albumin to creatinine ratio</td>
</tr>
<tr>
<td>BMI</td>
<td>Body mass index</td>
</tr>
<tr>
<td>BP</td>
<td>Blood pressure</td>
</tr>
<tr>
<td>Ca</td>
<td>Calcium</td>
</tr>
<tr>
<td>CAC</td>
<td>Coronary artery calcification</td>
</tr>
<tr>
<td>CKD</td>
<td>Chronic kidney disease</td>
</tr>
<tr>
<td>CKD+Ab</td>
<td>CKD animals with abdominal calcification</td>
</tr>
<tr>
<td>CKD+Th</td>
<td>CKD animals with thoracic calcification</td>
</tr>
<tr>
<td>CRP</td>
<td>C-reactive protein</td>
</tr>
<tr>
<td>CT</td>
<td>Computed topography</td>
</tr>
<tr>
<td>CVD</td>
<td>Cardiovascular Disease</td>
</tr>
<tr>
<td>DBP</td>
<td>Diastolic blood pressure</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified eagle medium</td>
</tr>
<tr>
<td>dP/dt\text{_max}</td>
<td>Maximum rate of pressure change</td>
</tr>
<tr>
<td>eGFR</td>
<td>Estimated glomerular filtration rate</td>
</tr>
<tr>
<td>ESKD</td>
<td>End stage kidney disease</td>
</tr>
<tr>
<td>FFQ</td>
<td>Food frequency questionnaire</td>
</tr>
<tr>
<td>FGF23</td>
<td>Fibroblast growth factor 23</td>
</tr>
<tr>
<td>GFR</td>
<td>Glomerular filtration rate</td>
</tr>
<tr>
<td>HR</td>
<td>Heart rate</td>
</tr>
<tr>
<td>K-DOQI</td>
<td>Kidney Disease Outcomes Quality Initiative</td>
</tr>
<tr>
<td>LVH</td>
<td>Left ventricular hypertrophy</td>
</tr>
<tr>
<td>LVI</td>
<td>Left ventricle index</td>
</tr>
<tr>
<td>LV:BW</td>
<td>Left ventricle to body weight ratio</td>
</tr>
<tr>
<td>MAP</td>
<td>Mean arterial pressure</td>
</tr>
<tr>
<td>MBD</td>
<td>Mineral bone disease</td>
</tr>
<tr>
<td>MGP</td>
<td>Matrix Gla protein</td>
</tr>
<tr>
<td>Pit-1/Pit-2</td>
<td>Type III sodium phosphate transporter</td>
</tr>
<tr>
<td>PO_4</td>
<td>Phosphate</td>
</tr>
<tr>
<td>PP</td>
<td>Pulse pressure</td>
</tr>
<tr>
<td>PTH</td>
<td>Parathyroid hormone</td>
</tr>
<tr>
<td>PWV</td>
<td>Pulse wave velocity</td>
</tr>
<tr>
<td>RVI</td>
<td>Right ventricle index</td>
</tr>
<tr>
<td>SBP</td>
<td>Systolic blood pressure</td>
</tr>
<tr>
<td>SGA</td>
<td>Subjective global assessment</td>
</tr>
<tr>
<td>SD</td>
<td>Sprague Dawley</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>VC</td>
<td>Vascular Calcification</td>
</tr>
<tr>
<td>VSMC</td>
<td>Vascular smooth muscle cells</td>
</tr>
<tr>
<td>25(OH)D</td>
<td>25-hydroxyvitamin D</td>
</tr>
</tbody>
</table>
Chapter 1

General Introduction
1.1 Chronic kidney disease and cardiovascular disease

Chronic kidney disease (CKD) is a progressive decline of kidney function over months or years with poor outcomes. CKD affects more than 25 million people in North America and this number is rising\(^1\). CKD has been classified into five stages, and at every stage the leading cause of mortality is linked to cardiovascular disease (CVD)\(^2\). In fact, individuals with CKD have been identified as one of the highest risk groups for developing cardiovascular disease, and in those patients who advance to End Stage Kidney Disease (ESKD; patients receiving dialysis or kidney transplants), cardiac mortality is reported to be 10 to 30 times higher than in the general population\(^3\). The traditional Framingham risk factors such as age, hypertension, smoking, diabetes mellitus and dyslipidemia present in this population do not completely account for the increased cardiovascular mortality. Non-traditional risk factors such as disordered mineral metabolism (phosphate, calcium and magnesium), appear to have key roles in predisposing CKD patients to vascular calcification, and the consequent circulatory dysfunction\(^4,5\).

1.2 Renal physiology

The kidney is an interesting organ that serves a number of regulatory functions including excretion of waste, urine production, regulation of electrolyte, mineral and acid-base balance, regulation of blood pressure, as well as hormone production (i.e. calcitriol, renin and erythropoietin). The functional unit of the kidney, the nephron, is comprised of renal corpuscle (i.e. glomerulus and Bowman’s capsule) and renal tubules capable of filtration, reabsorption and secretion, the sum of which equals renal excretion. Renal function is highly specialized, which means that breakdown products such as
creatinine are freely filtered, but do not get reabsorbed. In contrast, mineral balance is highly dependent on renal reabsorption. To maintain neutral mineral balance, about 88-98% of minerals must be reabsorbed by the renal tubules.

There is no single common cause of kidney disease, but the most common causes are diabetes, hypertension and age. Regardless of its etiology, there are multiple complications associated with the development and progression of CKD which greatly increase mortality in these patients.

1.3 Stages of CKD

The progression of CKD has been classified into five stages primarily based on the estimated glomerular filtration rate (GFR) (see table below). The most common and readily available method of estimating GFR is based on serum creatinine levels adjusted for age, gender, race and body size, and sometimes in conjunction with 24hr urine collection for creatinine clearance. Creatinine, which is a breakdown product of phosphocreatine found in muscle, is freely filtered by the glomerulus. CKD patients with reduced GFR have elevated serum creatinine levels. By stage five, patients have lost more than 90% of their kidney function and are at high risk for progression to ESKD at which point kidney replacement therapy becomes essential for survival.

Table 1.1 Stages of CKD

<table>
<thead>
<tr>
<th>Stage</th>
<th>GFR level (mL/min/1.73m²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy adult</td>
<td>120 – 130</td>
</tr>
<tr>
<td>CKD Stage 1</td>
<td>≥ 90</td>
</tr>
<tr>
<td>CKD Stage 2</td>
<td>60 – 89</td>
</tr>
<tr>
<td>CKD Stage 3</td>
<td>30 – 59</td>
</tr>
<tr>
<td>CKD Stage 4</td>
<td>15 – 29</td>
</tr>
<tr>
<td>CKD Stage 5</td>
<td>&lt; 15</td>
</tr>
</tbody>
</table>
Many factors limit the accuracy of creatinine as a biomarker for GFR, including body mass, protein intake, age, and accuracy of urine collection. The most accurate measure of GFR is taken by injecting compounds, such as inulin or iohexol, which are freely filtered without reabsorption and have a stable production rate without any influence from other pathological changes. However, these techniques are labor intensive, time consuming and costly. Therefore, other endogenous markers have been examined to estimate GFR.

In 1985, it was proposed that cystatin C could be used as a biomarker of GFR. Cystatin C is a low molecular weight protein which is produced by all nucleated cells at a constant rate. It is freely filtered across glomeruli and does not get reabsorbed. Several equations for GFR have been derived based on cystatin C; however, recent evidence suggests there are also limitations to this biomarker. Factors other than GFR, such as presence of melanoma, glucocorticoid therapy or thyroid dysfunction, impact cystatin C levels. Recent research in a large cohort of CKD patients has shown that cystatin C levels provide an alternative estimate of GFR that is not linked to muscle mass; instead, the most accurate estimation of GFR was based on an equation that combined creatinine, cystatin C, age, sex and race.

Equation: $eGFR = 177.6 \times \text{creatinine}^{-0.65} \times \text{cystatinC}^{-0.57} \times \text{age}^{-0.20} \times (0.82 \text{ if female}) \times (1.11 \text{ if black})$

In animal models of CKD, creatinine still remains the usual standard by which the severity of kidney failure is estimated. There may be fewer limitations in animal models of CKD because body mass and diet are usually controlled across all treatment groups. However, this assumption will need to be validated in future studies.
1.4 Mineral bone disorder

The term, “chronic kidney disease-mineral bone disorder” (CKD-MBD), as defined in 2006 is “a systemic disorder of mineral and bone metabolism due to CKD manifested by either one or a combination of biochemical abnormalities (phosphate, calcium, parathyroid hormone secretion or vitamin D metabolism), abnormalities in bone regulation and vascular calcification” \(^\text{18}\). There exist many parallels between the process of normal bone formation and vascular calcification. Disorders in mineral and bone metabolism are associated with increased risk for cardiovascular calcification, morbidity and mortality \(^\text{18}\). It is now well-recognized that hyperphosphatemia is a major risk factor for all-cause and cardiovascular morbidity and mortality in patients with CKD \(^\text{19}\). Impaired phosphate regulation is becoming increasingly linked to the development of cardiovascular disease \(^\text{20,21}\), and recent studies have also shown that even within the normal range of phosphate individuals at the high-normal end are at increased risk \(^\text{22}\). The underlying mechanisms in this linkage are not very well understood, but might be linked to the development and consequences of vascular remodeling and calcification, leading to changes in cardiovascular structure and function \(^\text{23,24}\).

1.4.1 Vascular calcification

Calcification may occur in both the intimal and medial layers of the arterial wall. Intimal calcification is associated with macrophages in lipid-rich areas of arteries and takes the form of atherosclerotic vascular disease \(^\text{25}\). Intimal calcification has been associated with classic Framingham risk factors, such as advancing age, diabetes, dyslipidemia, hypertension and smoking. In contrast, medial calcification occurs within the elastic regions of the arteries and is almost exclusively associated with vascular
smooth muscle cells (VSMC)\textsuperscript{26}. Although intimal calcification and medial calcification may occur simultaneously in CKD, medial calcification is a prevalent and early feature of the disease\textsuperscript{27}.

Medial vascular calcification is an active process involving a phenotypic change in VSMCs to osteoblastic like cells\textsuperscript{28}. Numerous \textit{in vitro} studies have suggested that phosphate is a key signaling molecule in this process. Elevated phosphate is mostly transported in the smooth muscle cells by the ubiquitous type III sodium phosphate transporters, also known as Pit-1 and Pit-2 transporters\textsuperscript{29–31}. High intracellular phosphate is linked to an increased expression of osteoblastic transcription factor, core-binding factor alpha 1 (Cbfa-1), leading to a phenotypic switch in VSMCs from a contractile phenotype characterized by markers of smooth muscle lineage (SMAD6, matrix Gla protein, smooth muscle actin) to an osteoblastic-like phenotype (sox-9, core-binding factor-1, osteocalcin)\textsuperscript{32–34}. This phenotypic change causes an increased deposition of calcium and phosphate crystals, similar in manner to bone formation\textsuperscript{35,36}. A few studies suggest that magnesium supplementation can prevent this phenotypic change\textsuperscript{37,38}, although the impact of magnesium on phosphate disposition and calcification has not been well elucidated.

Although phosphate is a key signaling molecule in development of vascular calcification, it is important to note that in vitro models do not account for the potential additive effects of a dynamic circulation. Furthermore, vascular calcification is a multifaceted process and evidence suggests that in addition to phosphate, apoptotic bodies and extracellular calcium also contribute to this process\textsuperscript{19,39–41}. \textit{In vivo}, vascular calcification involves a balance between activators and inducers (Figure 1.1). A number
of gene knockout and experimental animal models have confirmed that there are a number of vascular and bone-derived proteins that either inhibit or promote the calcification process. Selective gene knockout models for bone-associated proteins, such as osteoprotegerin\(^4\), or selective gene knockout models of VSMC proteins, such as smad6\(^4\) and matrix gla protein\(^4\), all develop varying degrees of arterial calcification. Experimental models of VC have confirmed other inhibitors of calcification including bone morphogenetic protein 7 (BMP7), pyrophosphate and fetuin. Treatment with BMP7 in a mouse model of CKD prevents vascular calcification possibly by preventing VSMC differentiation\(^4\). Fetuin, which is created by the liver and released in the circulation, has been shown to inhibit calcium-phosphate mineralization therefore preventing soft-tissue calcification\(^4\). Pyrophosphate is an endogenous molecule produced by numerous tissues including the aortas that prevents calcium and phosphate from crystallization, however tissue non-specific alkaline phosphatase hydrolyses pyrophosphate, thereby promoting calcification\(^4\). Taken together, in CKD, the uremic environment is believed to tip the balance towards activating the stimulators and deactivating the inhibitors of calcification, thus accelerating the process\(^4,4\).
Figure 1.1 Mechanism of vascular calcification.
Vascular calcification is an active process triggered by irregular phosphate regulation. The osteogenic transcription factor Cbfa-1 and bone morphogenetic protein 2 are upregulated which promote differentiation. Bone matrix protein such as osteopontin and osteocalcin are also upregulated and might be involved in the regulation of vascular calcification. In uremia, the balance between inhibitors and promoter is tipped towards inducing the promoters of calcification, thus accelerating the process.\(^{50}\)
1.5 Phosphate regulation

Eighty-five percent of total body phosphate is found in bone. Roughly 15% of total body phosphate is found within cells and less than one percent is found in the blood\textsuperscript{51}. Cellular phosphate participates in many processes including cell signaling and energy storage\textsuperscript{52–56}. However, circulatory phosphate functions as a mediator between bone and the kidneys, both of which are major players in the regulation of phosphate.

1.5.1 Hormonal regulation

The major hormonal regulators of phosphate homeostasis are vitamin D, fibroblast growth factor 23 (FGF-23) and parathyroid hormone (PTH). Although phosphate-sensing receptors have not been discovered and the mechanism by which phosphate imbalance is detected is still unknown, FGF-23 and PTH are released in the circulation to lower hyperphosphatemia. In contrast, the active form of vitamin D, 1,25 dihydroxyvitamin D\textsubscript{3} or calcitriol, a steroid hormone that is primarily synthesized in the kidneys, has a direct transcriptional effect on the gut as it increases phosphate uptake\textsuperscript{57,58}.

FGF-23 belongs to the FGF family of proteins. The N-terminus contains the FGF domain, but the c-terminus distinguishes it from other FGFs\textsuperscript{59,60}. That is, unlike other FGFs, FGF-23 is produced by osteoblasts and osteocytes and released into circulation\textsuperscript{59,60} where it has a direct effect on kidney re-absorption of phosphate. FGF-23 functions by binding to FGF receptors in the presence of a transmembrane protein, Klotho, on the basolateral membrane of renal tubules\textsuperscript{61,62}. This signaling mechanism leads to reduced expression of renal sodium-phosphate transporters (type IIa/c) in the proximal as well as distal tubules of nephrons\textsuperscript{61,62}. FGF-23 also reduces levels of calcitriol (the active form of vitamin D) by inhibiting the enzyme responsible for its production (1-alpha-
hydroxylase) and by activating the enzyme responsible for its breakdown (24-
hydroxylase)\(^ {64-67}\). The net effect is reduced gut absorption of calcium and phosphate (due
to \(\downarrow\)calcitriol)\(^ {68,69}\) and increased phosphate wasting by the kidneys (due to \(\downarrow\) phosphate
transporters)\(^ {61,63}\). Regulation of phosphate by PTH is similar to FGF-23. PTH decreases
sodium-phosphate transporters in proximal tubules\(^ {69-71}\). In comparison to FGF-23, the
effects of PTH may be more acute and short-term\(^ {72}\).

1.5.2 Prevalence of hyperphosphatemia

In CKD, hyperphosphatemia typically occurs by the fourth stage, and yet marked
changes in phosphate counter-regulatory hormones appear by stage two\(^ {73}\). As CKD
progresses, the concentration of phosphate in the exchangeable pools (i.e. mineralization
front and soft tissues) is elevated leading to positive phosphate balance. Conversely, the
positive phosphate balance triggers FGF-23 and PTH as a homeostatic response\(^ {73}\). Due
to loss of renal function, the capacity of calcitriol production is also decreased, leading to
decreased gut calcium absorption, hypocalcemia and a further stimulation of PTH
secretion. Chronically elevated levels of PTH cause excessive bone resorption and this
may, in fact, also contribute to hyperphosphatemia as the reservoir function of the
skeleton is lost. The vasculature and the soft tissues become a new reservoir for excess
phosphate, and through mechanisms discussed earlier, could ultimately lead to vascular
calcification\(^ {41,74}\).

Although phosphate levels in the blood may not be increased in the earlier stages
of CKD, FGF-23 levels have been found to be markedly elevated and can range from
100- to 1000-fold higher than healthy subjects\(^ {73}\). It is currently believed that the early
impact of FGF-23 on phosphaturia is homeostatic and beneficial; however, later in the
disease it may have direct organ effects leading to toxicity. For instance, recent studies have linked FGF-23 with left ventricular hypertrophy (LVH) and cardiovascular disease. FGF-23 has also been proven to be a strong and independent risk factor for all-cause mortality in CKD patients.

Recently, dietary phosphate was associated with five-year mortality in a cohort of patients with ESKD requiring hemodialysis even after adjusting for serum phosphate and the use of phosphate binders. The clinical guidelines for treatment of hyperphosphatemia recommend the use of a pharmacological phosphate lowering drug along with dietary phosphate restriction. However, positive phosphate balance can long precede an elevated serum phosphate level. Whether treatment should therefore begin at a much earlier stage is much debated. The impact of dietary phosphate on calcification progression in CKD patients will be explored in Chapter 5 of this thesis.

1.6 Calcium regulation

Much of the epidemiological and experimental evidence on calcification in CKD has focused on the role of elevated serum phosphate. However, recent evidence has shown an association between serum calcium and calcification in the CKD population. Serum calcium is also tightly regulated by vitamin D and PTH, as well as calcitonin. In brief, vitamin D increases serum calcium by increasing intestinal calcium absorption, decreasing renal calcium excretion and increasing calcium resorption from bone. PTH also increases serum calcium levels by promoting calcitriol production in the kidneys and decreasing renal excretion. Vitamin D is converted to calcitriol, the active form of vitamin D, in the kidney tubules by 1-alpha-hydroxylase. Therefore, the presence of CKD leads to early calcitriol deficiency because of reduced 1-alpha-
hydroxylase activity\textsuperscript{82}. Low calcitriol leads to hypocalcaemia, and together with the prevalent hyperphosphatemia, leads to increased PTH release and secondary hyperparathyroidism\textsuperscript{81}. The standard treatment for secondary hyperparathyroidism is calcitriol, but treatment, in some cases has been associated with increased vascular calcification presumably due, in part, to elevated gut absorption of calcium and phosphate (a direct effect of calcitriol) \textsuperscript{83–85}. Vitamin D analogues or calcimimetics have been used to avoid the adverse effects of calcitriol; there are, however, no randomized control trials that directly compare these newer agents to calcitriol. Furthermore, calcium-containing phosphate binders are used to treat hyperphosphatemia, which add to the calcium burden in these susceptible patients. The excess calcium from calcium-based phosphate binders has been linked to an increased risk of vascular calcification\textsuperscript{86–88}.

1.7 Magnesium regulation

Recent evidence suggests that low serum magnesium levels may be associated with cardiovascular and all-cause mortality \textsuperscript{89,90}. Studies have also shown an association between low serum magnesium and vascular calcification \textsuperscript{91}. Current understanding indicates that magnesium homeostasis depends primarily on a balance between intestinal uptake and renal excretion, rather than a hormonal system. Although ESKD patients often develop hypermagnesemia, it is recognized that serum magnesium levels do not accurately predict soft tissue or total body magnesium content \textsuperscript{6,92}. The relationship between tissue magnesium levels and vascular calcification is not known. Whether magnesium could be used as a potential therapeutic in the prevention of vascular calcification will be explored in Chapter 4.
1.8 Hemodynamic consequences of vascular calcification

The clinical consequences of vascular calcification are linked to loss of function of large elastic arteries. The major functional outcomes are elevated pulse wave velocity (PWV) and pulse pressure, both of which contribute to left ventricular hypertrophy and mortality in patients with CKD.

1.8.1 Pulse wave velocity as a marker for stiffness

Blood pressure is expressed as the product of total peripheral resistance and cardiac output. This definition identifies the mean arterial blood pressure as a constant throughout the cardiovascular system, but does not consider the pulsatile nature of blood flow. The large elastic arteries have a dual function in the cardiovascular system. They are conduit vessels directing blood to tissues and organs, but they also function as cushions, transforming the pulsatile flow generated from the heart to a steady flow at the periphery. That is, the conduit function of arteries delivers a continuous, steady and constant flow of blood required in the arterioles and capillary system to ensure efficient metabolic exchange. To overcome resistance to flow caused by friction and blood viscosity, a constant pressure must be applied (i.e. mean arterial pressure). For a given cardiac output, mean arterial pressure is determined by the peripheral vascular resistance.

The cushioning function of the arteries is critical in dampening the pressure oscillations generated by ventricular ejection. This function is determined by the elastic properties of large artery walls described in terms of compliance or stiffness. The primary pressure wave generated by left ventricular ejection travels down the aorta at a set speed. This process is referred to as pulse wave velocity (PWV), which increases with arterial
stiffening. The incident wave could be reflected at any point of discontinuity along the arterial tree, traveling back towards the heart. A higher PWV results in earlier return of these reflected waves. The sum of these changes is increased systolic blood pressure and pulse pressure and a decrease in diastolic blood pressure, causing increased left ventricular afterload and altered coronary perfusion. Elevated PWV has been shown to be a strong independent predictor of cardiovascular and all-cause mortality in ESKD. Although many factors such as aging and hypertension might contribute to arterial stiffening, in CKD-MBD associated vascular calcification is a major contributor to elevated pulse wave velocity and arterial stiffening of the large elastic arteries.

The shape of the wave generated by the left ventricle is influenced by cardiac function as well as arterial compliance. For instance, the rate of the upstroke in the wave might be steeper if the cushioning effect of the arteries is diminished. This area of research is relatively novel and will be explored in Chapter 3.

1.9 Clinical detection of vascular calcification

Vascular calcification and cardiac valve calcification are very common in patients with advanced stages of CKD and in patients on dialysis. The presence of vascular calcification and its progression are associated with an increased risk of cardiovascular events and mortality. In contrast, patients who do not have calcification have a good prognosis, with minimal or no calcification progression over an extended period of time. There are a number of noninvasive imaging techniques that have made it possible to detect cardiovascular calcification. These include computed topography (CT), planar x-ray, ultrasound and pulse wave velocity assessment. CT scans are the standard method...
of detecting coronary artery and aortic calcification and provide quantitative results. However, this technique cannot distinguish intimal and medial calcification, is expensive and is not readily available. Other methods of detection, such as planar x-ray and ultrasound, might be more cost effective, but only provide qualitative or semi-quantitative results, limiting the assessment of progression of calcification. Pulse wave velocity could be measured by arterial tonometry assessment, which measures pressure wave forms at various sites. This technique is not as readily available and only provides an indirect test for calcification. Given that calcification is highly prevalent in ESKD patients and progresses rapidly, early detection of calcification may ultimately improve the management of patients with CKD.

1.10 Animal models of CKD

Animal models of CKD demonstrate a range of severity in the vascular calcification phenotype. There are three major rodent models that have been used to study CKD. The first major rodent model was developed by surgically reducing the kidney mass by 5/6th. However, there are major limitations to the 5/6th nephrectomy model including the requirement for a cumbersome and high-risk surgery and the need to use either excessive dietary phosphorus or supraphysiologicial doses of vitamin D to induce vascular calcification. A mouse model of kidney disease was developed by Gagnon et al. (1983) by surgically electro-cauterizing one kidney and removing the second kidney, thus creating a remnant mouse kidney model. Similar to the 5/6th nephrectomy model, the rodents develop hallmark CKD complications, including elevated creatinine, hyperphosphatemia and hyperparathyroidism. A limitation to this procedure is that mice are not particularly susceptible to vascular calcification. More recently, this
approach has been combined with transgenic or knockout mouse strains to promote vascular calcification. For instance, to investigate the role of therapeutics in the prevention of atherosclerotic plaque formation and calcification, investigators have performed the remnant kidney procedure on an apolipoprotein E knockout strain. Unlike its wild type counterpart, this knockout strain develops atherosclerosis and calcification in the setting of CKD. Other major limitations of this mouse model include heterogeneity in kidney damage and the need for a high-risk surgery with a low survival rate.17

The third major CKD model is the adenine rat model. This model is a robust CKD model that develops cardiovascular complications similar to clinical setting. While assessing the metabolic fate of dietary purines in rats, In 1982, Yokozawa et al. discovered that high dietary adenine gets converted to 2,8-dihydroxyadenine, which is not soluble in water.116 The 2,8-dihydroxyadenine forms crystals and precipitates along the tubules and urinary tract causing nephrotoxicity and the development of signs and symptoms that are similar to those observed in humans with CKD.117 Without the use of vitamin D or a transgenic background, rats given an adenine diet develop moderate to severe CKD and vascular calcification.17 This model does not require a surgical procedure, however there are some limitations. Not all of the animals with CKD develop vascular calcification, although this heterogeneity is also observed in human CKD. The major limitation with this model is that 0.75% dietary adenine used by most studies is not highly palatable which leads to 30-50% weight loss in rats, and whether the complications that ensue are a result of malnutrition or CKD is not very well characterized.118–120 A modified version of this model which develops consistent vascular calcification without weight loss will be characterized in Chapter 2.
1.11 Rationale and statement of Hypothesis and objectives

Despite major advances in our knowledge of CKD and its pathophysiology, cardiovascular disease remains the leading cause of death in this population \(^2,3,121\). Vascular calcification contributes to this burden and has been associated with increased risk of cardiovascular events and mortality \(^94,122,123\). The following experimental and clinical research chapters focus on the development, prevention and treatment of vascular calcification in CKD.

The hemodynamic changes associated with the progression of vascular calcification are not very well characterised. For instance, studies in CKD patients from stages 2 to 5 have shown that pulse wave velocity is linked to arterial stiffness, but it is not clear if changes in pulse wave velocity are also linked to stiffness progression \(^105,124\).

Mineral imbalance in CKD has been linked to vascular calcification; however, hyperphosphatemia is a relatively late occurrence in the disease at which point calcification might already be present and progressing rapidly \(^73,125\). Studies that focus on mineral imbalance in CKD have traditionally characterized the impact of serum phosphate on vascular calcification, but have neglected the impact of dietary phosphate on calcification progression. Magnesium appears to play a significant role in the solubility of calcium and phosphate \(^126,127\); however, whether magnesium can prevent vascular calcification is unknown.

The hypotheses tested in the present experiments were:

1. A modified adenine diet (0.25%) will produce CKD and vascular calcification without significant weight loss.
2. Progression of vascular calcification will be temporally linked to functional changes of the cardiovascular system.

3. Magnesium treatment will prevent the initiation and progression of vascular calcification.

4. Dietary phosphate as well as the source of dietary phosphate in CKD patients is linked to calcification progression.

The studies in which these hypotheses were tested include:

**Chapter 2: Cardiovascular Disease in an Adenine-Induced Model of Chronic Kidney Disease; the Temporal Link between Vascular Calcification and Hemodynamic Consequences**

The objectives were:

1. To determine whether modifying the conventional 0.75% adenine to 0.25% adenine will minimize the risk of severe weight loss;

2. To determine the impact of duration of adenine feeding on:
   a. calcification of the major arteries;
   b. the blood pressure profile over time;
   c. the hemodynamic consequences;

3. To determine the impact of duration of adenine feeding on parameters of the CKD-MBD (phosphate, calcium and FGF-23) and to examine the association between these biochemical measures and the cardiovascular end-points.
Chapter 3: Regional Calcification Differentially Impacts Elevated Pulse Wave Velocity and Arterial $dP/dt_{\text{max}}$ in Chronic Kidney Disease Rats

The objectives were:

1. To determine the impact of regional calcification on PWV;
2. To determine the impact of PWV sensitivity to pressure changes with respect to vascular calcification distribution;
3. To characterize the effect of regional vascular calcification as well as cardiac structure on carotid and femoral arterial $dP/dt_{\text{max}}$.

Chapter 4: Magnesium Prevents Initiation of Soft Tissue Calcification \textit{in vitro} and in Chronic Kidney Disease

The objectives were:

1. To design an \textit{in vitro} model of thoracic aorta calcification using elevated phosphate as the stimulus for calcification;
2. To perform dose-response as well as time-dependent experiments with magnesium treatment \textit{in vitro} to prevent calcification;
3. To determine the effect of magnesium treatment on vascular and soft tissue calcification in the adenine CKD model;

Chapter 5: Association of Dietary Phosphate Intake and Phosphate to Protein Ratio with Progression of Coronary Artery Calcification in Patients with Stages 3 to 5 Chronic Kidney Disease

The objectives were:

1. To determine impact of total dietary phosphate on coronary artery calcification progression;
2. To determine the impact of the source of dietary phosphate on coronary artery calcification progression;
Chapter 2

Cardiovascular Disease in an Adenine-Induced Model of Chronic Kidney Disease; the Temporal Link between Vascular Calcification and Hemodynamic Consequences


2.1 Abstract

Medial vascular calcification is highly prevalent in chronic kidney disease (CKD), and it is a risk factor for mortality. This study characterizes the time course and the link between calcification of major arteries, changes in blood pressure (BP) and cardiac growth in experimental CKD. CKD (elevated serum creatinine and urea) was induced with a 0.25% adenine diet (5, 8 and 11 weeks). BP was measured by radiotelemetry in conscious rats or indwelling catheter under anesthesia. At each time point, serum biochemistry and tissue calcification was quantified. CKD was present in all animals by 5 weeks and the ensuing 6 weeks (11 weeks total). CKD animals developed elevated serum phosphate (5-8 weeks) and Fibroblast growth factor 23 (FGF-23; 5-11 weeks) levels. There was a 100% incidence of calcification at 11 weeks, 71% at eight weeks and 33% at five weeks, and distal arteries appeared more susceptible than proximal arteries. Calcification was associated with widening of pulse pressure (PP), and a higher pulse wave. Continuous radiotelemetry revealed a significant increase in systolic BP variability and an accelerated (<24hrs) elevation in PP of at least 10mmHg following eight weeks of CKD. This precipitous change was driven by a drop in mean diastolic BP rather than elevated mean systolic pressure. Pulse pressure, duration of CKD and FGF-23 levels correlated with left ventricular hypertrophy. The unique hemodynamic consequences of medial calcification, combined with the hormonal consequences of hyperphosphatemia (i.e. FGF-23), seem to have an exacerbated risk for left ventricular hypertrophy.

2.2 Introduction

Cardiovascular disease (CVD) is the leading cause of morbidity and mortality in patients with reduced kidney function. Non-traditional risk factors such as calcification
of the vascular media play a major role in increasing CVD risk. Vascular calcification (VC) is an active process involving a phenotypic change of vascular smooth muscle cells (VSMC) into bone lineage cells that are capable of laying down calcium phosphate minerals. This process is markedly accelerated in CKD patients.

Experimental approaches in rodents involving surgical ablation of the kidney have been shown to generate a representative model of mild to moderate CKD. On the other hand, administration of dietary adenine has been shown to generate a more severe model of CKD in rats. Excess dietary adenine saturates the normal adenine salvage pathway (adenine phosphoribosyltransferase pathway) and instead oxidizes to 2,8 dihydroxyadenine by xanthine oxidase. Two eight dihydroxyadenine is then excreted by the kidneys; however due to its low solubility it forms precipitates and crystals in the tubules causing tubular injury, inflammation, obstruction, and fibrosis. The phenotype in this model appears to be consistent with the complications of the chronic kidney disease-mineral bone disorder (CKD-MBD) observed in humans with CKD. That is, animals with adenine-induced CKD develop hyperphosphatemia, secondary hyperparathyroidism, renal osteodystrophy and vascular calcification. FGF-23 is a phosphaturic hormone that increases early in CKD to maintain normophosphatemia in the face of declining kidney function. There is a growing body of literature linking FGF-23 with cardiac hypertrophy and mortality in patients with CKD; however this relationship has not been studied in adenine-induced CKD.

The majority of published studies have reported ~50% incidence of calcification following the administration of 0.75% dietary adenine to rats for 4-6 weeks. By lowering the dietary protein content to 2.5%, Price et al were able to show elevated levels
of aortic calcium in 100% of CKD animals at the 4-week time point\textsuperscript{118}. However, the major confounding factor with this dietary regimen is that animals lose 30-50% of their initial body weight during the adenine intervention\textsuperscript{119,120}.

To date, it has not been studied in the adenine model of CKD whether the development of vascular calcification and its progression reflects the cardiovascular complications and changes within the circulation that are observed in humans with CKD. Therefore, we were interested in characterizing the changes in vascular compliance, blood pressure, and cardiac hypertrophy at three time points in a modified adenine model of CKD\textsuperscript{134,135}. Furthermore, it is unknown whether the presence of uremia or the duration of CKD impacts differentially upon calcification of major vessels (i.e. carotids, thoracic aorta, abdominal aorta, iliac and renal arteries).

The objectives of this study were three fold: 1) to modify the conventional 0.75% adenine to 0.25% adenine to minimize the risk of severe weight loss, 2) to determine the impact of duration of adenine feeding (5 weeks, 8 weeks and 11 weeks) on (i) calcification of the major arteries (carotid, thoracic aorta, abdominal aorta, iliac, renal), (ii) the blood pressure profile over time, (iii) the hemodynamic consequences (pulse wave velocity, left ventricular hypertrophy) and 3) to determine the impact of duration of adenine feeding (5 weeks, 8 weeks and 11 weeks) on parameters of the CKD-MBD (phosphate, calcium and FGF-23) and to examine the association between these biochemical measures and the cardiovascular end-points.
2.3 Methods

2.3.1 Experimental animals

Male Sprague-Dawley rats (Charles River, St. Constant, Quebec) weighing 375-400g at the start of the experiment were individually housed in standard polypropylene cages and maintained on a 12 h light-dark cycle (lights on at 7:00). Animals were allowed to acclimatize for at least 1 week prior to experimentation, and were provided with Purina Rat Chow (Test Diet) and water ad libitum. All procedures were in accordance with the guidelines of the Canadian Council on Animal Care, handling, and termination.

2.3.2 Study design

Rats were maintained on a specially formulated and nutritionally balanced diet (Harlan, Teklad, Madison, WI). This specially formulated diet contains 0.25% adenine, 1% phosphate, 1% calcium, 0.2 ppm vitamin K, 1 IU/g vitamin D, and 6% protein. Diets that contain 6% protein have been demonstrated to have no significant adverse impact on growth\textsuperscript{136}. The reduced adenine concentration of 0.25% was selected in order to increase palatability and prevent weight loss. Body weights and food intake were monitored on a daily basis. In a few instances animals were supplemented with a few pellets of normal chow if their weight loss reached $\geq 10\%$. Ten rats received normal rat chow (the control group), while the remaining animals received the 0.25% adenine diet for 5 weeks (n=6), 8 weeks (n=7), and 11 weeks (n=9). These time points were chosen to allow us to characterize a temporal relationship of adenine feeding, CKD and VC development. At each time point, blood pressure and pulse wave velocity (PWV) were determined under
anesthesia (ketamine 100mg/kg and xylazine 25 mg/kg) after which blood was collected from the inferior vena cava and spun (at 4°C, 4000g, 20 min). The heart was then excised, and the right ventricle was carefully separated from the left ventricle and the septum; left ventricle index (LVI) and right ventricle index (RVI) were determined as the ratio of left or right ventricle weight to body weight. Vessels were collected and carefully cleaned of extraneous connective tissue. A 5 mm-sections of the thoracic aorta, >1 cm inferior to the arch, was kept for histology, and the remaining aorta was snap frozen in liquid nitrogen and kept at -80°C for further analysis.

2.3.3 Blood biochemistry

Serum creatinine, urea, calcium, and phosphate levels were determined using a Roche Modular (Hytachi) in the Clinical Chemistry Core Laboratory (Department of Pathology and Molecular Medicine, Kingston General Hospital, Kingston, Ontario, Canada). Serum FGF-23 levels were measured using an FGF-23 enzyme-linked immunosorbent assay kit, according the manufacturer’s instructions (Kainos Laboratories, Inc., Tokyo, Japan).

2.3.4 Aortic calcium and phosphate content

Frozen iliac, renal, abdominal aorta, thoracic aorta and carotid vessels were thawed, weighed, and homogenized in 0.6 N hydrochloric acid for 24 h at 4°C. Samples were spun, and calcium content was determined colorimetrically using the O-cresolphthalein complexone method (Sigma). O-cresolphthalein colour reagent forms a purple complex with the calcium in the samples. The absorbance for this complex was measured for both standards and tissue homogenates at 540nm (SynergyHT Microplate Reader, Bio-Tek Instruments Inc, Winooski, VT, USA). Phosphate content was
determined using the malachite green method. The malachite green reagent was prepared as previously described\textsuperscript{137}. Upon addition of ammonium molybdate, a green complex is formed between malachite green, molybdate, and free phosphate. The absorbance for this complex was measured for both standards and tissue homogenates at 650nm.

2.3.5 Von Kossa method for visualizing vascular calcification

Five-mm sections of thoracic aorta were fixed in 10x neutral phosphate-buffered saline with 4% paraformaldehyde overnight. Sections were then embedded in paraffin blocks in the upright position to ensure that each aortic section could produce an average of 5 to 6 cross-sections (minimum three). Sections (3-4\(\mu\)m) were stained for calcification using the von Kossa method. Briefly, sections were first deparaffinized, rehydrated in distilled water, treated with 1% silver nitrate, and exposed to ultraviolet light for 20 min.

Then, sections were placed in 5% sodium thiosulfate for 2 min, and counterstained with nuclear Fast Red for 5 min. Areas of calcification appeared as dark brown regions in the medial wall of the aorta.

2.3.6 Radiotelemetry

Six rats in the 11 week group were implanted with a radiotelemetric pressure transducer (model TA11PA-C40, Data Sciences Inc) under isoflurane anesthesia as described previously\textsuperscript{138} and allowed to recover and acclimatize for two weeks prior to dietary adenine treatment. Systolic blood pressure (SBP), diastolic blood pressure (DBP), pulse pressure (PP), heart rate and activity were determined from data collected every 4 minutes (30 seconds, 150 Hz) by a digital radio signal received by units under each cage (model RA1010, RA1020, or RPC-1; Data Sciences) and transferred by a consolidation
matrix (BCM100, Data Sciences) to the data acquisition system (Dataquest LabPRO or Dataquest ART, Data Sciences). Given that rats are nocturnal, daily pressure averages were pooled from data collected from 8pm to 5am to reduce the impact of confounding factors that might increase the animals’ stress such as daily cage changes, and typical animal handling during work hours.

2.3.7 Hemodynamic measurements

PWV was assessed using the foot-to-foot method, as previously described\textsuperscript{139}. Specifically the method used determines the time for an aortic pulse to travel from the carotid artery to the iliac bifurcation. Two catheters were inserted at the superior (i.e. carotid) and inferior (i.e. femoral) ends of the aorta, and were used to measure blood pressure simultaneously. Blood pressure was recorded as a pulsatile waveform at a frequency of 1000Hz. The distance from the tip of the carotid catheter to the iliac catheter was measured. PWV was calculated using the following formula: \( PWV = \frac{\text{propagation distance}}{\text{propagation time}} \) (m/s) at a pressure between 80 and 90 mmHg. At least 10 normal and consecutive waveforms were individually analyzed, and averaged. Only three out of the seven animals at 8 weeks survived the full PWV procedure. However, systolic blood pressure, diastolic blood pressure, as well as pulse pressure from all animals were determined from the carotid catheter, and calculated using Chart version 5 software (ADInstruments). The six animals that had radiotelemetry also had their PWV measured under anesthesia using this method.
2.3.8 Statistical analysis

A stepwise linear regression analysis was performed on the following parameters: creatinine, urea, phosphate, calcium, FGF-23, tissue calcium and phosphate, heart weight and pulse pressure. All data from control and CKD rats were presented as means ± SD. Differences between groups were analyzed using a one-way analysis of variance (ANOVA) followed by a Newman-Keuls post hoc test. A P-value less than 0.05 was considered statistically significant. Statistical analyses were performed on GraphPad Prism version 5 (GraphPad Software, Inc).

2.4 Results

2.4.1 Effects of 0.25% dietary adenine on CKD progression

Animals on the adenine diet (0.25%) lost 2.3±2.4%, 12.2±1.6%, and 9.4±2.6% of their initial weight after 5, 8, and 11 weeks, respectively (Table 2.1). All animals developed CKD after 5 weeks of dietary exposure to 0.25% adenine. Serum creatinine was significantly elevated (>4.5-fold) at 5 weeks of CKD over control, and remained elevated at 8 (5.5-fold) and 11 weeks (5-fold) of CKD (Table 2.1). Serum urea was also significantly elevated at 8 weeks of CKD (4.4-fold), and remained elevated at 11 weeks of CKD (4.8-fold). Although serum phosphate was increased two-fold over control animals at week 5 and week 8 of CKD, this was not sustained at 11 weeks (Table 2.1). FGF-23 was significantly elevated at all 3 time-points (13-fold, 122-fold, and 63-fold increase at 5, 8 and 11 weeks, respectively). In contrast, serum calcium did not differ between controls or adenine-fed rats at any duration of CKD (Table 2.1).
2.4.2 Effect of 0.25% dietary adenine on VC

Calcification was determined by tissue calcium and phosphate content and as well as von Kossa staining. A vessel was considered calcified if it contained levels of calcium that were greater than 3 standard deviations above control vessel values. As demonstrated in the histological sections (Figure 2.1), calcification was localized to the media of the artery. The relationship between tissue calcium and tissue phosphate content was strongly correlated ($r^2=0.97$, $p<0.05$), and the calcium:phosphate ratio was 1.5±0.1.

In the 5-week group (n=6), two animals had elevated levels of calcium in the iliac artery but only one of these animals also had elevated levels of calcium in their other vessels (thoracic aorta, carotid, abdominal aorta, and renal artery) (Figure 2.2A). In the 8-week (n=7) and 11-week groups (n=9), a majority of animals had elevated levels of calcium in all vessels however, at both time points, the thoracic aorta and carotid artery were the least likely to be calcified compared to the more distal vascular beds (iliac, renal, abdominal aorta) (Figure 2.2B and C). That is, all animals with thoracic and carotid calcification (i.e. 57% and 78% of the animals at 8 and 11 weeks respectively) also had abdominal, renal and iliac artery calcification.
**Table 2.1 Weight loss and serum biochemistry of rats fed 0.25% adenine for 5, 8, and 11 weeks.**

<table>
<thead>
<tr>
<th></th>
<th>Control (7 wks)</th>
<th>5 weeks 0.25%Ad (n=6)</th>
<th>8 weeks 0.25%Ad (n=7)</th>
<th>11 weeks 0.25%Ad (n=9)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Weight Loss (%)</strong></td>
<td>0</td>
<td>2.3±2.4</td>
<td>12.2±1.6</td>
<td>9.4±2.6</td>
</tr>
<tr>
<td><strong>Serum Creatinine (μmol/L)</strong></td>
<td>50.0±4.9</td>
<td>232.1±30.3*</td>
<td>278.9±20.6*</td>
<td>254.4±19.2*</td>
</tr>
<tr>
<td><strong>Serum Urea (mmol/L)</strong></td>
<td>8.6±0.9</td>
<td>15.0±1.6</td>
<td>38.2±5.4†</td>
<td>40.8±4.1‡</td>
</tr>
<tr>
<td><strong>Serum Phosphate (mmol/L)</strong></td>
<td>2.4±0.2</td>
<td>5.3±0.5*</td>
<td>5.2±0.5*</td>
<td>2.9±0.3‡‡</td>
</tr>
<tr>
<td><strong>Serum Calcium (mmol/L)</strong></td>
<td>2.2±0.1</td>
<td>2.2±0.1</td>
<td>2.6±0.1</td>
<td>2.0±0.2</td>
</tr>
<tr>
<td><strong>Serum FGF-23 (ng/ml)</strong></td>
<td>0.4±0.05</td>
<td>5.1±1.7*</td>
<td>48.6±15.4‡‡</td>
<td>25.0±7.9*</td>
</tr>
</tbody>
</table>

* * p<0.05 versus Control  
† ‡ p<0.05 versus 5 weeks Adenine 0.25%  
‡‡ p<0.05 versus 8 weeks Adenine 0.25%  
Ad, adenine; FGF-23, fibroblast growth factor-23.
Figure 2.1 Effect of CKD on vascular calcification (VC) as shown by von Kossa stains of the thoracic aorta
Thoracic aorta without calcification (tissue calcium 8.11 nmol/µg and phosphate 0.55 nmol/µg) (A); thoracic aorta of CKD animal at week 8 (tissue calcium 604.4 nmol/µg and phosphate 123.5 nmol/µg) (B); C and D represent different sections along the thoracic aorta from the same calcified vessel (tissue calcium 518.1 nmol/µg and phosphate 310.8 nmol/µg).
Figure 2.2 Distribution and frequency of calcification in different vascular beds. Proportion of vessels calcified at 5 weeks (A), 8 weeks (B) and 11 weeks of CKD (C). Each data point represents one animal; dotted line represents the control mean.
2.4.3 Impact of 0.25% dietary adenine on blood pressure

Blood pressure was analyzed by radiotelemetry in conscious animals exposed to 11 weeks of 0.25% dietary adenine. Pulse pressure measured over a 24 hr time period was first significantly elevated at 8 weeks (42.9±4.2 mmHg to 51.0±4.7 mmHg, p<0.05) and then progressively increased through to 11 weeks (59.0±4.7mmHg) (Figure 2.3). Changes in pulse pressure were largely driven by a significant drop in diastolic blood pressure rather than by changes in systolic blood pressure (Figure 2.4A). DBP was significantly lower at week 9, 10 and 11 compared to all other weeks (-7.9±4.4, -15.9±3.8 and -11.1±2.4 mmHg respectively, p<0.05). Although there was no change in mean SBP at weeks 9-11 compared to earlier weeks, SBP demonstrated significantly greater variability from week 9 to week 11 compared to all other weeks (Figure 2.4B). Variability in DBP was only significantly greater at week 11 (Figure 2.4A). There was a general downward trend in heart rate, heart rate variability and activity with age. Specifically heart rate fell between 4 weeks and 11 weeks (380.1±3.6 to 323.2±4.0 bpm, p<0.001) in telemetry animals in a manner consistent with the reduction in activity (4.3±0.4 to 1.2±0.1 counts/min) (↓HR was significantly correlated with ↓activity, r²=0.77). HR variability fell between 4 and 8 weeks (36.8±1.5 to 27.7±2.0, p<0.0001), but did not change further in the last three weeks (24.8±1.3 at 11 weeks).

2.4.4 Impact of 0.25% dietary adenine on hemodynamic consequences

Blood pressure was analyzed in all rats under anesthesia via carotid catheters at 5, 8 and 11 week time points. Anesthetized blood pressure recording demonstrated elevated SBP in the 8 and 11-week groups, and decreased diastolic in the 11-week group (Figure 2.5A and B). Although pulse pressure was elevated in the 8-week group, it was only
Figure 2.3 Effect of CKD on the temporal development of elevated pulse pressure. Continuous radiotelemetry recordings (abdominal pressures) of pulse pressures revealed that pulse pressure was rapidly elevated at day 59 and progressed through to 11 weeks of CKD. All telemetry animals had vascular calcification at 11 weeks. Data presented as means ±SD.
Figure 2.4 Effect of CKD on systolic and diastolic pressures and mean weekly systolic and diastolic pressure variability.
Continuous radiotelemetry recording (abdominal pressures) revealed that the mean diastolic blood pressure (left y-axis) remained unchanged up to day 59 of CKD, and decreased progressively through to 11 weeks of CKD (A). Mean systolic blood pressure (left y-axis) remained the unchanged throughout the study period, however systolic variability was increased at weeks 9, 10 and 11 compared to all other weeks (B). Diastolic variability (right y-axis) was unchanged until the final week. *p<0.05 versus all other weeks. Data presented as means ±SD.
significantly elevated above control in the 11-week group (Figure 2.5C). Blood pressure had an impact on PWV measurements. That is, in animals without calcification (regardless of age or CKD status) there was a positive linear relationship between blood pressure (DBP, SBP and MAP) and PWV. In contrast, in animals with calcification this relationship was lacking (i.e. less than 1/3 of the slope of control animals). Nonetheless, PWV was found to be increased only in CKD animals with thoracic calcification at 5 (n=1), 8 (n=3) and 11 (n=7) weeks (Figure 2.6).

The severity of CKD (determined by serum creatinine level) predicted the LVI ($r^2=0.38$, $p=0.0001$), but not the RVI ($r^2=0.08$). LVI was also linked with the duration of adenine feeding, and was elevated the most at 11 weeks at which point all animals had VC (Figure 2.7). A stepwise linear regression of LVI with hemodynamic variables (i.e. PP, DBP, SBP, MAP, and PWV), tissue calcium and phosphate, and serum calcium, phosphate, and log (FGF-23) revealed that LVI was highly correlated with increased FGF-23 and PP (Figure 2.8A and B). All other variables either demonstrated a low or insignificant correlation with LVI.
Figure 2.5 Effect of CKD duration on blood pressure in anesthetized animals (carotid catheter).

Systolic blood pressure (A), diastolic blood pressure (B), and pulse pressure (C) of control, CKD rats at 5 weeks, 8 weeks and 11 weeks. Data presented as means ±SD. * p<0.05 vs control; † p<0.05 vs 5 week CKD; ‡ p<0.05 vs 8 week CKD
Figure 2.6 Effect of calcification on pulse wave velocity (PWV).
PWV was significantly elevated in animals displaying thoracic calcification (insert: PWV of calcified animals at 5, 8 and 11 weeks; dotted line represent mean PWV of control group). † p<0.05 vs other groups.
Figure 2.7 Left ventricular to body weight ratio (LV:BW) increased with the duration of CKD.

LV:BW was significantly elevated at week 8 over control, and at week 11 it was significantly higher than all other time points. * p<0.05 vs. control, *** p<0.05 vs. control, 5 and 8 week groups.
Figure 2.8 Serum fibroblast growth factor-23 (FGF-23) as well as pulse pressure linearly correlated with left ventricular weight.

Linear regression analysis between serum FGF-23 and left ventricular to body weight ratio (LV:BW) \(r^2=0.33, \ p<0.005\) (A), and pulse pressure and LV:BW \(r^2=0.66, \ p<0.0001\) (B).
2.5 Discussion

The present study establishes that i) lowering the dietary adenine concentration to 0.25% produces stable CKD at 5, 8 and 11 weeks but without severe weight loss, ii) extending the duration of adenine feeding from 5 to 11 weeks increases the propensity for VC and that distal vessels appear more susceptible to VC iii) medial calcification in this model has a unique hemodynamic profile which includes increased PP, lower DBP and increased variability in SBP and iv) abnormalities in phosphate homeostasis, reflected by elevated levels of FGF-23, are associated with LVH.

The severity of CKD was found to be robust in that all animals demonstrated a greater than 3.5-fold increase in creatinine levels after 5 weeks of adenine treatment. Furthermore, creatinine levels appeared to be relatively stable over time, such that between weeks 8 and 11, creatinine levels remained approximately 5-fold elevated over control animals. The frequency of VC increased over time; that is, at the 5 week time point 33% of rats exhibited VC but at the 11 week time point 100% of animals had calcification. Alterations in hemodynamic parameters were associated with the progression of CKD, as well as the onset and progression of VC. There was a progressive increase in LV mass that correlated with the duration of CKD, associated biochemical changes (e.g. ↑FGF-23), and hemodynamic alterations (↑PP). Lastly, our findings suggest there is a likely link between progression of calcification, hemodynamic changes, and changes in cardiac structure and function.

Vessels distal from the heart appeared to be most susceptible to VC, as calcification in these vessels consistently preceded that in the thoracic aorta and carotid artery. All vessels were exposed to identical CKD conditions and prevailing
concentrations of phosphate. That is, the exposure of vascular smooth muscle cells to the calcifying risk factors associated with the CKD environment would be the same. Therefore these data suggest that there may be regional differences in structural components of the vessel or in the local expression of key inhibitors of vascular calcification, such as matrix Gla protein (MGP), that impact upon susceptibility to VC. One possible explanation could relate to the fact that VSMCs in different arterial regions develop from different embryonic origins. Recently Leroux-Berger et al in a series of in vitro and in vivo experiments using aortas from MGP knockout mice demonstrated that susceptibility to calcification differed between the different regions of the aorta. Regional susceptibility to VC has not been well studied in the CKD population; most studies only report coronary or thoracic calcification. However, one study recently showed in obese subjects, that abdominal and iliac arteries were more frequently calcified than were thoracic aorta or carotid arteries.\textsuperscript{140}

Duration of CKD was linked to hemodynamic alterations within the circulation, although the greatest circulatory changes occurred in animals that also had thoracic calcification. That is, most of the circulatory changes were significant at 11 weeks (↑SBP, ↓DBP, ↑PP) reflecting the progression of calcification. Furthermore, the finding that PWV was only increased in animals with calcification of the thoracic aorta demonstrates the importance of calcification in determining vascular stiffness. These findings are consistent with CV complications reported in CKD patients. For example, CKD patients develop systolic hypertension and elevated PWV, both of which have been associated with VC.\textsuperscript{124,125,141,142}
In contrast to blood pressure assessments under anesthesia, the use of radiotelemetry eliminates the confounding influence of anesthetic agents on the circulation and allows for the examination of the temporal component of hemodynamic alterations in CKD rats. All of the animals implanted with radiotelemetry for 11 weeks developed VC. The initial and accelerated change in pulse pressure occurred at week eight and was progressive through to week 11. It was unexpected that the sudden rise in PP was associated with a drop in DBP, rather than a rise in SBP. Although mean SBP did not change throughout the 11 weeks study period, variability markedly increased between 9 and 11 weeks. Blood pressure variability has been consistently associated with end-organ damage in humans\textsuperscript{143–145}, but has never been studied in the context of vascular calcification in CKD. Although most previous work has considered the effect of 24 hour blood pressure variability, here we show chronic SBP variability in the awake hours (i.e. variability was not due to day/night dipping effect) which may be a better marker of LVH and vascular remodeling\textsuperscript{144}. The increase in SBP variability could be an indication of reduced compliance as well as increased stiffness. Since the changes in heart rate and heart rate variability did not account for the changes in blood pressure, the distinctive pressure profile, likely resulted from alterations in the mechanical properties (i.e. compliance) of the large elastic arteries due to medial wall calcification. Evidence for altered vascular properties in VC has previously been well established by Sutliff \textit{et al.} (2011)\textsuperscript{146}.

The observed changes in arterial compliance are likely a major contributing factor to the increased LV weight observed in the CKD animals. That is, the increased PWV and PP may be the physical stimulus contributing to LVH\textsuperscript{93}. However, in this model, the
presence of LVH is not fully explained by calcification-associated abnormalities generated within the circulation and we found an interesting link with FGF-23 levels. There is previous evidence linking FGF-23 with mortality and LVH in humans with and without CKD. It has been hypothesized that FGFs, including FGF-23, may stimulate myocardial cell growth and fibrosis via activation of FGF receptors which are expressed in adult myocardial cells. In the present study, we found an association between FGF-23 concentrations and LVH in CKD animals regardless of their calcification status. It is plausible that markedly elevated levels of FGF-23 in CKD may non-selectively activate FGF receptors in the heart, and thereby mediate myocardial cell enlargement and fibrosis however further studies are needed.

Clinical studies have shown that elevated serum phosphate levels in CKD are associated with coronary artery calcification, elevated FGF-23, and mortality\(^{21,147,148}\). CKD rats had an elevated serum phosphate at 5 and 8 weeks, but not at 11 weeks whereas, serum FGF-23 remained elevated throughout the study. Although phosphate is believed to be a key signaling molecule in the development of VC, an isolated serum phosphate value \emph{per se} in the late stage CKD animals did not predict this event. Other experimental models have also shown minimal or no elevations in serum phosphate, yet these animals also developed VC\(^{17}\). The observed decline in serum phosphate at 11 weeks could be due to multiple factors such as decreased food consumption related to deterioration of animal health, although increased tissue uptake of phosphate could also have occurred\(^{149}\). FGF-23 may therefore represent a more robust marker of the duration and severity of one of the key stimuli of VC and cardiovascular health in this model\(^{150}\).
The experimental approach of the present study attempted to model the development of CVD in the setting of a modified adenine CKD approach which minimizes weight loss. Our findings reveal that there is regional susceptibility to calcification and that a distinctive hemodynamic profile appears to be linked with its development. Finally, using this modified adenine-induced model of stable CKD, we established that prolonged elevation of FGF-23 was a better sentinel of both the positive phosphate balance (which impacts calcification) and its negative impact on cardiac structure than was the transient hyperphosphatemia.
Chapter 3
Regional Calcification Differentially Impacts Elevated Pulse Wave Velocity and Arterial $dP/dt_{\text{max}}$ in Chronic Kidney Disease Rats
3.1 Abstract

Vascular calcification (VC) is accelerated in patients with chronic kidney disease (CKD), an effect which leads to increased cardiovascular morbidity and mortality. VC development is heterogeneous, and so not all vascular beds calcify at the same rate. Advanced VC is widely acknowledged to compromise the circulatory and cardiovascular function, at least in part, as a result of hemodynamic loads such as increased pulse wave velocity (PWV). However, it is not known whether calcification of certain regions is critical in promoting the dysfunction. CKD was developed in all rats by administering adenine (0.25%) in the diet for 7 weeks. CKD animals accrued different amounts of calcification in the various vascular segments studied. Hemodynamic changes (blood pressure profile, PWV, dP/dt$_{\text{max}}$) were assessed in rats instrumented with indwelling catheters under anesthesia. All adenine rats had elevated serum creatinine at 7 weeks (5.6±1.7 fold vs. control). CKD animals had different amounts of calcification (no calcification n=9; only abdominal calcification n=9; both abdominal and thoracic calcification n=11). PWV was not affected by presence of CKD (539±65cm/s) or abdominal calcification (608±140cm/s), but was elevated in rats with thoracic calcification (1345±583cm/s; p<0.001). Although changes in blood pressure affected PWV ($r^2$=0.7; slope=0.84±0.06) in most rats, the presence of thoracic calcification significantly blunted this relationship ($r^2$=0.2; slope=0.36±0.09; p<0.0001 vs. control or CKD). In contrast, the presence of abdominal and thoracic calcification differentially affected proximal and distal arterial dP/dt$_{\text{max}}$. Femoral dP/dt$_{\text{max}}$ was elevated in animals with abdominal calcification (1.5 fold over control, p<0.001) and carotid dP/dt$_{\text{max}}$ was elevated in animals with thoracic calcification (2.1 fold over control, p<0.001). Changes
in both femoral ($r^2=0.4, \ p<0.001$) and carotid ($r^2=0.7, \ p<0.0001$) dP/dt$_{max}$ correlated with left ventricular index in CKD animals. The distal to proximal index of hemodynamic change and the ratio of carotid:femoral dP/dt$_{max}$, were also elevated in CKD animals without calcification ($p<0.05$). Hemodynamic changes, such as increased PWV, have long been a hallmark of vascular stiffness. However, this study revealed that specific changes in arterial dP/dt$_{max}$, but not PWV, predict the impact on both regional calcification and cardiac structure.

### 3.2 Introduction

Pulse wave velocity (PWV) is the most common parameter used to measure arterial stiffness in chronic kidney disease (CKD) patients, and it has been shown to be a strong independent predictor of all-cause and cardiovascular mortality in end stage kidney disease (ESKD) patients $^{23}$. One of the major contributors to elevated PWV in CKD is the accelerated development of medial vascular calcification (VC)$^4$. Studies have shown that PWV in CKD and ESKD patients is linked to calcification of large arteries including abdominal aorta, coronary arteries, common carotid arteries, thoracic aorta and femoral arteries $^{88,93,124,125,151}$. However, distribution of VC in patients has not been characterized, and so it is unclear if different vascular beds have differential impact on PWV. For instance, one study by Guerin et al. showed that out of the four vascular beds examined, PWV was elevated in patients when more than 2 vascular beds calcified.

VC is an active process in CKD involving the phenotypic change of vascular smooth muscle cells (VSMC) to osteochondrogenic-like cells. This phenotypic switch results in calcification and loss of elastic function in arteries $^{88}$. However, some vascular
beds are more prevalent to develop VC\textsuperscript{140,152}, and whether the regional profile of VC relates to circulatory readouts is not known.

The function of large, elastic arteries is to transform and dissipate the pulsatile pressure generated by the left ventricle to a steady flow at the periphery. VC hinders this function causing an elevation in PWV that leads to increased reflected waves, and ultimately left ventricular hypertrophy\textsuperscript{96}. Furthermore, the dynamics of a pulse wave generated by the left ventricle are influenced by both heart function and vascular compliance. For instance, the maximum rate of the upstroke in a given wave (dP/dt\textsubscript{max}) could be altered by irregular compliance along the aorta or left ventricle function\textsuperscript{106–108,153}.

Previously, we have shown that distal regions of the arterial system are more susceptible to VC in a rodent model of CKD\textsuperscript{152,154}. In this model, thoracic aorta is one of the last arteries to calcify. The current study looks at multiple circulatory markers of early to late stages of VC progression. Based on previous findings, we hypothesize that PWV is highly dependent on calcification of the thoracic aorta but the effect of VC on arterial dP/dt\textsubscript{max} is regional and cardiac dependent. The objectives of this study are to i) determine the impact of regional calcification on PWV ii) determine the sensitivity of PWV to changes in blood pressure with respect to VC distribution iii) characterize the effect of regional VC and cardiac structure on distal and proximal arterial dP/dt\textsubscript{max}.
3.3 Methods

3.3.1 Experimental animals

Male Sprague-Dawley rats (Charles River, St. Constant, Quebec) starting at 14 weeks of age were individually housed in standard polypropylene cages and maintained on a 12 h light-dark cycle (lights on at 7:00AM). Animals were allowed to acclimatize for at least 1 week prior to experimentation, and were provided with Purina Rat Chow (Test Diet) and water *ad libitum*. The animals were treated in accordance with the guidelines of the Canadian Council on Animal Care, handling and termination.

3.3.2 Generation of CKD

CKD was generated in the animals as previously described\(^{152}\). In brief, rats were given a specially formulated (but nutritionally balanced) diet (Harlan, Teklad, Madison, WI). The specially formulated diet contained either 0.25% adenine (CKD) or 0% adenine (control) along with 1% phosphate, 1% calcium, 0.2 mg/kg vitamin K, 1 IU/g vitamin D, and 6% protein. Health checks (weights and food intake, hydration status) were performed on a daily basis, and animals were supplemented with normal chow and/or Nutri-Cal or fluids if their weight loss reached 10%. Control animals were food-restricted by 20% of their regular food intake to maintain a steady body weight. Previously, we have observed that by 7 weeks the animals have developed CKD, but there is heterogeneity in the distribution of calcification, and so this time point was chosen in order to assess regional calcification. After 7 weeks of adenine feeding, rats were anesthetized with (30 mg/kg, Rogar/STB, London, Ontario, Canada) and inactin (thiobarbital sodium, 100 mg/kg, Sigma-Aldrich) and hemodynamic measurements were performed (see below). After these measurements, blood was drawn (~6-10mL) from the
left ventricle while the animals were still under anesthesia. The heart was excised and the right ventricle was carefully separated from the left ventricle and septum. The left ventricle index (LVI), the ratio between left ventricle weight and body weight, was calculated. Blood samples were spun (4°C, 4000 g, 20 min) using a BHG Hermle Z320K refrigerated centrifuge (Mandel Scientific Company, Gosheim, Germany). Aortic (abdominal and thoracic) tissue were removed and kept. All samples were snap-frozen in liquid nitrogen and stored at -80°C for further analysis.

3.3.3 Serum creatinine assessment

Previously, we have shown that rats generate CKD at 7 weeks \(^{152}\). Serum creatinine was analyzed using the QuantiChrom Creatinine Assay Kit (DICT-500) (BioAssay Systems, Hayward, CA).

3.3.4 Determination of aortic calcium content

Frozen abdominal aorta and thoracic aorta were thawed, weighed and homogenized in 0.6 N hydrochloric acid for 24 h at 4°C, at which point calcium and phosphate content were quantified as previously described \(^{152}\). In brief, calcium content was determined colorimetrically using the O-cresolphthalein complexone method (Sigma). O-cresolphthalein colour reagent forms a purple complex with the calcium in the samples, the absorbance for which can be measured at 540nm (SynergyHT Microplate Reader, Bio-Tek Instruments Inc, Winooski, VT, USA). Phosphate content was determined using the malachite green method. The malachite green reagent was prepared as previously described \(^{137}\). A green complex is formed between malachite green, molybdate, and free phosphate, the absorbance of which was at 650nm. The
validity of using these mineral values to determine the presence of calcification has been confirmed in our previous studies 152,154.

3.3.5 Hemodynamic assessments

Hemodynamic assessments were collected after a 10min stabilization period under anesthesia. To assess pressure changes, after the surgical procedures were completed animals that displayed at least a pressure range of 15mmHg were selected (see below). This pressure variability was probably as a result of the anesthetic, but most animals stabilized after a 10min period. The 15mmHg range was selected because individual correlations showed significance, but less than that there was no correlation. All hemodynamic variables (PWV, dP/dtmax, mean arterial pressure, systolic, diastolic, pulse pressure and heart rate) were determined at various pressure points in each animal. PWV was assessed using the foot-to-foot method, as previously described 152. This method determines the time for a given pulse generated by the heart to travel from the top of thoracic aorta (near the start of carotid artery) to the abdominal aorta (near the bifurcation). Two catheters were inserted at the carotid and femoral arteries and advanced into the aorta. Blood pressure was recorded as a pulsatile waveform at a frequency of 1000 Hz simultaneously from the two locations. The distance from the tip of the carotid catheter to the femoral catheter was measured. PWV was calculated using the following formula: PWV=propagation distance / propagation time (m/s). At least 10 normal and consecutive waveforms were individually analysed and averaged. In addition, the following hemodynamic variables were calculated at both catheter locations: heart rate, mean arterial pressure, dP/dtmax, systolic and diastolic pressures. All hemodynamic
measurements were completed using Chart version 7 software (ADInstruments, Colorado Springs, Colorado, USA)

Figure 3.1 – Example of \( \frac{dP}{dt_{\text{max}}} \).
\( \frac{dP}{dt_{\text{max}}} \) is defined by the maximum slope of a pressure curve measured in the carotid artery catheter or femoral artery catheter. This example shows three sample curves with low and high \( \frac{dP}{dt_{\text{max}}} \).

3.3.6 Statistical analysis

To analyze whether there was a correlation between hemodynamic parameters, linear regression analysis was performed for PWV, carotid and femoral \( \frac{dP}{dt_{\text{max}}} \) versus mean arterial pressure and heart rate. To account for the pressure heterogeneity between animals, PWV, \( \frac{dP}{dt_{\text{max}}} \), mean arterial pressure and heart rate ranges from each animal were normalized to the respective median value. All data were presented as mean±SD. The differences between the groups were analyzed using one-way analysis of variance followed by Newman–Keuls post hoc test to compare differences between groups or Bonferroni post hoc test to compare differences from control. Paired t-test was used to
compare the differences in PWV at 90mmHg and 120mmHg. P-values <0.05 were considered statistically significant. Analysis was performed using Graph Pad Prism v.6.

3.4 Results

It was found that all animals fed 0.25% adenine diet had a minimum of 3.8 fold increase in serum creatinine over control animals, but the average creatinine elevation was 5.6±1.7 fold (p<0.0001) (Table 3.1). This finding was considered as a uniform CKD formation across all animals and groups. The pressure profiles of individual animals revealed that arterial pressure had an impact on PWV (Figure 3.2). There was a high correlation between blood pressure and PWV in control animals (r²=0.9±0.08), but in CKD animals (r²=0.7±0.14) this relationship was altered (Figure 3.2A and B). There appeared to be two distinct groups of CKD animals: those with good correlation and those with low or no correlation (Figure 3.2; inserts). This led to grouping animals based on their regional calcification, that is CKD with no VC (CKD), CKD with abdominal VC only (CKD+Ab) and CKD with thoracic as well as abdominal VC (CKD+Th). In all cases, thoracic calcification was the last vessel calcified, since abdominal calcification was already present (Table 3.1).

The hemodynamic profiles of animals under anesthesia following a stabilization period are presented in Table 3.2. Mean arterial pressure was similar in all groups. In contrast, CKD animals with thoracic calcification had elevated pulse pressure manifested by an elevated systolic pressure and a decreased diastolic pressure. Similarly, the PWV was elevated only in CKD animals with thoracic calcification.
Table 3.1 Chronic kidney disease (CKD) groups based on vascular calcification

<table>
<thead>
<tr>
<th></th>
<th>Control N=17</th>
<th>CKD N=9</th>
<th>CKD+Ab N=9</th>
<th>CKD+Th N=11</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum creatinine (μmol/L)</td>
<td>57.1±13.0</td>
<td>304.7±102.6</td>
<td>367.0±79.8</td>
<td>292.4±98.8</td>
</tr>
<tr>
<td>Thoracic Aorta Calcium (nmol/mg)</td>
<td>4.3±1.9</td>
<td>6.5±1.8</td>
<td>21.1±26.7</td>
<td>1323.3±446.4</td>
</tr>
<tr>
<td>Abdominal Aorta Calcium (nmol/mg)</td>
<td>5.6±2.0</td>
<td>7.3±1.2</td>
<td>280.4±414.3</td>
<td>837.6±656.5</td>
</tr>
</tbody>
</table>

CKD+Ab, CKD animals with abdominal calcification; CKD+Th, CKD animals with thoracic and abdominal calcification.
Figure 3.2 Impact of arterial pressure on pulse wave velocity (PWV).

There was a high association between arterial pressure and PWV in control animals ($r^2=0.9\pm0.08$)(A). The association between arterial pressure and PWV was mixed in CKD ($r^2=0.7\pm0.14$) (B) (inset: example of individual animals in each group). The regression curve in (A) and (B) graphs is an average slope of their respective individual rats.
Table 3.2 Blood pressure profiles under anesthesia (after stabilization).

<table>
<thead>
<tr>
<th></th>
<th>Control (N=17)</th>
<th>CKD (N=9)</th>
<th>CKD+Ab (N=9)</th>
<th>CKD+Th (N=11)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart rate (bpm)</td>
<td>342.4±36.5</td>
<td>305.3±25.7</td>
<td>323.5±28.1</td>
<td>331.6±36.7</td>
</tr>
<tr>
<td>Mean Arterial pressure (mmHg)</td>
<td>108.9±11.5</td>
<td>112.2±14.6</td>
<td>110.2±12.6</td>
<td>100.0±5.096</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>127.8±12.0</td>
<td>131.0±14.4</td>
<td>128.8±11.5</td>
<td>148.2±21.4^a</td>
</tr>
<tr>
<td>Diastolic blood pressure (mmHg)</td>
<td>92.2±11.4</td>
<td>94.5±15.2</td>
<td>92.5±14.9</td>
<td>61.0±17.0^a</td>
</tr>
<tr>
<td>Pulse pressure (mmHg)</td>
<td>35.6±3.7</td>
<td>36.6±5.4</td>
<td>36.3±8.7</td>
<td>87.2±60.6^a</td>
</tr>
<tr>
<td>Pulse wave velocity (cm/s)</td>
<td>582.7±170.3</td>
<td>538.5±65.0</td>
<td>608.0±140.1</td>
<td>1345.4±582.9^a</td>
</tr>
<tr>
<td>Left ventricular index (g/kg)</td>
<td>1.8±0.1</td>
<td>2.0±0.2^b</td>
<td>2.0±0.3^b</td>
<td>2.4±0.5^a</td>
</tr>
</tbody>
</table>

^a p<0.05 vs. all other groups; ^b p<0.05 vs. control

CKD+Ab, CKD animals with abdominal calcification; CKD+Th, CKD animals with thoracic and abdominal calcification.
3.4.1 PWV is linked to blood pressure and thoracic calcification

The overall association of arterial pressure and PWV was not affected by the presence of CKD or the development of abdominal calcification. The change in arterial pressure was significantly correlated to PWV in control ($r^2=0.7; p<0.0001$), CKD ($r^2=0.7; p<0.0001$) and CKD+Ab ($r^2=0.8; p<0.0001$) animals (Figure 3.3A, B and C). In contrast, change in arterial pressure had a reduced impact on PWV in CKD animals with thoracic calcification ($r^2=0.2; p<0.01$) (Figure 3.3D). In fact, most of the animals with thoracic calcification (67%) did not display a significant relationship between arterial pressure and PWV (Figure 3.3D). The slope of the pressure-PWV relationship in the thoracic calcification group was significantly lower than all other groups (Figure 3.3 E and F, $p<0.0001$). There was no relationship between heart rate change (HR) and PWV change in control ($r^2= 0.03, p=0.1$) or CKD+Th ($r^2=0.01, p=0.5$) (data not shown).

Assessment of differences in PWV after normalizing to a common blood pressure was important to account for the basis of change. Although PWV remained unchanged between control, CKD and CKD+Ab groups at fixed pressures, PWV was significantly higher at 120mmHg versus 90mmHg in these groups (Figure 3.4). However, PWV in the CKD+Th group at 90mmHg and 120mmHg was not significantly different. Furthermore, PWV in the CKD+Th group was elevated above all other groups at both pressures ($p<0.0001$) (Figure 3.4).

The CKD animals had elevated LVI which was exacerbated by the presence of thoracic calcification (Table 3.2). Despite this outcome, there was no significant correlation between LVI and PWV ($r^2= 0.007$ control, $r^2=0.06$ CKD; $p>0.05$) (data not shown).
Figure 3.3 Thoracic calcification impacts the relationship of change in arterial pressure and PWV.

There was a high association between ∆Mean arterial Pressure and ∆PWV in control (A; $r^2=0.7$), chronic kidney disease (CKD) (B; $r^2=0.7$) and CKD with abdominal aorta calcification (+Ab) (C; $r^2=0.8$). The association was blunted in CKD with thoracic aorta calcification (+Th) (C; $r^2=0.2$). The sensitivity to blood pressure was lower in the CKD+Th group (D and F). The CKD animals with thoracic calcification had a significantly lower slope than other groups (F). *$p<0.05$ vs all other groups.
Figure 3.4 Pulse wave velocity (PWV) of common pressures under anesthesia.
In control, chronic kidney disease (CKD) without calcification, CKD with abdominal calcification (+Ab) pair matched groups, PWV was higher at 120mmHg as compared to 90mmHg. PWV was not different in CKD with thoracic calcification (+Th) animals at 90 and 120mmHg. CKD+Th animals had elevated PWV as compared to other groups at both pressures (p<0.05). *p<0.05 vs pair matched group.
3.4.2 dP/dt\textsubscript{max} of carotid and femoral blood pressures are linked to both calcification and LVI

Carotid and femoral dP/dt\textsubscript{max} associated differently with blood pressure. Carotid dP/dt\textsubscript{max} had an inverse association with blood pressure change in control animals and CKD animals without VC (Figure 3.5A). In contrast, femoral dP/dt\textsubscript{max} was positively associated with change in blood pressure (Figure 3.5C). Despite this reversal for proximal to distal pressure, both carotid and femoral dP/dt\textsubscript{max} in CKD+Th group had a strong positive association with blood pressure (Figure 3.5A and C). Furthermore, CKD+Th rats were more sensitive to blood pressure change, as depicted by their steeper regression slopes compared to all other groups (Figure 3.5B and D).

Carotid and femoral dP/dt\textsubscript{max} were elevated in the CKD+Th group, and femoral dP/dt\textsubscript{max} was also elevated in CKD+Ab group (Figure 3.6). However, unlike PWV, Carotid dP/dt\textsubscript{max} \((r^2=0.7, \ p<0.0001)\) and femoral dP/dt\textsubscript{max} \((r^2=0.4, \ p<0.0005)\) significantly correlated with LVI in CKD animals (Figure 3.7).
Figure 3.5 Relationship between blood pressure and arterial dP/dt(max) in CKD. Carotid dP/dt(max) had an inverse relationship with blood pressure in control (r²=0.3, p<0.01) and chronic kidney disease (CKD) (r²=0.3, p<0.01) animals, but CKD animals with abdominal calcification (CKD+Ab) did not show an association (r²=0.01, p=0.6) (A). CKD animals with thoracic calcification (CKD+Th) had a positive association with dP/dt(max) at both locations (carotid: r²=0.4, p<0.01. Femoral; r²=0.5, p<0.01) (A, C). Pressure relationship with femoral dP/dt max was mixed (control: r²=0.2, p<0.01. CKD: r²=0.04, p=0.23. CKD+Ab: r²=0.3, p<0.01), but none of the groups had an inverse relationship (C). The slope of CKD+Th was higher than other groups (B, D). *p<0.05 CKD+Th vs. all other groups, †p<0.05 CKD+Th vs CKD and Control.
Figure 3.6 The effect of calcification on carotid (A) and femoral (B) \( \frac{dP}{dt_{\text{max}}} \). Carotid and femoral \( \frac{dP}{dt_{\text{max}}} \) were elevated in chronic kidney disease animals with thoracic calcification (CKD+Th). Femoral \( \frac{dP}{dt_{\text{max}}} \) was also elevated in CKD animals with abdominal calcification (CKD+Ab). All values were calculated at pressures reported in Table 2. *p<0.05; \(^a\)p<0.05 vs. all groups; \(^b\)p<0.05 vs control.
Figure 3.7 The association of dP/dt\textsubscript{max} at carotid and femoral arteries with left ventricle index (LVI).
In control animals, carotid ($r^2=0.2$, $p=0.05$) (A) or femoral ($r^2=0.02$, $p=0.6$) (B) did not predict LVI. However, CKD animals, both carotid ($r^2=0.7$, $p<0.0001$)(C) and femoral ($r^2=0.4$, $p<0.001$)(D) dP/dt\textsubscript{max} predicted LVI.
3.5 Discussion

This study reveals that there are differential consequences of regional aortic calcification on PWV and arterial dP/dt\textsubscript{max} in animals suffering from adenine induced CKD. Specifically, increased PWV and carotid dP/dt\textsubscript{max} were linked to the development of calcification of the thoracic aorta, one of the last vessels segments to calcify. In contrast, calcification of the distal segments of the aorta (abdominal) occurred earlier in CKD, but was not associated with increased PWV or carotid dP/dt\textsubscript{max}. Calcified distal segments were, however, linked to increased femoral dP/dt\textsubscript{max}. Variations in blood pressure differentially impacted PWV and dP/dt\textsubscript{max}, depending on presence or absence of thoracic calcification. In the absence of thoracic calcification, PWV was highly dependent on changes in blood pressure, whereas dP/dt\textsubscript{max} was less dependent. Thoracic calcification altered the relationship between blood pressure and PWV and dP/dt\textsubscript{max}. Whereas the sensitivity of PWV to changes in blood pressure was lost with thoracic calcification, it was markedly increased for dP/dt\textsubscript{max}. Although both elevated PWV and dP/dt\textsubscript{max} predicted calcification, PWV elevation likely reflected altered vessel function, whereas dP/dt\textsubscript{max} was affected by a combination of cardiac and vascular factors.

Increased PWV was dependent on the location of the calcification. Although the full length of the aorta has been reported to be responsible for propagating pulse waves generated by the heart, these findings indicate that structural and functional changes specifically associated with thoracic calcification may be more important. In the adenine model of CKD, thoracic calcification is a relatively late occurrence, such that increases in PWV would be expected to occur relatively late in the disease process, not as an early sentinel of impending pathogenesis.
The current study revealed that dP/dt_{max} is a robust and early marker of regional vascular calcification. Increased femoral dP/dt_{max} and carotid dP/dt_{max} corresponded to calcified abdominal and thoracic aorta, respectively. Studies have shown that arterial dP/dt_{max} is linked to cardiac contractility and ejection fraction \(^{106,107}\), although factors that affect vascular compliance are also likely involved \(^{108}\). Changes in carotid and femoral dP/dt_{max} were linked to both LVI and regional calcification in the CKD animals indicating that there is likely a combined effect on dP/dt_{max}. The finding that abdominal calcification was required for changes in femoral dP/dt_{max} and thoracic calcification was required for changes in carotid dP/dt_{max} indicates that these hemodynamic consequences, but not PWV *per se*, are predictive of regional calcification in CKD.

The differential association observed between changes in blood pressure and carotid-femoral dP/dt_{max} suggests that along the aorta, different factors contribute to the rate of change of pressure within each pressure curve. For instance, carotid dP/dt_{max} might be influenced partially by the cardiovascular reflex mechanisms. Previous studies have shown that cardiac function has a similar inverse relationship with blood pressure \(^{156}\), suggesting that increased carotid dP/dt_{max} might predict left ventricle dP/dt_{max} \(^{106,107}\). Furthermore, calcification causes a reduction in baroreceptor sensitivity in CKD patients \(^{157}\), a finding that agrees with the current data. Specifically, the inverse relationship observed in the control animals was lost in the presence of thoracic calcification. In fact, vascular calcification markedly increased the sensitivity of blood pressure and dP/dt_{max} relationship at both carotid and femoral locations. This effect suggests that loss of vascular compliance has a major impact on dP/dt_{max}, resulting from an elevated elastic modulus (E_{inc}, a measure of elastic properties) and decreased distensibility \(^{88,158}\).
PWV was found to be much more sensitive to changes in arterial pressure than $dP/dt_{\text{max}}$. In particular, variations in blood pressure in control and CKD animals without thoracic VC significantly impacted PWV, whereas this relationship was blunted in the presence of thoracic calcification. Normally, elastic arteries under high pressure of cardiac systole distend and physiologically stiffen, whereas, under low pressure, vessels regain some compliance\textsuperscript{158,159}. That is, normally during cardiac diastole, the elastic walls recoil, thus releasing kinetic energy accumulated during systole and thereby propelling this volume to the periphery. At higher blood pressures more kinetic energy is released, which in part explains the linear relationship between PWV and BP\textsuperscript{97,160}. In contrast, calcification reduces the elastic properties of thoracic aorta\textsuperscript{93,146}, thereby influencing the difference in PWV response to low and high pressure conditions.

Elevated PWV in CKD animals with thoracic calcification might also explain the observed change in cardiac structure. These animals had altered systolic, diastolic, pulse pressure and an elevation of LVI compared to the other CKD animals. A known consequence of elevated PWV is increased reflected waves from the periphery, contributing to an increased systolic pressure, pulse pressure and cardiac afterload\textsuperscript{134,161}. In the CKD animals, it is likely that these calcification-induced hemodynamic changes significantly contributed to the exacerbated LVI. CKD animals without thoracic calcification only had a slight increase in LVI over control animals, consistent with our previous work suggesting there might be other metabolic complications in CKD\textsuperscript{152}.

The impact of blood pressure on circulatory markers of calcification suggests that PWV, more so than $dP/dt_{\text{max}}$, should be measured at a normalized constant blood pressure across all subjects to avoid false positive results. Previous research has also shown a
strong correlation between blood pressure and PWV\textsuperscript{162,163}. However, there have also been contradictory results for the relationship between heart rate and PWV\textsuperscript{104,164,165}. In the current study, changes in heart rate did not impact PWV measurements. This result is most likely because there were only small fluctuations in the resting heart rate that did not have a significant impact on blood pressure and PWV.

An important limitation to assessing hemodynamic changes in CKD results from the progressive nature and regional heterogeneity of calcification. For example, abdominal calcification progresses three-fold in animals that lack thoracic calcification compared to animals with thoracic calcification. However, the impact of this abdominal calcification per se on hemodynamics is not well described. The novel findings in the current study indicate that selectively increased femoral dP/dt\textsubscript{max} versus carotid dP/dt\textsubscript{max} distinguished the degree and the regional heterogeneity of aortic calcification, whereas changes in PWV did not. Specifically, the present findings provide the first evidence of sentinel changes indicating vascular pathogenesis that precedes aortic calcification by way of assessment of the carotid to femoral dP/dt\textsubscript{max} index. This concept is supported, at least in part, by previous studies showing that changes in arterial structure and function are associated with the distribution of calcification\textsuperscript{88}.

In conclusion, the present experimental CKD study revealed that regional VC has a differential impact on PWV and arterial dP/dt\textsubscript{max}. Specifically, PWV predicted thoracic calcification, whereas femoral and carotid dP/dt\textsubscript{max} predicted both abdominal and thoracic calcification, respectively. Changes in dP/dt\textsubscript{max} appear to be predictive of progression of calcification throughout the aorta, whereas changes in PWV appear to be linked to advanced aortic vascular calcification. Moreover, arterial dP/dt\textsubscript{max} appears to be
influenced by both vascular compliance and cardiac function. Given the serious consequences of the development of vascular calcification in CKD patients, it may be that non-invasive assessments of hemodynamic change (combined PWV and arterial dP/dt_{max} measurements) could provide important early information that could then benefit treatments that would impact disease progression.
Chapter 4

Magnesium Prevents Initiation of Soft Tissue Calcification \textit{in vitro} and in Experimental Chronic Kidney Disease
4.1 Abstract

Vascular calcification (VC) is accelerated in patients with chronic kidney disease (CKD), resulting in increased risk of cardiovascular disease and mortality. Mineral imbalance in CKD contributes to this process, and elevated phosphate is a major risk factor. Magnesium plays an important role in bone regulation, and has been recently shown to be a modifier of VC. Vascular calcification was assessed \textit{in vivo} and \textit{in vitro}. CKD was generated by giving Sprague-Dawely rats an adenine diet (0.25%) for five weeks with (0.2%) or without (0.05%) increased levels of magnesium. Calcification was assessed by tissue calcium levels and confirmed with von Kossa staining. In a separate \textit{in vitro} study, aortas collected from healthy rats (~400g) were incubated in pro-calcification media containing various levels of magnesium (0.8-2.5mM). Adenine-fed rats developed CKD as indicated by elevated serum creatinine. The severity of CKD was not altered by increasing the level of dietary magnesium (394.2±80.7µM and 389.5±57.0µM in low and high magnesium diets). However, the high magnesium diet decreased the frequency and severity of calcified vessels compared to rats fed a normal magnesium diet (p<0.001). In the \textit{in vitro} study, all aortic rings incubated for 4-6 days showed evidence of calcification in high phosphate media (>3.0mM). Increasing the magnesium levels (0.8mM to 1.2mM, 1.6mM, 2.0mM and 2.5mM) blunted calcification significantly (49±15%, 63±4%, 89±7% and 93±4%, respectively). Delaying high magnesium treatment was only effective if the supplementation occurred prior to day 4 of the 6 day incubation (addition of magnesium at day 2: 59.2±40.9nmol/mg and day 3: 113.4±88.0nmol/mg compared to control 175.5±54.3nmol/mg, p<0.05). A two day pretreatment with magnesium (2.5mM) followed by normal magnesium (0.8mM) also prevented calcification at day 6. That is,
incubation of these vessels for only two days in 2.5mM magnesium increased tissue magnesium (12.6±1.2nmol/mg vs 2.4±.8nmol/mg, p<0.05) and prevented calcification when transferred to pro-calcification media without high magnesium (+4 days) (tissue calcium: 10.5±1.3nmol/mg vs 175.5±28.9nmol/mg in control). Thus, increasing dietary magnesium \textit{in vivo} and local concentration of magnesium \textit{in vitro} both effectively attenuate vascular calcification. Low magnesium status is an under-recognized and potentially modifiable risk factor in CKD.

4.2 Introduction

Traditional risk factors do not adequately explain the high risk for cardiovascular disease in patients with chronic kidney disease (CKD). These patients are also at risk of non-traditional risk factors, such as accelerated vascular calcification (VC). Calcification, particularly in the media of blood vessels, is associated with mineral imbalance in this population and leads to increased cardiovascular disease and death\textsuperscript{4,166}.

Experimental data have now confirmed that VC is an active process that involves the transdifferentiation of vascular smooth muscle cells (VSMC) to osteoblast-like cells\textsuperscript{167}. These osteoblast-like cells mineralize in a process analogous to bone formation. Chronic kidney disease-mineral bone disorders (CKD-MBD), which are characterized by abnormalities in phosphate and calcium homeostasis, have been consistently linked with VC in this population\textsuperscript{147,148,168}. However, there is a growing body of epidemiological evidence to suggest that low serum magnesium levels may be associated with the pathophysiology of VC in patients with end stage kidney disease (ESKD)\textsuperscript{91}. Furthermore, low serum magnesium levels also predict cardiovascular-associated and all-cause
mortality in non-uremic populations\textsuperscript{89,90}. Despite this association, few studies have focused on magnesium and its role in the development of VC.

Magnesium plays an important role in the regulation of vascular tone, heart rhythm and skeletal and mineral metabolism. Although the role of magnesium in the calcification process is unclear, the association between hypomagnesemia and calcification\textsuperscript{91} suggests that supplementing magnesium might decrease the risk of calcification. It is well known that ionic magnesium can directly inhibit precipitation of calcium-phosphate as apatite at physiological pH\textsuperscript{126,127}. On the other hand, \textit{in vitro} studies using VSMC lines have shown that higher concentrations of magnesium can prevent the transdifferentiation of smooth muscle cells into osteoblast-like cells, as well as upregulating the synthesis of inhibitory proteins involved in the prevention of calcification\textsuperscript{37,38}. However, whether magnesium treatment prevents calcification in experimental CKD is unknown.

Therefore, the following experiments were designed to determine whether magnesium treatment prevents soft tissue calcification \textit{ex vivo} and \textit{in vivo}. We incubated thoracic aortas in high phosphate media and performed dose-response and time-dependent experiments with magnesium. For the \textit{in vivo} studies, the effect of magnesium treatment on the development of vascular and soft tissue calcification was assessed in adenine-induced CKD\textsuperscript{152} at a dose designed to minimize the adverse effects of hypermagnesemia\textsuperscript{169–172}.
4.3 Methods

4.3.1 Experimental animals

Male Sprague-Dawley rats (Charles River, St. Constant, Quebec) weighing 375-400g at the start of the experiment were individually housed and maintained on a 12 h light-dark cycle (lights on at 7:00). Animals were allowed to acclimatize for at least 1 week prior to experimentation, and were provided with Purina Rat Chow (Test Diet) and water ad libitum. All procedures were in accordance with the guidelines of the Canadian Council on Animal Care, handling and termination.

4.3.2 Ex vivo vascular calcification

The ex vivo culture protocol adapted from Lomashvili et al.,\textsuperscript{173} involves incubating aortic rings in serum free Dulbecco’s Modified Eagle’s Media (DMEM) (at 37°C and 5% CO₂) for 4-6 days. In brief, rats were perfused with saline via the left ventricle, after which thoracic aortas were carefully isolated. The adipose tissue and the adventitia were removed carefully under a dissecting microscope and the aortas were cut into 2-4mm segments. Aortic segments were incubated in DMEM containing 3.0mM or 3.8mM phosphate with either normal (0.8mM) or elevated magnesium (1.2-2.5mM). The media change occurred every two days, and at the end of the incubation period the aortic segments were collected for calcium and phosphate analysis.
4.3.3 Experimental CKD in rats

CKD was generated using the adenine model as previously described\textsuperscript{152}. Sixteen rats were maintained on a nutritionally balanced adenine diet (0.25% adenine, 1% phosphate, 1% calcium, 0.2 ppm vitamin K, 1 IU/g vitamin D, and 6% protein (Harlan, Teklad, Madison, WI)) for 5 weeks. Although previously we showed that at 5 weeks of CKD not all of the animals develop calcification\textsuperscript{152}, this time period was chosen to determine the impact of magnesium on both calcified and non-calcified CKD blood vessels, but prior to the generation of severe calcification. This adenine diet contained either normal (0.05%, \(n=8\)) or high (0.2%, \(n=8\)) magnesium. Body weights, food intake and general animal health (i.e. checking for diarrhea and dehydration) were monitored on a daily basis. At the end of the experiment, rats were fasted for 12hrs before hemodynamic measurements (see below) and euthanization, at which point blood serum, heart, kidneys and vessels (thoracic aorta, abdominal aorta, renal artery and pudendal artery) were collected and snap frozen with liquid nitrogen and stored in -80°C for further analysis.

4.3.4 Hemodynamic measurements

Animals were anesthetized with ketamine (30 mg/kg, Rogar/STB, London, Ontario, Canada) and thiobarbital sodium (100 mg/kg, Sigma-Aldrich). Body temperature was maintained at 37±0.5°C using a thermistor, temperature controller and heating pad (Yellow Springs Instruments, Yellow Springs, OH). A heparinized saline–filled (50 IU/ml) catheter (PE-50) was inserted in the carotid artery to measure blood pressure as a pulsatile wave form at a frequency of 1000Hz. A second catheter was inserted in the femoral artery and advanced to the abdominal aorta to measure regional hemodynamic
changes in the abdominal aorta. Blood pressure profiles of all animals were determined using a pressure transducer (CDX3, Cobe) connected to a PowerLab 8/30 (ADInstruments) data acquisition system and calculated using Chart version 5 software (ADInstruments).

4.3.5 Blood biochemistry

Serum creatinine was measured using a QuantiChrom creatinine assay kit (BioAssay Systems, Hayward, CA, USA). Serum fibroblast growth factor 23 (FGF-23) levels, the endogenous phosphaturic factor, were measured using an FGF-23 enzyme-linked immunosorbent assay kit (Kainos Laboratories, Inc., Tokyo, Japan). Serum magnesium was determined using a colometric assay according the manufacturer’s instructions (Point Scientific, Inc, Canton, MI, USA). Serum phosphate was determined as described below.

4.3.6 Calcium and phosphate content

Previously, we have shown that the distribution of calcification in this model changes from distal to proximal\textsuperscript{152}, and so calcification of numerous arteries (thoracic aorta, abdominal aorta, renal artery and pudendal artery) were determined. This process allowed us to characterize the overall impact of magnesium in different vascular beds. Frozen tissues were thawed, weighed and homogenized in 1 N hydrochloric acid for 24 h at 4°C. Samples were spun and calcium content was determined colorimetrically using the O-cresolphthalein complexone method. O-cresolphthalein colour reagent forms a purple complex with the calcium in the samples. The absorbance for this complex was measured for both standards and tissue homogenates at 540nm (SynergyHT Microplate Reader, Bio-Tek Instruments Inc, Winooski, VT, USA). Phosphate content was
determined using the malachite green method. The malachite green reagent was prepared as previously described\textsuperscript{137}. Upon addition of ammonium molybdate, a green complex is formed between malachite green, molybdate and free phosphate. The absorbance for this complex was measured for both standards and samples at 650nm.

### 4.3.7 Von Kossa method for visualizing vascular calcification

Five-mm sections of thoracic aorta were washed in phosphate-buffered saline and then embedded in Cryomatrix (Thermo Scientific, Rochester, NY, USA) in the upright position and frozen with liquid nitrogen. Sections (3-4\textmu m) were stained for calcification using the von Kossa method as previously described\textsuperscript{152}. Briefly, sections were treated with 1\% silver nitrate and exposed to ultraviolet light for 20 min. Then, sections were placed in 5\% sodium thiosulfate for 2 min, and counterstained with nuclear Fast Red for 5 min. Areas of calcification appeared as dark brown regions in the medial wall of the aorta.

### 4.3.8 Statistical analysis

All data from \textit{ex vivo} calcification protocol or CKD rats were presented as means ± SD. Differences between groups were analyzed using a one-way analysis of variance (ANOVA) followed by a Newman-Keuls or Bonferroni post hoc test. The classification of a tissue as being severely calcified was based on tissue calcium of above 100nmol/mg, as at this level von Kossa stainable crystal formation was clearly evident in all cases. A chi-squared test was performed to determine the distribution of severe calcification in CKD animals. To characterize the overall impact of magnesium on vascular calcification, different vascular beds (thoracic aorta, abdominal aorta, renal artery and pudendal artery) were pooled for each animal. A p-value less than 0.05 was considered statistically
significant. Statistical analyses were performed on GraphPad Prism version 5 (GraphPad Software, Inc).
4.4 Results

4.4.1 In vitro aortic calcification is inhibited by magnesium

Aortic segments incubated in DMEM (0.8mM Mg++, 1.6mM Ca++) for four days calcified when exposed to phosphate concentration of 3.0mM or higher (Figure 4.1). Although higher concentrations (i.e. >3.8mM) of phosphate were assessed in preliminary studies, the maximum phosphate used in routine analysis was 3.8mM since this concentration provided a robust level of calcification. Von Kossa staining of the segments incubated in 3.8mM phosphate confirmed the mineralization, as there was punctate calcification of the tunica media (Figure 4.2). Increasing the magnesium (from 0.8mM to 1.2-2.5mM) concentration-dependently inhibited the high phosphate-induced (3.0mM) calcification (Figure 4.3A), achieving full inhibition at the highest concentration used (2.5mM). Tissue magnesium was similarly elevated in the aortic segments when incubated in higher concentrations of magnesium (≥1.2mM, Figure 4.3B).

4.4.2 Delaying in vitro magnesium treatment modifies calcification

Incubation of aortic segments in the highest phosphate media (3.8mM) for six days resulted in markedly elevated tissue calcification (Figure 4.4A). To determine whether magnesium could inhibit the progression of calcification, the highest concentration of magnesium (2.5mM) was added on four different days (0, 2, 3 or 4) and the changes in tissue minerals were assessed on day 6 (Figure 4.4A). Magnesium treatment blunted calcification and had the greatest impact when added earlier. For instance, when magnesium was added at day 4 of 6, tissue calcium was not different from the control (no Mg++ added) group. In contrast, tissue magnesium was highest in the day
Figure 4.1 Effect of phosphate on aortic segments incubated in DMEM media for four days.
DMEM contains 0.9mM phosphate, but elevating the phosphate to ≥3.0mM resulted in elevation of tissue calcium. Data presented as means ±SD. *p<0.05 versus all other groups
Figure 4.2 Effect of phosphate on *in vitro* calcification of aortic rings as shown by von Kossa staining. Thoracic aorta incubated for 4 days in 3.8mM phosphate DMEM with medial calcification (A and B).
Figure 4.3 Magnesium inhibits *in vitro* calcification.
Aortic rings incubated in 3.0mM phosphate for 4 days had elevated tissue calcium, however increasing the magnesium concentration in the media from 0.8 to 2.5mM reduced calcification (A). Elevated magnesium concentration in the media resulted in elevated tissue magnesium at day 4 of incubation (B). Data presented as means ±SD. *P*<0.01 versus 0.8mM magnesium media; "P"<0.01 versus 1.2mM magnesium media.
**Figure 4.4** Progression of calcification is altered by delayed magnesium treatment. Magnesium (Mg$$^{++}$$; 2.5mM) was added at day 0, 2, 3, and 4 of a 6 day incubation period in 3.8mM phosphate. Tissue calcium (Ca$$^{++}$$) in aortic rings was lower if media Mg$$^{++}$$ was elevated early (i.e., day 0, day 2, and day 3) (A). Tissue Mg was elevated in all calcified tissues (B). Similar experiments were carried out in 3.0mM phosphate media to see the effect of magnesium on amorphous calcium-phosphate accumulation (C & D). Tissue Mg in the late Mg treatment (D; day 4 of 6) is significantly increased despite not impacting calcium levels. *p<0.05 vs No Mg group. Ψ p<0.05 vs Day 4 group.
4 group, most likely reflecting magnesium incorporated into the crystal (Figure 4.4B). In fact, tissue magnesium levels of calcified tissues were higher than the non-calcified tissue (i.e. all other groups vs. day 0 of 6; p<0.05) (Figure 4.4B). In another experiment, a lower level of phosphate (3.0mM) was used. This concentration caused only a moderate elevation of tissue calcium (47.9±14.9 vs 175.5±28.9 nmol/mg in 3.8mM phosphate, p<0.001) and likely indicated an instance of calcium-phosphate interaction that occurred prior to crystal formation. However, addition of magnesium to the media at day 2 significantly decreased tissue calcium build-up in this condition as well (Figure 4.4C). Tissue magnesium was similar in all of the groups except that it was elevated when magnesium was added late, at day 4 (Figure 4.4D).

In the case of all of the calcified tissues exposed to high media phosphate (3.8mM), except for the group which had no calcification (i.e. magnesium treatment at day 0), there was a positive correlation between tissue calcium and phosphate. This stoichiometry reflected, at least in part, the various stages and types of crystal formation. The slope of the calcium vs. phosphate graphs was greatest in the calcified groups (i.e. No Mg++ added r^2=0.90, slope=1.69±0.001; Mg++ added Day 2 r^2=0.88, slope=1.1±0.003; Mg++ added Day 3 r^2=0.92, slope=1.5±0.03; Mg++ added Day 4 r^2=0.7, slope=1.2±0.02). Similarly, tissue calcium vs. magnesium did not correlate in groups without calcification (Mg++ added day 0 and Mg++ added day 2), but the No Mg++ added group accumulated magnesium and had a positive calcium vs. magnesium correlation (r^2=0.96, slope = 19.2±1.2). If Mg was added late, magnesium still accumulated in the tissue alongside calcium, and the calcium-magnesium relationship was linearly correlated (Mg Day 3, r^2=0.66, slope=11.8±3.0 and Mg day 4 r^2=0.99, slope=7.4±0.3).
4.4.3 Pre-treatment with magnesium in vitro prevents calcification

Pre-treatment for the first 2 days with 2.5mM magnesium (back to 0.8mM magnesium for days 3 to 6) persistently inhibited VC until day 6 for both phosphate levels (3.0 and 3.8mM)(Figure 4.5A and B). This magnesium pre-treatment for two days resulted in elevated tissue magnesium at day 6 (Figure 4.5D). Tissues incubated in 3.8mM phosphate also had elevated tissue magnesium as compared to 3.0mM phosphate, but this was due, at least in part, to increased mineralization in these tissues (Figure 4.5C).

4.4.4 Dietary magnesium modifies soft tissue calcification in CKD rats

Animals given dietary adenine (0.25%) to induce CKD combined with normal magnesium (0.05%) for five weeks lost 3% of their body weight (408.7±24.8g down to 395.5±22.4g; p<0.05). In contrast, animals with adenine-induced CKD given high magnesium (0.2%) did not lose body weight (429.6±13.1g to 445.2±22.6g; p>0.05) and did not develop diarrhea. Serum creatinine (>7 fold), serum phosphate (>2 fold) and serum FGF-23 (>500 fold) were significantly elevated in all adenine animals (Table 4.1). There was no difference in the degree of CKD, serum phosphate or serum FGF-23 in the normal and high magnesium diet groups. Dietary magnesium resulted in significantly elevated serum magnesium (Table 4.1).

Although the frequency of thoracic calcification was similar to the study in Chapter 2 (2 out of 8 in CKD, and 1 out of 8 in CKD with magnesium), the various vessels were differentially responsive to magnesium supplementation. In 41% and 28% of the vessels in CKD rats and CKD plus magnesium treated rats, respectively, tissue calcium was elevated. However, in the CKD group not supplemented with magnesium,
vessel calcium was significantly higher than in the CKD group treated with magnesium (Figure 4.6A). That is, out of all the calcified vessels, supplemental magnesium treatment attenuated progression of calcification (38% had severe calcification in CKD vs. 6% in magnesium treated CKD, p<0.01). In addition, although both groups had similar renal failure (Table 4.1 Serum biochemistry of rats fed 0.25% adenine for 5 wks ± normal (0.05%) or high dietary Mg++ (0.2%), the kidney tissue of the CKD group had significantly elevated tissue calcium levels compared to the magnesium treated group (Figure 4.6B). In contrast, the tissue calcium levels of the hearts (left ventricle wall) were relatively low and were not different between the groups (5.0±2.1 vs 5.4±1.7 nmol/mg).

Renal failure did not significantly affect the blood pressure profiles of the animals. All groups had similar mean arterial pressure, diastolic pressure, systolic pressure and pulse pressure (Table 4.2). Heart rate was also similar among all groups. However the distal to proximal index of hemodynamic change, the ratio of carotid:femoral dP/dt_max, was significantly higher in the CKD group (Table 4.2).
Figure 4.5 Pre-treatment (Pre-Tx) of the aortic rings in vitro with magnesium inhibits calcification.
Aortic rings were incubated in high phosphate (3.0 or 3.8mM P DMEM) for 6 days. Magnesium treatment for the first 2 days (Mg Pre-Tx) resulted in inhibition of calcification in both P concentrations (A; B). Magnesium pre-treated rings in 3.0mM P had sustained elevated tissue magnesium at day 6 (D). In contrast tissue magnesium in both 3.8mM phosphate was elevated (C). Data presented as means ±SD. *P<0.001 versus HP from the same group.
Table 4.1 Serum biochemistry of rats fed 0.25% adenine for 5 wks ± normal (0.05%) or high dietary Mg$^{++}$ (0.2%)

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<th>Control</th>
<th>CKD</th>
<th>CKD Mg$^{++}$</th>
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<tr>
<td><strong>Serum Creatinine (μM)</strong></td>
<td>50.0±4.9</td>
<td>394.2±80.7*</td>
<td>389.5±57.0*</td>
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<tr>
<td><strong>Serum Phosphate (mM)</strong></td>
<td>2.4±0.2</td>
<td>5.03±1.1*</td>
<td>5.12±0.6*</td>
</tr>
<tr>
<td><strong>Serum FGF-23 (pg/ml)</strong></td>
<td>0.4±0.05</td>
<td>289.2±226.2*</td>
<td>218.9±145.5*</td>
</tr>
<tr>
<td><strong>Serum Magnesium (mM)</strong></td>
<td>0.5±0.1</td>
<td>0.5±0.3</td>
<td>0.8±0.2$^\dagger$</td>
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*p<0.01 versus control group
$p<0.05$ versus CKD group
CKD, Chronic kidney disease; Mg$^{++}$, high magnesium (0.2%) diet; FGF, Fibroblast growth factor
Figure 4.6 Dietary magnesium attenuates soft tissue calcification. CKD rats on dietary (0.2%) magnesium (CKD-Mg++) had lower vessel calcium levels (A) and kidney calcium levels (B) than untreated CKD rats (CKD). *p<0.05 vs CKD; dotted line represents control calcium levels.
Table 4.2 Indwelling catheter measurements of blood pressure and heart rates of rats fed 0.25% adenine for 5 weeks with normal (0.05%) or high magnesium (0.2%) diets

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<tbody>
<tr>
<td>Mean arterial pressure</td>
<td>109.8±14.8</td>
<td>109.2±14.7</td>
<td>109.7±16.9</td>
</tr>
<tr>
<td>(mmHg)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Systolic blood pressure</td>
<td>127.4±16.1</td>
<td>121.2±18.5</td>
<td>128.0±18.8</td>
</tr>
<tr>
<td>(mmHg)</td>
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<tr>
<td>Diastolic blood pressure</td>
<td>94.0±13.3</td>
<td>96.3±12.6</td>
<td>93.4±15.0</td>
</tr>
<tr>
<td>(mmHg)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pulse pressure</td>
<td>33.5±9.3</td>
<td>30.2±10.3</td>
<td>34.6±4.8</td>
</tr>
<tr>
<td>(mmHg)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heart rate</td>
<td>328.8±48.5</td>
<td>302.8±40.1</td>
<td>289.8±25.8</td>
</tr>
<tr>
<td>(bpm)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Carotid:Femoral</td>
<td>1.65±0.23</td>
<td>2.50±0.90*</td>
<td>1.54±0.39</td>
</tr>
<tr>
<td>dP/dt_{max}</td>
<td></td>
<td></td>
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</tbody>
</table>

*p<0.05 vs CKD Mg++

CKD, Chronic kidney disease; Mg++, high magnesium (0.2%) diet
4.5 Discussion

This study has both *in vivo* and *in vitro* components and provides evidence that treatment with magnesium attenuates soft tissue calcification. The *in vivo* rodent model used dietary adenine to induce stable CKD, whereas *in vitro* the uremic environment was modeled using progressive increases in phosphate concentration\(^\text{173}\). Calcification in the *in vivo* CKD model was observed within five weeks, whereas aortas incubated in high phosphate developed a similar pattern of medial calcification in four days. In CKD rats, a four-fold increase in dietary magnesium over five weeks resulted in only a ~20% elevation in serum magnesium. However, this elevation was associated with significantly reduced calcification without impacting hyperphosphatemia. Although magnesium treatment attenuated calcification, it did not alter arterial pressure and heart rate, indicating potential benefits to both heart function and vascular compliance\(^\text{169,171,174}\). Alternatively, the addition of magnesium *in vitro* showed that prevention of aortic calcification with magnesium occurs in a concentration dependent manner, but that increasing the concentration of magnesium could not reverse calcification once it had developed. Furthermore, in order to prevent the initiation and proliferation of hydroxyapatite crystals, additional magnesium needed to be added early in the protocol. Taken together, these *in vitro* and *in vivo* findings suggest magnesium is beneficial in preventing calcification and the ensuing cardiovascular complications, but appears to be less efficacious in reversing or hindering VC once it has developed.

*In vitro*, the inhibitory actions of magnesium on calcification were concentration, timing and stage of calcification dependent. To inhibit calcification, magnesium had to be present at least by day 3 of a 6 day experiment, since addition at day 4 did not prevent
calcification. This timing likely reflects the influence of the dissolved magnesium on the formation of calcium and phosphate nanocrystals in order to prevent nucleation and further crystallization\textsuperscript{127,175}. Formation of nanocrystals has been proposed to be a signaling mechanism of vascular smooth muscle differentiation and calcification\textsuperscript{176,177}; however, several studies have shown that magnesium stabilizes calcium-phosphate in solution, thereby preventing crystal formation and deposition\textsuperscript{126,127}. The expected ratio of calcium to phosphate in hydroxyapatite is 10 to 6 (1.66), which is similar to the composition found in the \textit{in vitro} calcified aortas (Ca:PO\textsubscript{4} = 1.69), but markedly different in the magnesium treated aortas (Ca:PO\textsubscript{4}<1.5). This data suggests that hydroxyapatite was the predominant crystal being formed when magnesium was not altered. However, in the presence of magnesium, not only was the total mineralization lessened, but the altered ratio of minerals suggested precursors of hydroxyapatite, such as amorphous calcium-phosphate or octacalcium-phosphate(Ca:P\textsubscript{0}4 ratios 1.2-1.5), were likely forming\textsuperscript{176,178}.

The minimal impact of late magnesium treatment on calcification could result from the rapid sequestration of magnesium in the developing crystal (i.e. it does not remain in solution at sufficient inhibitory concentrations). This agrees with Boskey \textit{et al.}\textsuperscript{127} who showed that once a complex containing calcium, phospholipids and phosphate is formed \textit{in vitro}, magnesium is not as effective at preventing proliferation. The present \textit{in vitro} findings confirm that magnesium, in a concentration dependent manner, can prevent the formation of hydroxyapatite in aortic tissue, but does not reverse this process.

Magnesium likely inhibits calcification by directly altering the interaction between soluble calcium and phosphate, in addition to its cellular inhibitory and stimulatory mechanisms\textsuperscript{37,38}. The two day pre-treatment with high magnesium in the
media was sufficient to prevent calcification for at least a six day period, an effect which was associated with sustained increases in tissue magnesium. This finding indicates that transient magnesium treatment can induce prolonged decreases in tissue susceptibility to calcification, potentially by promoting increased bioavailable intracellular and extracellular concentrations. In addition, VC is widely acknowledged to be an active process involving, at least in part, osteogenic transdifferentiation of VSMC \(^{167}\). High magnesium treatments of both bovine and rat VSMCs exposed \textit{in vitro} to a pro-calcification environment showed that osteogenic changes were attenuated \(^{37,38}\). The mechanism for this inhibition has been suggested to be mediated by the magnesium transporter, TRPM7 (member of the melastatin-related transient receptor potential (TRPM) ion channel) \(^{37}\). This ion channel is permeable to divalent ions (including calcium), but has a high affinity for magnesium \(^{179,180}\). TRPM7 is different from other ion channels in that it contains a kinase domain. Recent evidence suggests that the kinase domain of TRPM7 is regulated by cytoplasmic magnesium and phosphorylates itself, as well as calcium channels such as annexin \(^{180–182}\). Therefore, the sustained elevated magnesium might have also had an impact on the phosphorylation of these channels, preventing calcium transport and protecting the aortas from calcification. Taken together, the inhibitory actions of elevated tissue magnesium on calcification likely involve modulation of multiple processes. Our data suggest that in vascular tissue, there is likely both a direct effect of magnesium on calcium-phosphate crystal formation, as well as an effect on the regulation of cation transport, in general.

\textit{In vivo}, dietary magnesium had an effect on the frequency and degree of calcification. The inhibitory effect of magnesium was found to be independent of serum
levels of calcium and phosphate. Both CKD groups developed positive phosphate balance, as indicated by elevated serum phosphate and FGF-23 levels. That is, the dose of magnesium in the current animal study did not significantly effect phosphate homeostasis, despite the fact that clinical data suggest magnesium is an effective phosphate binder. FGF-23 levels rise in early stages of CKD, which is believed to be a homeostatic response to positive phosphate balance. Lowering serum phosphate with phosphate binders has been shown to decrease FGF-23 levels as well as vascular calcification. However, in the present study, magnesium treatment reduced soft tissue mineralization, but did not impact circulating levels of either calcium or phosphate. Despite this lack of change on circulating levels, the frequency and degree of calcification was lower, suggesting that magnesium most likely prevents initiation and growth of hydroxyapatite by modifying the microenvironment of soft tissues, a concept confirmed in the in vitro experiments.

The degree of inhibition of VC by magnesium in vivo is most likely dose dependent. However, hypermagnesemia in rats has been associated with numerous health issues. Dietary magnesium of 0.5% in rats has been shown to inhibit bone formation, and supraphysiological doses of magnesium given to vitamin-D treated rats actually increased soft tissue calcification. Furthermore, severe hypermagnesemia (i.e. serum levels > 3mM) could result in cardiovascular complications such as hypotension and bradycardia. Although a higher dose of magnesium might have completely inhibited calcification in the CKD rats, it was important to choose a moderate dose that would not have adverse effects, and would be more translational.
The magnesium treated CKD group did not develop altered blood pressure or increased heart rate. In fact, the beneficial anti-calcification profile of magnesium might have also had a beneficial effect on heart function. Numerous studies have shown that the dp/dt of pressure waves has a high correlation to ejection fraction and could potentially be used as a non-invasive method of determining heart function \cite{107,108,189}. As previously reported, in this adenine model, CKD rats have progressive changes in cardiovascular function starting at seven weeks that are associated with vascular calcification \cite{151,152,190}. In this study, CKD rats had increased calcification and higher distal to proximal index of hemodynamic change, carotid:femoral dP/dt_{max} ratio (See Chapter 3), suggesting an imminent cardiovascular complication. However, magnesium treatment mitigated these cardiovascular risks, and the dP/dt_{max} ratio was not different from control animals.

Taken together, the \textit{in vivo} and \textit{in vitro} findings suggest that appropriately timed magnesium treatment, at certain doses, can have beneficial effects in modifying some, but not all aspects, of the abnormalities associated with CKD. Specifically, the positive profile of magnesium action appeared to be soft tissue specific, as it did not impact homeostasis of circulating minerals. That is, magnesium supplementation attenuated initiation and progression of blood vessel calcification \textit{in vitro} and \textit{in vivo}, and positively impacted hemodynamic consequences \textit{in vivo}. Further knowledge of local tissue regulation of magnesium could potentially reveal one of the pathogenic mechanisms involved in cardiovascular disease progression in CKD patients. Regardless, the present findings suggest that moderate magnesium supplementation, strategically administered, might be able to attenuate extra-osseous mineralization in this population, an effect which should benefit their negative, highly progressive risk profile.
Chapter 5

Association of Dietary Phosphate Intake and Phosphate to Protein Ratio

with Progression of Coronary Artery Calcification in Patients with

Stage 3 to 5 Chronic Kidney Disease
5.1 Abstract

Serum phosphate is linked to increased vascular calcification and mortality in patients with end stage kidney disease (ESKD). However, at earlier stages of CKD, serum phosphate is not elevated, despite marked changes in levels of the counter-regulatory hormones that are known to be involved in phosphate homeostasis and the total burden of phosphate on calcification progression is unknown. The usual dietary phosphate intake might, therefore, represent a better indicator of risk of calcification progression associated with positive phosphate balance. Coronary artery calcification (CAC) was measured by multislice computed tomography scan in 86 patients with CKD stages 3-5 (excluding dialysis). Calcification progression was defined as >15% increase in the Agatston score in four years. A food frequency questionnaire was used to assess ‘usual’ phosphate intake and phosphate intake to protein intake ratio. Dietary phosphate intake, but not phosphate:protein ratio, was strongly correlated with total energy (r=0.8, p<0.001) and protein (r=0.9, p<0.001). CAC progression was observed in 59 patients. Multivariable logistic regression models revealed that total dietary phosphate was associated with CAC progression after incremental adjustment for case-mix, laboratory and dietary variables, phosphate binder use and baseline CAC (OR: 1.5; CI[1.0-2.2]; p<0.05). In contrast, phosphate:protein ratio was associated with increased risk of CAC progression in the unadjusted analysis, as well as in the incremental models (OR:1.4 CI[1.0-2.0]; p<0.05). Increased dietary phosphate and dietary phosphate to protein ratio are both associated with risk of calcification progression in stage 3-5 CKD patients even after adjusting for phosphate binder use and total protein and energy intake.
Interventions targeting dietary phosphate may be important at early stages of CKD and before serum levels of phosphate become abnormal.

5.2 Introduction

High levels of serum phosphate have been consistently linked to vascular calcification progression and mortality in patients with end stage kidney disease (ESKD)\textsuperscript{19,148,191,192}. However, the relationship between levels of phosphate and outcomes is less clear in earlier stage CKD patients where serum phosphate levels are often maintained in the normal range by a variety of compensatory mechanisms\textsuperscript{73}. At earlier stages of CKD, serum phosphate levels may not reflect the extent of phosphate dysregulation and ‘normal’ may not be ‘optimal’\textsuperscript{73}. Indeed, there is a growing body of evidence from the general population linking higher serum phosphate levels well within the ‘normal’ range with substantially increased risks of cardiovascular disease\textsuperscript{22,193}.

In addition to levels of counter-regulatory hormones, an assessment of the usual dietary phosphate intake might also reflect the overall phosphate burden at earlier stages of CKD. The bioavailability of phosphate as a consequence of preservatives and animal protein content is very high in a typical Western diet\textsuperscript{194,195}. However, there are marked differences in the absorption of dietary phosphate depending on the protein source\textsuperscript{196}. That is, phosphate derived from animal-based sources are easily hydrolyzed in the gastrointestinal tract and absorbed into the circulation. In contrast, plant-based phosphate has lower bioavailability based on the absence of the degrading enzyme in humans. Following the administration of equivalent amounts of phosphate, urinary excretion appears to be higher with meat-based foods in healthy humans\textsuperscript{197}. 
Higher dietary phosphate intake is independently associated with mortality in ESKD patients. However, studies also indicate that a low dietary intake of phosphate if accompanied by low protein and macronutrient intake is also linked with mortality. Therefore an index of 'phosphate to protein ratio' might better predict important clinical outcomes. Indeed, Noori et al examined the relationship between the 'phosphate to protein ratio' and mortality in ESKD patients and found that this ratio was independently associated with mortality even after adjustment for serum phosphate, phosphate binders, dietary energy, protein and potassium intake. The current Kidney Disease Outcomes Quality Initiative (K-DOQI) guidelines recommend up to 1000 mg/day dietary phosphate for patients with CKD. However, given that the bioavailability of phosphate differs based on the protein source, a measure of the total phosphate intake may not be the most important benchmark.

The severity and progression of vascular calcification can be determined by computed tomography where the coronary artery calcium (CAC) score measures the calcium burden in the three major coronary arteries. Elevated CAC scores are observed in over 80% of prevalent hemodialysis patients and higher CAC scores are consistently associated with mortality in this patient group. Furthermore, progression of CAC over time is predictive of clinically important outcomes in the general population and in patients with earlier stage CKD.

In this regard, the objective of this study was to assess the usual intake of phosphate and the phosphate to protein ratio in 89 patients with stage 3-5 CKD and to examine the progression of coronary artery calcification over four years. We hypothesized that dietary phosphate, and in particular the intake of foods with higher
phosphate to protein ratio, would be independently associated with the progression of coronary artery calcification.

5.3 Methods

5.3.1 Participants

Patients presenting for follow-up to the Kingston General Hospital Chronic Kidney Disease clinic (Kingston, Ontario, Canada) between July, 2005 and December, 2006 were initially invited to participate in this study. Eligibility criteria included: age > 18, stages 3-5 CKD (not requiring renal replacement therapy), the absence of the use of a bisphosphonate and the absence of any lifetime exposure to warfarin. All patients provided informed consent according to the Declaration of Helsinki. All procedures were in accordance with the ethical standards of Queen’s University. Seven hundred and ninety-one patients were screened for enrollment. Patients were not enrolled for the following reasons: unwilling to participate (n=217), warfarin or bisphosphonate exposure (n=104), not stage 3-5 CKD (n=260). Two hundred and ten patients consented for enrollment and 185 patients were enrolled in the study. All available subjects were then re-approached in 2009 and 89 subjects consented to a repeat CAC score. Reasons for not repeating the CAC score in 2009: deceased (n=27), no longer attending the CKD clinic for follow-up (n=22), now requiring renal replacement therapy (n=29), unwillingness to participate (n=17) and unknown (n=1). Clinical data were abstracted by patient interview and chart review. Weight and height were collected from each individual to calculate body mass index (BMI). Current smokers were defined as patients smoking at least one cigarette/day during the previous six months.
5.3.2 Assessment of nutritional status

A subjective global assessment of nutritional status was performed by a Registered Dietician. The overall and individual elements of the subjective global assessment (SGA) classification incorporated the examiner’s clinical judgment and were scored out of 7: 6-7, normal (well-nourished); 3-5, mild-to-moderate malnutrition; and 1-2, severely malnourished. The SGA involves a detailed history of weight loss over the past six months and two weeks, nutritional intake and gastrointestinal symptoms. Weight loss over the last six months of > 10% is severe, 5-10% is mild-moderate and <5% is considered normal. Weight stability over the past two weeks is rated as ‘normal’ if weight is stable and ‘severe’ if weight is increasing or decreasing. Nutritional intake is considered ‘usual’ or ‘not usual and decreasing’. Gastrointestinal symptoms consider those that have been persistent for more than two weeks and include none, anorexia, nausea, vomiting and diarrhea. The physical examination includes an assessment of loss of subcutaneous fat (thickness of skinfold over the tricep and bicep) and muscle loss (temporalis muscle, prominence of clavicle, visibility of scapula and ribs, protrusion of interosseous muscle between thumb and forefinger and amount of quadriceps and calf muscle mass).
5.3.3 Laboratory measures

Laboratory measures included ionized calcium (mmol/L), phosphate (mmol/L), intact parathyroid hormone (iPTH) (pmol/L), alkaline phosphatase (mmol/L), serum albumin (g/L) and 25-hydroxyvitamin D (25(OH)D; nM/L). iPTH was assessed by electrochemiluminescence (Roche, Basil, Switzerland) modular analytics E170 immunoassay. A Roche modular bromoscesol green method was used to measure serum albumin. Serum creatinine (Creatinine Plus Modular assay; Roche Basil, Switzerland) on the day of study enrollment was used in the 4-variable Modification of Diet in Renal Disease (MDRD) Study equation to determine estimated glomerular filtration rate (eGFR)\textsuperscript{210}. All of these aforementioned analyses were performed in the laboratory at Kingston General Hospital to minimize inter-laboratory variability. Plasma 25(OH)D was measured by radioimmunoassay (DiaSorin, MDS Laboratory Services, Toronto, ON, Canada). In a sub-set of patients with stored biological material sufficient for analysis, Fibroblast Growth Factoer 23 (FGF-23) was measured by the c-terminal Enzyme-Linked ImmunoSorbent Assay (Immutopics, Inc., San Clemente, CA, USA).

5.3.4 Assessment of dietary intake

Measurements were derived from a comprehensive food frequency questionnaire (FFQ) designed to capture an individual’s ‘usual’ dietary intake. At baseline, the usual dietary intake for the previous year was assessed using a semi-quantitative 126-item FFQ\textsuperscript{211,212}. The questionnaires were filled out at the time of the clinic appointment with the help of a Registered Dietician. This FFQ consists of a list of foods with a standard serving size and a selection of nine frequency categories ranging from never or < 1 serving/month to > 6 servings/day. Subjects were asked to report the estimated frequency
of consumption over the past 6-12 months. The nutrient intake is determined by multiplying food consumption by the nutrient content of the specified standard portion.

Dietary information was judged as unreliable and therefore not used in the analysis if reported energy intake was < 600 kcal/day or > 4000 kcal/day for women and > 4200 kcal/day for men and if ≥ 12 food items were left blank.

5.3.5 Evaluation of coronary artery calcification

In 2005, CAC scores were evaluated using The Toshiba Aquillion computed tomography (CT) multislice scanner (four sets of detectors) and VScore analytical software package. The scan thickness is 3 mm x 4 slices simultaneously over 12 mm per rotation, and the field of coverage is 12 cm. Images were acquired with prospective gating technique using a discrete algorithm213.

In 2009, the CAC scores were evaluated using a General Electric VCT multislice CT (64 slice) helical scanner and using Smartscore software (version 3.5, GE Medical Systems). The slice thickness is 2.5 mm x 8 slices acquired over 2 cm per revolution. Total CAC score was generated as per the Agatston method214.

5.3.6 Statistical analysis

All statistical tests (PASW Statistics version 18.0 (IBM Corporation, Somers, NY)) were 2-sided and unadjusted P-values of 0.05 or less were considered significant. Chi-square tests (Pearson’s or Fisher’s Exact as appropriate) and independent samples t-tests (or Mann-Whitney U in the event of non-normal distributions) were used as appropriate. Multiple variable logistic regression modeling was performed to determine risk factors associated with CAC progression.
5.4 Results

The baseline demographic, clinical and laboratory variables in 89 patients with stage 3-5 CKD are shown in Table 5.1. The mean age of the patients was 60.8±14 and 58\%(n=52) of patients were male.

Table 5.2 shows the correlation coefficients of relevant measures with total dietary phosphate intake and phosphate to protein ratio. Total dietary phosphate intake was very strongly correlated with total energy, protein, potassium and calcium intake, but not with serum phosphate levels or FGF-23. There were also strong correlations between total phosphate intake and the intake of protein from both animal and dairy sources. There was no association between total dietary phosphate intake and the baseline CAC score. In contrast, the dietary phosphate to protein ratio was not correlated with total energy or total protein intake and was only moderately associated with potassium and calcium intake. There was a very strong correlation between the phosphate to protein ratio and the intake of protein from dairy sources as well as with the percentage of total protein intake that was derived from dairy sources. In contrast, there was a negative correlation between the phosphate to protein ratio and the intake of protein from animal protein sources as well as the percentage of total protein intake that was derived from animal sources.

CAC progression, defined as a CAC score increase of ≥15% between 2005 and 2009, was observed in 59 patients (Table 5.1). The prevalence of diabetes mellitus was higher in patients with CAC progression; however, baseline CAC score, eGFR and nutritional status scores were similar between the two groups. Although there was no difference in total dietary phosphate intake between groups in the unadjusted analysis, the
dietary phosphate to protein intake ratio was significantly higher in those with CAC progression.

The association between dietary phosphate and dietary phosphate to protein ratio with >15% CAC progression was examined with four progressive multivariable adjusted logistic regression models. As shown in Table 5.3, total dietary phosphate was associated with a >15% increase in CAC score once adjusted for case-mix, laboratory variables, dietary variables (total energy, protein and potassium) and baseline CAC score (OR: 1.5 CI 1.0-2.2; p<0.05).

As shown in Table 5.4, the dietary phosphate to protein ratio was associated with a significantly increased risk of >15% CAC progression in the unadjusted (OR: 1.3 CI [1.0-1.6]; p=0.03) and adjusted models (OR: 1.4 CI [1.0-2.0]; p<0.04).
Table 5.1 Baseline, demographic, clinical and laboratory values in 89 patients with stage 3 to 5 CKD and according to change in CAC score between 2005 and 2009.

<table>
<thead>
<tr>
<th>Variables</th>
<th>All patients N=89</th>
<th>CAC progression &lt; 15% (n=30)</th>
<th>CAC progression &gt;15% (n=59)</th>
<th>P for trend</th>
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</thead>
<tbody>
<tr>
<td><strong>Clinical Characteristics</strong></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>60.8 (14.0)</td>
<td>57.3 (16.0)</td>
<td>62.5 (11.9)</td>
<td>0.08</td>
</tr>
<tr>
<td>Gender (male) (%)</td>
<td>52 (58%)</td>
<td>18</td>
<td>34</td>
<td>0.51</td>
</tr>
<tr>
<td>Diabetes mellitus (%)</td>
<td>34 (38%)</td>
<td>7 (23)</td>
<td>27 (46%)</td>
<td>0.03</td>
</tr>
<tr>
<td>Cardiovascular disease (%)</td>
<td>11 (12%)</td>
<td>4 (13)</td>
<td>7 (12%)</td>
<td>0.55</td>
</tr>
<tr>
<td>History of smoking (%)</td>
<td>52 (58%)</td>
<td>16</td>
<td>36</td>
<td>0.32</td>
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<td><strong>Kidney Function Measures</strong></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>eGFR</td>
<td>29.1 (12)</td>
<td>30.4 (11.8)</td>
<td>28.5 (12.2)</td>
<td>0.48</td>
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<tr>
<td>CKD stage 3 (%)</td>
<td>35 (39%)</td>
<td>14 (47%)</td>
<td>21 (36%)</td>
<td>0.60</td>
</tr>
<tr>
<td>CKD stage 4 (%)</td>
<td>44 (49%)</td>
<td>13 (43%)</td>
<td>31 (53%)</td>
<td></td>
</tr>
<tr>
<td>CKD stage 5 (%)</td>
<td>10 (11%)</td>
<td>3 (10%)</td>
<td>7 (12%)</td>
<td></td>
</tr>
<tr>
<td><strong>Calcification Measures at baseline</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CAC score (median, quartiles)</td>
<td>217 (9, 796)</td>
<td>285 (10, 1089)</td>
<td>288 (9, 823)</td>
<td>0.33</td>
</tr>
<tr>
<td><strong>Nutritional Parameters</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMI, kg/m² (continuous)</td>
<td>31.4 (7.6)</td>
<td>31.2 (7.4)</td>
<td>31.5 (8.3)</td>
<td>0.97</td>
</tr>
<tr>
<td>SGA, score out of 7</td>
<td>6.2 (0.9)</td>
<td>6.1 (1.0)</td>
<td>6.3 (0.8)</td>
<td>0.30</td>
</tr>
<tr>
<td><strong>Laboratory measures</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25(OH)D (mM)</td>
<td>72.9 (27.3)</td>
<td>69.0 (27.3)</td>
<td>75.1 (27.3)</td>
<td>0.33</td>
</tr>
<tr>
<td>Total cholesterol (mM)</td>
<td>4.6 (1.3)</td>
<td>4.6 (1.1)</td>
<td>4.6 (1.3)</td>
<td>0.94</td>
</tr>
<tr>
<td>Phosphate (mM)</td>
<td>1.3 (0.3)</td>
<td>1.2 (0.4)</td>
<td>1.3 (0.3)</td>
<td>0.14</td>
</tr>
<tr>
<td>Albumin (g/L)</td>
<td>42.1 (4.6)</td>
<td>42.0 (4.0)</td>
<td>42.1 (4.9)</td>
<td>0.96</td>
</tr>
<tr>
<td>Calcium (mM)</td>
<td>2.3 (0.2)</td>
<td>2.4 (0.1)</td>
<td>2.4 (0.2)</td>
<td>0.94</td>
</tr>
<tr>
<td>PTH (median, quartiles) (pg/mL)</td>
<td>8.3 (5.2, 13.8)</td>
<td>7.0 (4.9, 11.7)</td>
<td>9.2 (5.4, 15.8)</td>
<td>0.32</td>
</tr>
<tr>
<td>Alkaline phosphatase</td>
<td>82.9 (32.2)</td>
<td>79.1 (23.6)</td>
<td>84.9 (35.9)</td>
<td>0.43</td>
</tr>
<tr>
<td>CRP (median, quartiles) (mg/L)</td>
<td>2.7 (1.3, 6.0)</td>
<td>2.0 (1.2, 6.5)</td>
<td>3.1 (1.3, 6.0)</td>
<td>0.44</td>
</tr>
<tr>
<td>Urinary ACR (median, quartiles) (mg/L)</td>
<td>22.3 (2.9, 3.4)</td>
<td>13.7 (3.4)</td>
<td>27.9 (2.8)</td>
<td>0.24</td>
</tr>
<tr>
<td>Calcium phosphate binder (%)</td>
<td>40 (22.9)</td>
<td>14 (24.1)</td>
<td>16 (27.6)</td>
<td>0.38</td>
</tr>
<tr>
<td>Total energy intake</td>
<td>1904 (622)</td>
<td>1978 (715)</td>
<td>18566 (572)</td>
<td>0.43</td>
</tr>
<tr>
<td>Protein intake</td>
<td>83.4 (26.4)</td>
<td>86.4 (29.3)</td>
<td>82.0 (24.9)</td>
<td>0.46</td>
</tr>
<tr>
<td>Potassium intake</td>
<td>3169 (1094)</td>
<td>3220 (1274)</td>
<td>3143 (1002)</td>
<td>0.76</td>
</tr>
<tr>
<td>Phosphate intake</td>
<td>1323 (431)</td>
<td>1308 (452)</td>
<td>1331 (423)</td>
<td>0.81</td>
</tr>
<tr>
<td>Calcium intake</td>
<td>1021 (543)</td>
<td>984 (491)</td>
<td>1040 (571)</td>
<td>0.65</td>
</tr>
<tr>
<td>Phosphate:protein ratio (mg/g)</td>
<td>16.0 (2.3)</td>
<td>15.2 (2.0)</td>
<td>16.4 (2.4)</td>
<td>0.03</td>
</tr>
</tbody>
</table>

Values represent mean (SD), n (%) or median (quartiles) as appropriate. ACR, albumin to creatinine ratio; BMI, body mass index; CRP, C-reactive protein; PTH, parathyroid hormone; SGA, subjective global assessment; 25(OH)D, 25-hydroxyvitamin D
Table 5.2 Bivariate association between dietary intake of phosphate and dietary phosphate:protein ratio and baseline, demographic, clinical and laboratory values 89 patients with stage 3-5 CKD.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Dietary phosphate (mg/day)</th>
<th>Phosphate:protein ratio (mg/g/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Correlation coefficient or t-statistic(^a)</td>
<td>p-value</td>
</tr>
<tr>
<td>Clinical Characteristics</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age, yrs</td>
<td>-0.026</td>
<td>0.81</td>
</tr>
<tr>
<td>Gender (male)(^a)</td>
<td>0.52</td>
<td>0.61</td>
</tr>
<tr>
<td>Diabetes mellitus(^a)</td>
<td>-0.042</td>
<td>0.97</td>
</tr>
<tr>
<td>Cardiovascular disease(^a)</td>
<td>-0.92</td>
<td>0.36</td>
</tr>
<tr>
<td>Peripheral vascular disease(^a)</td>
<td>0.39</td>
<td>0.70</td>
</tr>
<tr>
<td>Renovascular disease(^a)</td>
<td>-0.67</td>
<td>0.51</td>
</tr>
<tr>
<td>History of smoking(^a)</td>
<td>0.11</td>
<td>0.92</td>
</tr>
<tr>
<td>Kidney Function Measures</td>
<td></td>
<td></td>
</tr>
<tr>
<td>eGFR</td>
<td>0.115</td>
<td>0.29</td>
</tr>
<tr>
<td>Calcification Measures at baseline</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CAC score (spearman)</td>
<td>-0.029</td>
<td>0.79</td>
</tr>
<tr>
<td>Nutritional Parameters</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMI, kg/m2 (continuous)</td>
<td>0.063</td>
<td>0.56</td>
</tr>
<tr>
<td>SGA, score /7 (^a)</td>
<td>0.12</td>
<td>0.28</td>
</tr>
<tr>
<td>Laboratory measures</td>
<td></td>
<td></td>
</tr>
<tr>
<td>25(OH)D, nmol/L</td>
<td>0.008</td>
<td>0.94</td>
</tr>
<tr>
<td>Total cholesterol, mmol/L</td>
<td>0.170</td>
<td>0.11</td>
</tr>
<tr>
<td>Phosphate, mmol/L</td>
<td>-0.076</td>
<td>0.48</td>
</tr>
<tr>
<td>Albumin, g/L</td>
<td>-0.11</td>
<td>0.31</td>
</tr>
<tr>
<td>Calcium, mmol/L</td>
<td>-0.07</td>
<td>0.54</td>
</tr>
<tr>
<td>PTH, pmol/L (Spearman)</td>
<td>-0.148</td>
<td>0.051</td>
</tr>
<tr>
<td>FGF23 IU/mL</td>
<td>-0.037</td>
<td>0.82</td>
</tr>
<tr>
<td>Alkaline phosphatase IU/ml</td>
<td>-0.11</td>
<td>0.30</td>
</tr>
<tr>
<td>CRP mg/L (Spearman)</td>
<td>0.071</td>
<td>0.52</td>
</tr>
<tr>
<td>Urinary ACR (Spearman)</td>
<td>-0.023</td>
<td>0.83</td>
</tr>
<tr>
<td>Calcium phosphate binder (%)(^a)</td>
<td>0.60</td>
<td>0.55</td>
</tr>
<tr>
<td>Total energy intake</td>
<td>0.83</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Protein intake</td>
<td>0.90</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Potassium intake</td>
<td>0.86</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Calcium intake</td>
<td>0.52</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Animal protein intake</td>
<td>0.81</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Dairy protein intake</td>
<td>0.73</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Animal protein/Total protein (%)</td>
<td>0.09</td>
<td>0.43</td>
</tr>
<tr>
<td>Dairy protein/total protein (%)</td>
<td>0.27</td>
<td>0.012</td>
</tr>
</tbody>
</table>

Values represent correlation coefficients (Pearson or Spearman) or t-statistics\(^a\) as appropriate. ACR, albumin to creatinine ratio; BMI, body mass index; CRP, C-reactive protein; PTH, parathyroid hormone; SGA, subjective global assessment; 25(OH)D, 25-hydroxyvitamin D.
Table 5.3 Association of dietary phosphate intake with a greater than 15% increase in CAC score between 2005 and 2009 in 89 patients with stage 3 to 5 CKD

<table>
<thead>
<tr>
<th></th>
<th>OR (95% CI)</th>
<th>P for trend</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unadjusted</td>
<td>1.0 (1.0-1.0)</td>
<td>0.8</td>
</tr>
<tr>
<td>Case-mix (^a) adjusted</td>
<td>1.0(0.9-1.1)</td>
<td>0.8</td>
</tr>
<tr>
<td>Case-mix + laboratory variables (^b)</td>
<td>1.0(0.9-1.1)</td>
<td>0.9</td>
</tr>
<tr>
<td>Case-mix + lab variables + diet (^c) + binder use</td>
<td>1.5(1.0-2.2)</td>
<td>0.046</td>
</tr>
<tr>
<td>Case-mix + lab variables + diet + baseline CAC score</td>
<td>1.5(1.0-2.2)</td>
<td>0.045</td>
</tr>
</tbody>
</table>

\(^a\) case-mix variables include age, eGFR, diabetes, SGA and gender  
\(^b\) laboratory variables include serum phosphate, cholesterol and PTH  
\(^c\) diet includes calorie, protein and potassium intakes
Table 5.4 Association of phosphate:protein intake ratio with a greater than 15% increase in CAC score between 2005 and 2009 in 89 patients with stage 3 to 5 CKD

<table>
<thead>
<tr>
<th></th>
<th>OR (95% CI)</th>
<th>P for trend</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unadjusted</td>
<td>1.3 (1.0-1.6)</td>
<td>0.03</td>
</tr>
<tr>
<td>Case-mix a adjusted</td>
<td>1.3 (1.0-1.6)</td>
<td>0.04</td>
</tr>
<tr>
<td>Case-mix + laboratory variables b</td>
<td>1.3 (1.0-1.6)</td>
<td>0.04</td>
</tr>
<tr>
<td>Case-mix + lab variables + diet c + binder use</td>
<td>1.4 (1.0-1.9)</td>
<td>0.04</td>
</tr>
<tr>
<td>Case-mix + lab variables + diet + baseline CAC score</td>
<td>1.4 (1.0-2.0)</td>
<td>0.04</td>
</tr>
</tbody>
</table>

a case-mix variables include age, eGFR, diabetes, SGA and gender
b laboratory variables include serum phosphate, cholesterol and PTH
c diet includes calorie, protein and potassium intakes
5.5 Discussion

The results of this study indicate that total dietary phosphate intake and the ratio of dietary phosphate to protein are associated with CAC progression over four years in patients with stage 3-5 CKD. Although experimental data have consistently identified phosphate as a key signaling molecule in the development and progression of vascular calcification, the association between serum levels of phosphate and calcification burden at early stages of CKD in observational studies have been inconsistent. To our knowledge, this is the first study to show an association between dietary phosphate intake and the phosphate to protein ratio, in relation to the calcification progression in patients with stage 3-5 CKD. These findings suggest that the assessment of usual phosphate intake could be an indicator of overall phosphate burden in comparison to routine biochemical measures and should be specifically targeted for intervention.

The results of the current study demonstrate that a usual diet with a higher amount of phosphate per gram of protein was linked to calcification progression in both unadjusted and adjusted models. There was no association between the phosphate to protein ratio and the various indices of nutritional status (i.e. SGA, or serum albumin) in this cohort, and unlike total dietary phosphate intake, the ratio was not sensitive to total calorie intake. It was, however, highly correlated with dairy protein intake and with the percentage of total protein intake derived from dairy sources. These data lend support to the notion that the source of protein consumed is important (e.g. animal versus dairy versus vegetable-based) in patients with CKD or alternatively that the burden of phosphate from low-protein containing foods (e.g. processed meats, refrigerated bakery items, colas) could be playing a role. Inorganic phosphate additives, such as those found
in carbonated beverages and processed meats, are highly bioavailable (>90%)\(^{195}\). The latter may not be accounted for in the estimated phosphate content of processed foods because it is not yet mandatory to list their quantities. It is important to note that the dietary phosphate burden from phosphate-containing preservatives has increased dramatically in a decade from an average of 470 mg/day in the 1990s to more than 1000 mg/day for a typical American diet in recent years\(^{221}\). Foods with phosphate additives are generally of lower quality and cost compared to fresh produce without additives. Patients with a lower socioeconomic status have significantly higher levels of serum phosphate despite lower protein intake in the United States\(^{222}\).

It is recognized that the restriction of dietary phosphate intake is most easily accomplished by reducing protein intake\(^{223}\). In the current study, dietary phosphate intake was highly correlated with total protein intake as expected, but, unlike reports in ESKD patients\(^{198,200}\) was not associated with the clinical examination of nutritional status. However, the majority of patients enrolled in this study were classified as ‘well-nourished’ and no cases of malnutrition were identified. In patients with ESKD, it is well-recognized that care must be taken to maintain adequate dietary protein as the presence of protein-energy wasting is a strong risk factor for death in this group\(^{198}\). A normal phosphate level in the setting of inadequate protein intake has been linked to mortality in observational studies\(^{198}\). Moreover, Lynch \textit{et al.} recently reported that dietary phosphate restriction in ESKD patients showed a trend towards poorer nutritional status and no benefit on survival\(^{200}\). Indeed, K-DOQI recommends that protein sources with the least amount of phosphate be prescribed to patients with CKD in order to reduce phosphate but maintain dietary protein intake. This guideline from 2003 introduced the
concept of the phosphate to protein ratio per standard food serving. For example, a single serving of a chicken breast contains 7.3 mgs of phosphate per gram of protein, whereas a single serving of yogurt contains almost 30 mgs of phosphate per gram of protein. We found a very strong positive correlation between the phosphate to protein ratio and the percentage of total protein derived from dairy sources; the relationship between the phosphate to protein ratio and animal-based protein intake was also significant, but in a negative direction. In addition to the absolute amount of phosphate per gram of protein, another consideration is that the bioavailability of phosphate depends on the food source. More specifically, phosphate present in vegetable-based protein has very low bioavailability based on the absence of the enzyme phytase in humans. A study of rats with CKD demonstrated that rats fed a grain-based diet had significantly lower serum phosphate, urinary phosphate excretion and serum levels of FGF-23 compared to rats fed a casein-based diet 224, despite equivalence of protein content. Clinical evidence also suggests that the source of dietary phosphate has a significant impact on phosphate homeostasis. That is, studies have shown that vegetarian diets tend to mitigate elevated serum phosphate and FGF-23 compared to animal-based diets196,225. Finally, bioavailability can also vary by vitamin D status, activation of intestinal vitamin D receptors and the presence of compounds that can bind to phosphate and in so doing inhibit its absorption (e.g. calcium, magnesium and aluminum).196,201. The associations that we reported between dietary phosphate and the phosphate to protein ratio were independent of calcium-based phosphate binder use.

It is recognized that phosphate retention occurs early in the progression of CKD, and yet serum phosphate levels remain within the normal range until much later in the
disease course. Rising levels of counter-regulatory hormones (e.g. PTH and FGF-23) are early occurrences that maintain serum phosphate levels in the normal range\textsuperscript{73}. However, compared to healthy subjects, the homeostatic response that allows an increase in phosphate excretion following an oral phosphate load is attenuated in patients with CKD\textsuperscript{226}. This result may allow the excess post-prandial phosphate to enter pools (i.e. soft tissues) that are not readily exchangeable with serum phosphate, a process that may pose a significant risk for the development and progression of vascular calcification. More specifically, the uptake of phosphate by vascular smooth muscle cells is a key signaling factor in the conversion of cell phenotype to an osteo/chondroblastic-like cells, which then undergoes calcification\textsuperscript{81}. In the current study, multiple biomarkers of mineral metabolism including serum phosphate, serum calcium and serum iPTH did not correlate with CAC severity at baseline or CAC progression over four years. It is likely that one isolated serum level of phosphate or iPTH - both subject to recent dietary intake - may be insensitive markers of a cumulative risk factor in a study of this many patients. That is, a measure of the ‘usual dietary intake’ may better reflect total phosphate burden over time in patients with early stage CKD.

The Kidney Disease Improving Global Outcomes 2009 clinical practice guidelines do not specifically address limiting dietary phosphate at earlier stages of CKD in the absence of hyperphosphatemia\textsuperscript{227}. However, the K-DOQI guidelines do recommend restricting dietary phosphate to 800 to 1000 mg/day when blood levels of PTH begin to rise (i.e. approximately CKD stage 2) and/or when serum phosphate levels are elevated\textsuperscript{228}. The results of the current study support the approach recommended by K-DOQI, as in this observational study, increased dietary phosphate intake appears to
predict CAC progression in CKD patients with normal levels of serum phosphate. However, Russo et al. (2007) reported that dietary phosphate restriction alone did not prevent CAC progression in a very small group of early stage CKD patients over two years of observation\textsuperscript{229,230}. To our knowledge, this represents the only randomized controlled trial that has evaluated the impact of dietary phosphate restriction on a calcification-related outcome.

In this study, the association between dietary phosphate intake and the phosphate to protein ratio with CAC progression was independent of phosphate binder use. Very few studies have evaluated phosphate binder use in early stage CKD. However, Block et al reported in a randomized controlled trial of approximately 150 patients with an estimated GFR between 20 and 40 ml/min, that phosphate binders successfully lowered serum phosphate and urinary phosphate and attenuated the progression of secondary hyperparathyroidism, but promoted the progression of coronary artery calcification\textsuperscript{231}. These data support the notion that phosphate binders (both calcium and non-calcium-based) could induce a positive calcium balance in patients with CKD, directly as with calcium ingestion, or indirectly by enhancing the availability of free intestinal calcium. Therefore, the safety and efficacy of phosphate binders at low GFR is uncertain and until further data is available dietary intervention should remain the primary focus in the clinical care of patients.

The FFQ used in the current study calculates nutrient intake using a temporal method of nutrient quantification, thereby off-setting the lack of direct quantitative assessment which may either over- or under-estimate at the individual level\textsuperscript{232}. To our knowledge, there have been very few studies in CKD patients that have used an FFQ as
an assessment tool and none that have been validated for phosphate intake. For instance, consumption of processed meats and refrigerated bakery items are captured by the FFQ used in this study; however, it cannot resolve the total phosphate intake by these various sources. Furthermore, the FFQ does not capture the phosphate salts that are present in medications that are commonly prescribed to this patient group. Future studies should address these issues as these ‘uncaptured’ items could undermine dietary counseling and phosphate binder therapy in this group of patients. An optimal method to quantify the source and amount of dietary phosphate-based on bioavailability is of paramount importance in providing optimal care to CKD patients. Moreover, there is a growing body of literature that suggests that phosphate is a risk factor for left ventricular hypertrophy, calcification and mortality in the general population, suggesting that the importance of targeting dietary phosphate may extend beyond those individuals with advanced kidney failure.

CAC progression is an important outcome to consider as it has been shown to be a better predictor of cardiovascular events than baseline CAC scores. In the Multi-Ethnic Study of Atherosclerosis, a graded relationship of CAC progression with coronary heart disease event risk was demonstrated, with greater CAC progression associated with greater risk. The authors examined the threshold at which annualized relative (percent change) progression was best associated with coronary heart disease events and a greater than 15% annual increase (HR: 1.4, p<0.05) in CAC score was identified. Furthermore, it has been reported that a CAC score change (either increase or decrease) greater than 15% may be considered evidence of true change as opposed to scoring variability. Therefore, the degree of change in CAC score used in the current study may be clinically
relevant. In this study, the first series of CAC scans were completed on an earlier
generation of scanner (4 slice helical) versus the second series of scans completed on a
current generation of scanner (64 slice helical). The current scanner results in decreased
motion artifact, improved spatial resolution and potentially more precise CAC results.
The first series of scans could have resulted in an over-estimation of CAC scores due to
motion artifact, which would be less in the second series of scans. These differing
measurement techniques would potentially bias results towards the null, and so we are
confident the change in CAC scores detected between the two series of scans is depicting
an accurate reflection of CAC progression. Further limitations include the small sample
size for a dietary study.

In conclusion, dietary phosphate and dietary phosphate to protein ratio predict
CAC progression in stage 3-5 CKD patients. This relationship was independent of SGA
score, nutritional status and protein intake. Furthermore, the results support that the
source of dietary phosphate should be considered in the management of total phosphate
burden and calcification progression at early stages of CKD when serum phosphate levels
may be normal.
Chapter 6

General Discussion
Mineral imbalance in patients with CKD often results in the development of vascular calcification, an outcome which may indicate an important pathology linking kidney dysfunction to increased cardiovascular risk in this population. More than 60% of CKD patients (stage 3) have coronary artery calcification, and among ESKD patients, prevalent calcification is much higher (>80%), progresses rapidly and is associated with increased mortality. Even young dialysis patients (<30 years old) with few traditional cardiovascular risk factors have extensive vascular calcification. Although vascular calcification was once thought to be a passive process of calcium and phosphate precipitation in soft tissues as it related to aging, it is now widely accepted that vascular calcification is an active process. There are a number of non-modifiable risk factors associated with vascular calcification – such as advanced age and time on dialysis- but, phosphate is a key modifiable risk factor which can greatly impact the management of CKD and calcification progression. In fact, many experimental studies have now confirmed that phosphate is a key signaling molecule in initiation and progression of vascular calcification. However, elevated serum phosphate levels in CKD occurs late in the disease progression, and by this time calcification might already be present and progressing. Although regional calcification might not directly impact blood pressure, the consequences of coronary and aortic calcification are linked to elevated pulse wave velocity (PWV) and cardiovascular morbidity and mortality in CKD patients.

In this regard, the studies in this thesis were performed to characterize the development, consequences and treatment of vascular calcification in CKD. The results presented in this thesis demonstrate the impact of mineral imbalance on cardiovascular
change in CKD and, for the first time, the time-course of the hemodynamic consequences of vascular calcification. Specifically, the results show that i) hyperphosphatemia and elevated FGF-23 are associated with vascular calcification and cardiac hypertrophy (Chapter 2 and 4), ii) there is heterogeneity in the hemodynamic response to vascular calcification (Chapter 2 and 3), iii) regional calcification is associated with regional hemodynamic alterations (Chapter 3), and iv) magnesium impacts the initiation and progression of vascular calcification (Chapter 4). Finally, findings in the clinical chapter (Chapter 5) revealed the importance of dietary phosphate and its source on the progression of vascular calcification in CKD patients.

6.1 Cardiovascular consequences of vascular calcification in CKD

Previously, experimental CKD approaches in rodents attempted to model mild to moderate CKD, however vascular calcification was not a prominent feature. More recently, administration of dietary adenine was shown to generate a more severe model of CKD in rats, but with significant weight loss. Therefore, there was a need to develop a stable model of CKD with vascular calcification. The studies in Chapter 2 demonstrate that lowering the concentration of adenine from 0.75% to 0.25% was key in producing stable CKD without significant weight loss. Previous studies using 0.75% adenine induced CKD consistently report about 50% incident of calcification, whereas the current modification of the adenine model resulted in 100% calcification in animals at 11 weeks of CKD. This increase in animal health and improved experimental outcome provided us with a valid approach for our other studies.

Although the hemodynamic consequences of calcification are well known with respect to increases in PWV and pulse pressure, for the first time the time-course of these
hemodynamic consequences of calcification were revealed in continuous radiotelemetry studies. That is, calcification was associated with a sudden rise in pulse pressure (PP) which resulted, at least in part, from a drop in diastolic blood pressure (DBP).

Furthermore, calcification progression was linked to increased systolic blood pressure variability. Systolic variability has been associated with end-organ damage, stroke and cardiovascular disease\(^{124,125}\). These novel, distinctive and temporal blood pressure analyses revealed that hemodynamic factors other than hallmark measures of elevated PWV and pulse pressure are associated with the development of vascular calcification. Indeed, PWV was also elevated in CKD animals with calcification, but regional calcification had a differential impact on PWV.

### 6.1.1 Hemodynamic consequences of regional calcification

CKD patients are at a higher risk of developing vascular calcification at every stage of the disease\(^{124,238,241}\). Although PWV could also be elevated at any stage\(^{124}\), presence of vascular calcification does not always result in elevated PWV\(^{88}\). As this thesis demonstrates, this disparity is most likely due to differential regional calcification. Although most CKD studies only measure coronary artery calcification and thoracic calcification, a recent study in obese patients revealed a more frequent susceptibility to calcification in the abdominal and iliac arteries versus thoracic aorta or carotid arteries\(^{141}\). The distribution of calcification in the adenine model of CKD in this thesis has a similar pattern whereby distal vessels (i.e. femoral artery, abdominal aorta, renal artery) calcified early while the calcification of proximal vessels (thoracic aorta and carotid arteries) lagged behind. The difference in susceptibility to vascular calcification is not well understood, but could be related to structural and functional differences, embryonic
origin and differential expression of local inhibitors. In the current studies (Chapter 3), the distinct heterogeneity in distribution of calcification resulted in a late elevation in PWV and regional specific hemodynamic alterations. Analyses of the rate of change in pressure, $dP/dt_{\text{max}}$, revealed that this hemodynamic variable is likely influenced by regional vascular compliance\(^{108}\). That is, marked calcification of the thoracic aorta resulted in increased PWV, but regional calcification resulted in regional changes in $dP/dt_{\text{max}}$. Specifically, thoracic calcification was linked to carotid $dP/dt_{\text{max}}$ and abdominal aorta calcification was linked to femoral $dP/dt_{\text{max}}$. Furthermore, a composite index of carotid:femoral $dP/dt_{\text{max}}$ appeared to precede aortic calcification, agreeing with previous findings showing that changes in arterial structure and function are associated with initiation and distribution pattern of calcification \(^{40,88,242}\). Taken together, changes in $dP/dt_{\text{max}}$ are predictive of progression of calcification throughout the aorta, whereas changes in PWV are linked to advanced aortic vascular calcification (i.e. thoracic calcification only). Furthermore, the heterogeneity in PWV and the changing $dP/dt_{\text{max}}$ relationship to blood pressure suggested that PWV and $dP/dt_{\text{max}}$ are highly influenced by vascular dysfunction brought on by calcification, but arterial $dP/dt_{\text{max}}$ appears to also be influenced by changes in cardiac function and structure.

### 6.1.2 Left ventricular hypertrophy in CKD

One clinical outcome of aortic stiffness is left ventricular hypertrophy (LVH). LVH is highly prevalent at all stages of CKD and it is a powerful independent predictor of mortality in dialysis patients.\(^{243-245}\). In the current adenine model of CKD (Chapter 2 and 3), animals without calcification had LVH, and as expected LVH was higher in CKD animals with vascular calcification. The observed changes in arterial compliance
are a major contributing factor to elevated LVH. For instance, elevated pulse pressure and PWV are linked to an increase in afterload causing a physical stimulus for development of LVH\textsuperscript{93,100,101}. Although other studies have shown that elevated pulse pressure and PWV are associated with LVH\textsuperscript{243,246}, the studies in this thesis also revealed an association with both carotid and femoral dP/dt$_{\text{max}}$. As demonstrated in Chapter 3, this hemodynamic marker is elevated early in CKD, and therefore might predict cardiac structure and function earlier than PWV or PP changes. Given that more than 50\% of CKD patients at stage 1 and 2 might already have LVH\textsuperscript{243} and that regression of LVH is associated with decreased cardiovascular events and better survival\textsuperscript{247,248}, early diagnosis of cardiac dysfunction is critical. These novel and non-invasive assessments of hemodynamic change (combined PWV and arterial dP/dt$_{\text{max}}$ measurements) could provide important information in the development of vascular calcification and LVH in CKD patients which may ultimately improve the management of the disease.

In CKD, LVH is not fully explained by the hemodynamic abnormalities associated with vascular calcification. Mineral imbalance has been recognized as a major risk factor for cardiovascular morbidity and mortality in CKD patients\textsuperscript{19–21}. Although the underlying link is, in part, associated with increased calcification due to phosphate imbalance, data from Chapter 2 suggests that fibroblast growth factor 23 (FGF-23) might also play a role. FGF-23 is a key hormone in kidney regulation of phosphate, but it has been linked to mortality and LVH in CKD\textsuperscript{75,77}. Recent evidence has shown it might have a direct effect on cardiac structure via the FGF receptors in the heart\textsuperscript{76}. In the study in Chapter 2, serum FGF-23 remained elevated even though the serum phosphate level was only elevated transiently. FGF-23 was also associated with LVH regardless of
calcification status. It is possible that prolonged elevation of FGF-23 in the CKD animals caused a non-selective activation of FGF receptors in the heart, thus causing LVH even in CKD animals without calcification.

6.2 Impact of magnesium on calcification

Vascular calcification is a complex and multifactorial process involving many inducers and inhibitors (see Chapter 1). To elucidate the role of mineral imbalance in vascular calcification, an ex vivo model of calcification was developed in Chapter 4. This model allows for development of calcification in the aorta in isolation from the dynamic physical influences and circulatory factors of an in vivo environment. In this setting, elevated phosphate was a key promoter in the calcification of thoracic aorta, a process which was dose-dependently and temporally blocked by addition of magnesium. That is, addition of magnesium in vitro prevented aortic calcification in a concentration dependent manner. To prevent initiation and proliferation of calcification crystals, magnesium needed to be added early in the process. In contrast, increasing magnesium late did not reverse pre-existing calcification. These studies suggest that magnesium most likely inhibits calcification by stabilizing the calcium phosphate solubility in the tissue, thereby preventing nucleation and crystallization\(^{127,175}\). In vivo, using the same adenine model of CKD, dietary magnesium also attenuated calcification. Although the in vivo mechanism of magnesium inhibition is unknown, it likely shares similarities with the mechanisms from the in vitro model of calcification. Given that magnesium treatment in vivo did not impact systemic hyperphosphatemia (\(\uparrow\)serum phosphate and \(\uparrow\)FGF-23), calcification inhibition was likely a result of magnesium directly affecting the tissue
calcium-phosphate interaction. Regardless, the findings suggest that moderate magnesium supplementation could impede soft tissue mineralization.

### 6.3 Impact of dietary phosphate on calcification progression in CKD patients

Elevated phosphate has been consistently linked to vascular calcification and mortality in ESKD patients\(^{19,148,191,192}\), however this association at early stages of CKD has been inconsistent\(^{215-220}\), most likely because hyperphosphatemia occurs relatively late in the disease progression\(^{73}\). There is also a growing body of evidence linking higher serum phosphate levels well within the ‘normal’ range to a substantially increased risk of cardiovascular disease\(^{22,193}\). Although a good source of phosphate is found in animal protein, in a typical Western diet the bioavailability of phosphate from preservatives and carbonated beverages is very high\(^{194,195}\). Dietary phosphate intake might therefore better reflect phosphate burden at earlier stages of CKD when hyperphosphatemia is not as prevalent. In this regard, the impact of dietary phosphate on calcification progression in patients with stage 3-5 CKD (excluding dialysis) was investigated in Chapter 5. Total dietary phosphate intake was associated with coronary artery calcification (CAC) progression after adjusting for various variables (see Chapter 5). However, a higher amount of phosphate per gram of protein was also linked to CAC progression in both unadjusted and adjusted models. These data support the notion that the source of phosphate may indeed be important such that low-protein containing foods (e.g. processed meats, refrigerated baked goods, colas) or preservative-rich diets could increase the phosphate burden in CKD patients. These results suggest that the source of dietary phosphate should be considered in the management of total phosphate burden and calcification progression at early stages of CKD.
6.4 Summary

The studies in this thesis contribute to the knowledge regarding the detection, management, prevention and treatment of vascular calcification in CKD. The pathogenesis of the modified adenine CKD model closely parallels the human condition with hallmark cardiovascular outcomes. The progression of calcification in this model is linked to key hemodynamic consequences and the findings herein have identified new methods of characterizing the consequences of regional calcification in CKD. Although the findings in Chapter 4 indicated that magnesium treatment has the potential for calcification prevention, magnesium status in CKD patients is under-recognized and could potentially be a modifiable risk factor. Finally, the clinical chapter (Chapter 5) identified the significant impact of dietary phosphate and the source of dietary phosphate in the progression of vascular calcification in early stage CKD patients.

6.5 Limitations

Although the approaches, methodologies and experimental designs were carefully considered for the studies reported in this thesis, future studies would be improved if a number of issues are addressed as indicated below:

- Use solid state catheters instead of fluid filled catheters to assess hemodynamic changes
- Assess differences in the susceptibility to calcification in Sprague-Dawley rats and the sub-strains, in other rat strains as well as mouse models.
- Assess gender differences in CKD induced changes.
- Expand the assessment of VC to all vascular beds in both in vitro and in vivo
6.6 Future directions

A definitive mineral imbalance characterized by transient hyperphosphatemia, prolonged elevated FGF-23, and culminating in vascular calcification develops in adenine-induced CKD. This observation suggests that altered phosphate kinetics are likely associated with every stage of CKD. It appears likely that soft tissue changes are key to altered phosphate disposition in CKD, an involvement which ultimately leads to vascular calcification. Therefore, future studies should focus on the acute and chronic kinetics of phosphate handling in this CKD model (to both oral and/or intravenous phosphate loads). Further investigation is required for the finding that serum calcium levels remain unchanged in the progression of CKD, while the hormones which regulate calcium are altered (↑PTH, ↓vitamin D). Specifically, since vitamin D bioactivation occurs in the kidney and has a direct effect on calcium and phosphate regulation, its role in the progression of calcification requires further study.

The in vitro model of calcification developed in Chapter 4 elucidated the impact of phosphate and magnesium on aortic calcification. The data indicated that elevated tissue magnesium prevented phosphate induced calcification. Even when the media magnesium was lowered, a persistent increase in tissue magnesium prevented calcification. These data suggest that cellular influx and efflux are not necessarily equal. The role of the magnesium channel, TRPM7, in this process will need to be characterized.

The novel hemodynamic findings in Chapter 3 support the notion that regional calcification could be predicted non-invasively. The detection of the hemodynamic alteration associated with calcification presented in this chapter could possibly be
implemented in the management of patients using non-invasive techniques that use oscillometric recordings\(^ {108}\). Future studies are needed to validate the dynamic alterations in the arterial pulse profile associated with regional calcification in a cohort of early stage CKD patients.

The findings in the clinical chapter (Chapter 5) emphasize increased dietary phosphate burden in patients who may otherwise have normal serum phosphate levels. This approach supports the idea that dietary phosphate and, more specifically, the source of dietary phosphate should be considered in the management of total phosphate and calcification progression at early stages of CKD. Whether proper dietary phosphate restriction, which is to say restriction that does not compromise dietary protein and macronutrients, has a beneficial impact on calcification outcome in CKD patients requires further investigation.
References


