CHARACTERIZATION OF VASCULAR CALCIFICATION IN A RODENT MODEL OF CHRONIC KIDNEY DISEASE

by

Navid Seyed Shobeiri

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Abstract

Chronic kidney disease (CKD) is a worldwide health problem with rising incidence and high cardiovascular mortality. CKD compromises cardiovascular function, in part, characterized by vascular calcification (VC), elevated pulse wave velocity (PWV) and pulse pressure (PP). Through manipulation of dietary adenine, we produced a model characterized by graded severity of CKD, VC and hyperphosphatemia. To our knowledge, we are the first to explore the relationship between aortic calcium content and changes in circulatory function in rodents with CKD. Fourteen-week old Sprague-Dawley rats received a diet containing an adenine concentration (0.25-0.75%) plus high-normal dietary phosphate (1%), for up to 10 weeks. Circulatory changes were determined by arterial radiotelemetry (n=6) and by assessment of aortic pulse wave velocity (PWV, n=32). VC was assessed using the calcium-O-cresophthalein-complexone assay. At sacrifice, kidney function (creatinine (μmol/L)) was worst in the group with VC (251.3±60.2 μmol/L), compared to non-calcifying CKD (200.3±68.8 μmol/L) or control (50.0±16.2 μmol/L). PWV (cm/s) adjusted for blood pressure (BP) was markedly elevated in animals with VC (3.23±0.33 log(cm/s)) versus non-calcifying CKD (2.85± 0.12 log(cm/s)) or control (2.96±0.08 log(cm/s)). Arterial pressure radiotelemetry revealed that there was an increase in pulse pressure (38±4.7 mmHg to 58 ±15.2 mmHg) during the development of VC. Systolic pressure remained relatively stable throughout (129±8.7 mmHg), diastolic pressure fell during weeks 9 and 10 of the study (91±6.0 mmHg down to 74±9.1 mmHg), a fall that almost fully accounted for the changes in pulse pressure. The calcifying CKD animals also exhibited left ventricular hypertrophy

(LVH) compared to CKD or control animals (2.32±0.3 vs 2.03±0.2, 1.80±0.1 g/kg respectively). Manipulating dietary adenine produces a graded severity of CKD with calcification which impact circulatory changes (PP and PWV). These altered circulatory functions are likely to be key factors in the enhanced LVH. This model appears to be a useful for the study of CKD-associated VC.

Co-Authorship

The following thesis was performed and written by Mr. Navid Shobeiri with the following co-authorships and assistance:

- Chapter 1: co-authored by Dr. R.M. Holden and Dr. M.A. Adams
- Chapter 2: co-authored by Dr. R.M. Holden and Dr. M.A. Adams
- Chapter 3: co-authored by Dr. R.M. Holden and Dr. M.A. Adams with technical assistance from Mrs. R. Phelan for animal handling and sacrificing, and from Mrs. J. Pang for preparing histological slides.
- Chapter 4: co-authored by Dr. R.M. Holden and Dr. M.A. Adams with technical assistance from Mr. D. Beseau for tissue calcium analysis, from Mrs. R. Phelan for animal handling and sacrificing, and from Ms. C. Smallegange for telemetry implantations

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Table of Contents

List of Figures

List of Tables

List of Abbreviations

ApoE	Apolipoprotein E
Ad	Adenine
BMP-7	Bone morphogenic protein 7
BW	Body Weight
Ca	Calcium
$CaCO_3$	Calcium carbonate
Calc	Calcitriol
Cin	Cinacalcet HCL
CKD	Chronic kidney disease
Cr	Creatinine
CVD	Cardiovascular Disease
DBP	Diastolic blood pressure
eGFR	Estimated glomerular filtration rate
ESKD	End stage kidney disease
Eti	Etidronate
FGF23	Fibroblast growth factor 23
GFR	Glomerular filtration rate
KGH	Kingston General Hospital
LDLR	Low density lipoprotein receptor
LVH	Left ventricular hypertrophy
LV	Left ventricle
MGP	Matrix Gla protein
NA	Not available
Nx	Nephrectomy
OCT	22-oxacalcitriol
P	Phosphorous
PP	Pulse pressure
PTH	Parathyroid hormone
PTx	Parathyroidectomy
PWV	Pulse wave velocity
RV	Right ventricle
SBP	Systolic blood pressure
SD	Sprague Dawley
SEM	Standard error of the mean
Sev	Sevelamer
VC	Vascular Calcification
VSMC	Vascular smooth muscle cell
Wt	Weight
Wks	Weeks

Chapter 1

Introduction

Chronic kidney disease (CKD) affects more than 25 million people in North America (including 2 million Canadians)[1]. Although the two leading causes of CKD are diabetes and hypertension, there are multiple co-morbidities associated with CKD independent of its etiology[2]. Recently it has been reported that CKD is an independent risk factor for cardiovascular disease[3]. In fact, individuals with CKD have been identified as one of the highest risk groups for developing cardiovascular disease, and those who finally 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[4, 5].

In 2002, the National Kidney Foundation Kidney Disease Outcome Quality Initiative defined CKD and classified the progression of the disease into 5 different stages primarily based on the estimated glomerular filtration rate (eGFR)[6]. eGFR is calculated based on serum creatinine levels, age, gender, race and body size. In brief, all other variables being constant, high serum creatinine levels reflect the low golmerular filtration rates (i.e. damaged kidneys). Normal GFR in young adults is approximately 120 to 130 mL/min/1.73 m^2 and declines with age. Stage 1 CKD is described as kidney damage with normal or increased GFR (>90mL/min/1.73m^2), stage 2 is eGFR from 60-89 mL/min/1.73 m^2 and with each successive stage eGFR declines further, ultimately reaching stage 5 with an eGFR <15mL/min/1.73m^2 in which case kidney replacement

therapy might be essential for survival. Complications associated with CKD stem long before ESKD and the etiology is not always known for these complications. The term ESKD describes patients who are treated with dialysis or kidney transplantation. In the United States of America 23 million people are estimated to have CKD and more than 400000 have ESKD and among patients receiving maintenance dialysis, 1 year mortality rates have been estimated as 22% in the US and 16% in Europe[5]

Cardiovascular disease can occur at any stage of CKD progression and is the leading cause of mortality in these patients[4, 5]. In fact, individuals with CKD are more likely to die of a cardiovascular event before they require renal replacement therapy. The traditional Framingham risk factors such as age, hypertension, smoking, diabetes mellitus and dyslipidemia present in this population does not completely account for the increased cardiovascular mortality. Non-traditional risk factors which develop after the onset of CKD, specifically metabolic imbalance, are associated with vascular calcification and arterial stiffness, which may decrease arterial compliance, and cause an increase in cardiovascular and all-cause mortality[7, 8]. A major sequelae of reduced kidney function is dysregulated divalent ion mineral metabolism, characterized in particular by a sustained increase in serum phosphorus[9, 10]. These abnormalities occur by the third stage of CKD whereby renal impairment limits phosphate excretion and causes an increase in serum phosphate levels, which together with low ionized calcium levels and hyperparathyroidism, contributes to the development of the chronic kidney disease-mineral bone disorder (CKD-MBD)[11]. CKD-MBD manifests clinically with bone disease

(pain and fracture) and vascular calcification (cardiovascular and peripheral vascular occlusive events). Furthermore, treatment of hyperparathyroidism with vitamin D sterols (Calcitriol) increases intestinal calcium and phosphorus absorption with subsequent development of hypercalcemia and hyperphosphatemia and further aggravation of VC with implications on cardiovascular disease[12].

Arterial calcification could be intimal or medial. Intimal occurs in atherosclerosis disease and is associated with inflammatory cells[36], whereas medial calcification is almost exclusively involved with vascular smooth muscle cells (VSMC)[66]. Medial calcification is common in patients with advanced renal disease, and is thought to result in part, from increased serum phosphate concentrations. The progression of VC is complicated and there are numerous enzymes involved in the process. To date, there have been many enzymes recognized as inhibitors of calcification, and many enzymes which promote calcification (see table below). Although it is important to study the mechanism involved in VC, the purpose of this thesis was not to elucidate such mechanisms, but to establish a robust model and investigate the functional changes which occur when VC is present in CKD.

Inducers	Inhibitors
Phosphate/calcium	Fetuin
Bone morphogenic-2	Osteoprotegerin
Osteocalcin	Bone morphogenic-7
Vitamin D (calcitriol)	Matrix Gla protein
Osteopectin	Pyrophosphate

The VC-linked changes in arterial compliance generate complications in cardiovascular function, such as increased pulse pressure and pulse wave velocity (PWV), higher systolic blood pressure (SBP) and lower diastolic blood pressure (DBP), thereby causing increased left ventricular (LV) afterload and altering coronary perfusion[13, 14]. The principal outcomes of these changes are left ventricular hypertrophy (LVH), aggravation of coronary ischemia, and increased fatigue of arterial wall tissues[15]. Higher SBP and pulse pressure, lower DBP, and LVH have been identified as independent risk factors of cardiovascular morbidity and mortality in the general population[16] as well as in ESKD patients[17, 18]. Recent studies have also shown increased PWV to be an independent risk factor for all-cause and cardiovascular mortality in ESRD patients[19, 20].

Vascular calcification in CKD patients may start with an early onset of medial wall calcification, which is a major contributor to altered compliance and arterial stiffness[14, 21]. Animal models of CKD which develop vascular calcification have contributed to our understanding of the biology responsible for this vascular disease. Remarkably research in progressive nephropathies has identified major consistencies between rodent and human findings, however each rodent model developed to date has focused on different aspects of kidney disease (see Chapter 2). My goal was to investigate the functional implications of vascular calcification in CKD; therefore it was imperative that the correct model was chosen for development of medial wall calcification in CKD. The systematic review of the rodent models in Chapter 2 describes

and analyses the animal models available in this field. In general there are three commonly used models: i) the electrocautery mouse model, ii) the 5/6th nephrectomy rat model and iii) the adenine rat model. Each model has its own weaknesses and strengths; however they all contribute to the ongoing research in progressive nephropathy.

Rationale and approach:

There is evidence that altered phosphate metabolism is mechanistically linked with cardiovascular disease in CKD[9, 22-24]. *In vitro* studies show that phosphate is taken up by vascular smooth muscle cells which develop calcification with subsequent cardiovascular disease[25]; however the mechanism *in vivo* is not well understood. Since calcification may occur at any stage of kidney disease, systemic phosphate fluctuations may play a major role in development of calcification and cardiovascular disease. Using a rat model of CKD with calcification, my goal was to expand on describing the link between mineral metabolism (i.e. phosphate), VC and altered cardiovascular function.

With the variety of rodent models from which to choose, we selected a robust CKD model which would develop extensive vascular calcification with cardiovascular complications similar to clinical setting. We used the adenine induced CKD rat model to develop graded CKD, which allowed us to evaluate the cardiovascular outcomes at different stages of kidney disease. In doing so, we had to alter the previously defined adenine model[26].In previous reports, using a 0.75% adenine diet for 4 weeks, Sprague

Dawley (SD) rats developed severe kidney disease. However due to the high adenine concentration, these rats would lose more than 30% of their initial body weight[26, 27]. Therefore the first step was to design a study to develop CKD in rats without severe weight loss. Once the model was developed, I characterized the hemodynamic changes in CKD rats by aortic catheterization under anesthesia as well as radiotelemetry blood pressure recordings.

The studies were designed to test the following hypotheses in order:

1. A 0.75% adenine diet given to rats daily will produce chronic kidney disease with elevated serum creatinine and urea.
2. Lower adenine concentrations in the diet (0.5% and 0.25%) will produce kidney disease with less than 15% weight loss
3. CKD rats which develop hyperphosphatemia will also exhibit aortic calcification
4. The degree of increase in left ventricular mass and alterations in blood pressure and pulse wave velocity will depend on the level of chronic kidney disease as well as the level of calcification

To test these hypotheses, the experiments are briefly outlined as follow. An artificial 0.75% adenine diet was provided to adult SD rats (300-400g) *ad libitum* for four weeks plus supplements when needed in order to minimize weight loss. The control diet contained no adenine. Blood samples were taken intermittently via the saphenous vein to determine creatinine, urea and plasma mineral levels (markers of kidney failure), and

once again at the end of the experiment. CKD animals would be expected to have elevated creatinine levels compared to control. The level of calcification in aorta was determined spectrophotometrically by measuring absolute tissue calcium concentrations, and histologically using von Kossa staining on cross sections of aorta. (N.B. serum creatinine alone is used to measure degree of kidney damage. Since all animals are of the same age, same gender, same genetic background (Sprague Dawley) and same size, eGFR is not necessary to quantify degree of kidney damage)[6].

A second set of studies using lower adenine concentrations for a longer duration were used to assess the hemodynamic changes associated with the presence of CKD and/or calcification. The blood pressure profile in some animals was assessed using radiotelemetry. At the end of the experiments blood pressure parameters were determined via aortic catheterization under anesthesia (i.e. PWV, PP, SBP, and DBP). Heart weights of all animals were determined post mortem. Plasma creatinine, urea, phosphate and calcium were also determined at an intermediate time point as well as at the end.

In our pilot study we found that the high adenine concentration (0.75%) was not palatable, and rats consumed less than 20% of their daily food intake, which caused severe weight loss. We sought to modify this model using adenine concentrations of 0.5% and 0.25% for different durations. End points based on blood creatinine concentration (blood collected via saphenous vein) were used to determine the severity of CKD development in SD rats. Hydration status as well as weight loss were monitored

closely and supplements were provided when needed. Using this modified adenine diet, we developed a CKD model with severe vascular calcification in 6-11 weeks without severe weight loss. Chapter three describes the development procedure in full detail, and chapter four sums up the cardiovascular and metabolic implications of vascular calcification in CKD.

Chapter 2

Rodent models of chronic kidney disease and vascular calcification: a systematic review

2.1 Introduction

Chronic kidney disease is a worldwide health problem with a rising incidence and poor outcomes. Cardiovascular disease is recognized as an important cause of morbidity and mortality in patients with CKD. In end stage kidney disease patients, the presence and extent of arterial calcification is independently predictive of subsequent cardiovascular disease and mortality beyond established conventional risk factors [14, 14, 15]. New advancements in technology, despite their limitations, have increased our recognition of the extent of vascular calcification in patients with CKD; noninvasive techniques such as computed tomography have enabled us to quantify the degree of calcification. Studies consistently demonstrate that the majority of ESKD patients have significant calcification in the coronary arteries and aorta, and nearly 50% have valvular calcification [28].

Current techniques used clinically to quantify calcification lack the ability to distinguish between medial or intimal calcification. While calcification of the media and intima may occur simultaneously in the same patient, medial wall calcification is a consistent and early feature of CKD-associated vascular calcification. The pathophysiology of medial wall vascular calcification in CKD is complex and CKD-associated abnormalities in mineral metabolism (hyperphosphatemia, hyperparathyroidism, hypocalcemia and vitamin D deficiency) contribute. Although

observational studies have contributed to our understanding of the various risk factors associated with calcification in humans with CKD, there is a lack of randomized controlled trial evidence to indicate which risk factors predominate and which therapies might benefit patients most. Animal models, therefore, allow us to evaluate clinical observations made in humans within a controlled environment and, in doing so, will allow us to better understand the contributors to and the biological significance of vascular calcification in the setting of CKD. The objective of this review therefore is to discuss the animal models ($5/6^{th}$ nephrectomy, murine and adenine models) that have been employed to study vascular calcification outcomes in the setting of CKD.

2.2 Contributions of animal models to vascular calcification as an active process

Calcification occurs at two sites in the arterial wall: at the intima and the media. Intimal calcification is patchy, associated with VSMCs and macrophages in lipid rich areas of arteries and takes the form of atherosclerotic vascular disease. 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 almost exclusively associated with VSMCs [29]. Intimal and medial calcification may occur independently of each other and therefore are believed to represent different pathologic processes. In young adult CKD patients, medial wall calcification predominates almost exclusively [21] and its presence has been linked to abnormalities in mineral metabolism that occur by stage 3 CKD (hyperphosphatemia) and its treatment (calcium-based phosphate binders and vitamin D).

There is growing *in vitro* and *in vivo* evidence that hyperphosphatemia and extracellular calcium stimulate phenotypic transformation of VSMCs[29, 30]. VSMCs have been found to exhibit distinctive phenotypes including a contractile phenotype characterized by markers of smooth muscle lineage (SMAD6, matrix gla potein, α-SMA) as well as phenotypes characterized by an osteochondrogenic-like differentiation and bone formation (sox-9, cbfa-1, osteocalcin)[31, 32]. In the setting of elevated phosphorus levels, as occurs in CKD, the latter phenotype becomes more prevalent. A number of animal knockout experiments have confirmed that there are VSMC-derived and bone-derived proteins that either inhibit or promote the calcification process. Selective gene knockout models for bone associated proteins such as osteoprotegerin and osteocalcin [33] or selective gene knockout models of VSMC proteins such as smad6[34] and matrix gla protein[35] all develop varying degrees of arterial calcification. Whether a final common pathway exists in the development of vascular calcification will require more extensive experimentation in animal models of CKD.

2.3 Animal models of CKD which develop vascular calcification

Research regarding the progression of changes in CKD has identified a remarkable consistency of phenotypes between animals and humans. Common to all models is an increased plasma creatinine, increased blood urea nitrogen, hyperparathyroidism and hyperphosphatemia. However, these rodent models of CKD generate a range of severity in the vascular calcification phenotype possibly due to a lack

of consistency in genetic background, degree of kidney damage, time course of study, and dietary regimen.

Since the first publication in 1889, numerous investigators first studied kidney disease by surgically reducing kidney mass by 2/3 or 3/4 in various animal species. In 1932 Chauntin and Ferris then developed the 5/6 nephrectomy (5/6Nx) rat model which has been used ever since [36]. The hallmark of all these studies is the development of uremia, although other complications similar to the human condition have also been evident. To the best of our knowledge, the first report of vascular calcification, using the 5/6Nx model, was published in 1979 [37]. Subsequently, the first mouse model of CKD was reported by Gagnon *et al.* in 1987[38], an approach that has been used by several groups to study vascular calcification outcomes. In this model, similar to the 5/6Nx, most of the mass of one kidney is reduced by electrocautery, leaving the renal hilum and the adrenals intact, followed by contralateral nephrectomy. A third model, first reported in 1982[39] but which has received significant attention recently, is the induction of CKD using dietary administration of the renal toxin, adenine, in rats. While studying the metabolic fate of dietary purines, Yokozawa's group discovered that, in the setting of high dietary adenine, 2,8-hydroxyadenine is formed via oxidation by xanthine oxidase and due to its low solubility in water forms precipitates along the tubules and urinary tract causing nephrotoxicity and the development of symptoms that are similar to clinical CKD.

Although there are inherent differences between these three models, each challenge generates a certain level of CKD after an initial acute insult to the kidneys, and

the development of vascular calcification may or may not result in the following 4-36 weeks (tables1-4). The degree of vascular calcification that develops within these models has typically been detected via tissue chemical analysis followed by a qualitative tissue staining procedure for localization. In most cases tissue calcium is eluted in acid and the supernatant is measured using the cresolphthalein o-complexone method or atomic absorption. However the digestion of tissue with acid results in destruction of the tissue and whether increased calcium content is representative of actual calcification or just calcium excess requires confirmation via a von Kossa stain of previously fixed aortic sections. Although various visualization techniques have been used including Alizarin Red staining of whole tissue mounts, chemical quantification alone is insufficient to evaluate calcification of aortic tissue.

2.4 The 5/6 nephrectomy model of CKD

All variations of this model reduce the total mass of the kidney by $5/6^{th}$. The most common technique is a two step procedure which requires removal of the two poles of one kidney ($2/3^{rd}$ of the first kidney) followed by full nephrectomy of the opposite intact kidney one week later. Table 1 refers to vascular calcification outcome studies that have compared the 5/6 nephrectomy animal to SHAM controls. The primary differences between studies have been the duration of CKD used in the study, and dietary concentration of calcium and phosphate.

The $5/6^{th}$ Nx model has been found to produce serum creatinine levels which are on average about 2.2 fold higher than control animals (Table 1). Without the concurrent

use of vitamin D, the 5/6th nephrectomy model results in phosphorus levels that range up to 3.8-fold higher than control animals under dietary regimens containing between 0.9 and 1.2% phosphorus by weight. Hyperparathyroidism is a consistent feature of this model with levels increasing 2.4 to 77-fold over control animals, whereas serum calcium is minimally altered.

Ejerblad *et al.* (1979), using 5/6th Nx, were the first to report aortic calcification after 12-36 weeks using a 1% by weight phosphorus-containing diet (Table 1)[37]. Interestingly, the data suggested that without a high phosphorus diet, the commonly used Sprague Dawley rat strain was resistant to vascular calcification. For example, out of the eight studies that quantified aortic calcium content, in only one study (Cozzolino *et al.* [24]) was there even a minor increase in aortic calcium content using a diet that contained less than 1% phosphorus (Table 1 and 2). Therefore, either high dietary phosphorus (i.e. 1.2%) or the use of an agent that promotes arterial calcification, such as 1,25$(OH)_2D_3$ (calcitriol), has become a common constituent of this model.

Table 2 has data summarizing the results from various studies that allows for comparison of various treatment regimens (calcitriol, vitamin D analogues, phosphate binders, calcimimetics) in animals with CKD. In the 5/6 Nx model, concurrent administration of vitamin D appears to result in circulating phosphorus concentrations that are about 1.6-fold increased over sham-treated animals. Only mild elevations in calcium (less than 10%) are observed and yet there is substantial reduction in parathyroid hormone (PTH) when compared to CKD animals not receiving concurrent 1,25$(OH)_2D_3$ (from 37% to 83% reduction). With the administration of vitamin D (either calcitriol or

an analogue), vascular calcification appears to be accelerated in this animal model with at least a doubling of aortic calcium concentration and in one case a 21-fold increase over CKD animals not receiving vitamin D[40]. In general, if one also considers those studies which only demonstrate calcification with von Kossa staining (i.e. no vessel calcium concentrations), it is evident that in the 5/6 Nx CKD animal model, vitamin D-treated animals consistently demonstrate greater severity of calcification without causing major hyperphosphatemia as seen in the adenine model.

The major limitations of using the 5/6th Nx model for the study of vascular calcification have been the requirement of a two-step surgical procedure and the need to use either excessive dietary phosphorus or an accelerating agent such as vitamin D. Further, even with reduction of 5/6th of the renal tissue, SD rats are not prone to develop severe kidney damage, and as a result these animals do not routinely calcify in studies lasting less than 12 weeks (table 2). In contrast, there is clear evidence of calcification 24 weeks after surgery (table 1), even with dietary phosphorus less than 1%. That is, without high dietary phosphorus (≥1.2%) or supplementary calcitriol treatment young SD rats do not appear to develop calcification unless CKD is substantially prolonged.

2.5 Evidence regarding use of the 5/6Nx model to study the treatments of vascular calcification in CKD

Vitamin D causes a reduction in parathyroid hormone production, and for decades, calcitriol (active form of vitamin D) has been used to treat hyperparathyroidism. In the 5/6th Nx animal model, the administration of calcitriol promotes

hyperphosphatemia, mild hypercalcemia and increases the prevalence of vascular and tissue calcification, although the mechanism is not very well understood (Table 2). New vitamin D analogues (22-oxacalcitriol, paricalcitol, cinacalcet) have been developed that minimize the development of hypercalcemia while treating hyperparathyroidism as effectively as calcitriol. However, no clinical trial in humans have compared calcification outcomes between these newer vitamin D analogues and calcitriol; the only comparative studies have been performed in 5/x6 Nx animal models and consistently demonstrate the adverse effect of calcitriol on calcification in this model. (Table 2)

In 2003, Hirata *et al.* [41] compared the effect of calcitriol (0.125 ug/kg/day) and 22-oxacalcitriol (vitamin D analogue) (6.25 ug/kg/day) on serum PTH and aortic calcification in 5/6 Nx rats. Although both agents lowered PTH similarly, calcitriol produced a 7-fold increase in aortic calcium content whereas there was no effect in the 22-oxacalcitriol treatment group. Similar results were reported by Wu-Wong *et al*[42], Cardús *et al*[40] and Lopez *et al*, [43] all of whom compared the vitamin D analogue, paricalcitol with calcitriol. There was either no increase in aortic calcium content with paricalcitol over CKD controls (Wu-Wong) or a minimal increase in calcification (Cardus, Lopez) compared to the substantive impact seen in the calcitriol-treated groups. Henly *et al.* [44] (2005) compared calcitriol (0.25 to 0.28 ug/kg/day) to the calcimimetic, cinecalcet, and reported significantly increased calcification in all animals that received any calcitriol treatment. Interestingly, concomitant treatment with a calcimimetic (cinacalcet) reduced vascular calcification. Calcimimetics bind to calcium-sensing

receptors to increase their sensitivity to calcium, thereby reducing PTH production and secretion without inducing hypercalcemia.[44]

Other studies have employed the 5/6 Nx model plus concurrent calcitriol treatment to study the effect of bisphosphonates and ammonium chloride on the progression of vascular calcification. In one study calcitriol (1 ug/kg/day) was given to 5/6Nx animals, however the primary purpose was to investigate the dose-response effect of the bisphosphonate, etidronate on vascular calcification [45]. In another study the effects of metabolic acidosis (ammonium chloride) on vascular calcification was studied using 5/6Nx animals on calcitriol (1 ug/kg/day) [46]. Both groups were able to produce extensive accelerated calcification within 2-5 weeks, and consequently demonstrated a reduction in calcification with etidronate or ammonium chloride treatment respectively.

2.6 Mouse electrocautery model of CKD

The CKD model in mice using surface electrocautery of the kidney was developed by Gagnon *et al.* in 1987[38]. More recently this approach has been combined with transgenic mouse models to promote vascular calcification in the setting of either the metabolic syndrome (LDLR-/-) or accelerated atherosclerosis (apoE-/-) (Table 3). In this model, CKD is induced by surgical ablation of the kidneys. This is a two step procedure; initially the cortex of one kidney is electrocauterized paying careful attention not to destroy the adrenals and the hilum of the kidney. One week later, once the animals have recovered, the second kidney is nephrectomized. This procedure appears to produce variable severity of CKD with blood urea levels ranging from 1.5 to 4 fold higher than

normal animals (table 3). These animals also develop hyperparathyroidism (~3 fold PTH increase) but significant hyperphosphatemia is not always present and at most is a 1.5 fold increase over normal even when combined with high dietary phosphorus (1.65% phosphorus by weight). One interesting feature of this model, that differentiates it from the 5/6th Nx model, is the consistent development of mild hypercalcemia (1.1 to 1.2-fold increase over normal) in the CKD animals.

To the best of our knowledge, vascular calcification has not been reported in wild-type mice with CKD. However, extensive calcification develops in LDLR-/- and apoE-/- transgenic models using this electrocautery model. The LDLR-/- CKD model resembles clinical uremia with an associated metabolic syndrome as these mice have obesity, hypertension, insulin resistance and early type 2 diabetes. The LDLR-/- mice without CKD have a predisposition to accelerated vascular calcification and atherosclerosis on a high fat diet, however the addition of CKD produces a 1.5 to 2.6 fold increase in aortic calcium content (Table 3). Similarly, the apoE-/- fed a regular diet generates accelerated atherosclerosis in the form of increased intimal disease as well as medial calcification that becomes modestly elevated when CKD is induced in these animals.

A major limitation of the mouse electrocautery model is the high mortality rate of the animals, the wide variability in the severity of the CKD that is generated, and the impact of the consistent feature of mild hypercalcemia. Where reported, mortality ranges between11% and 30% and is in part due to surgery and anaesthesia as well as the complications of CKD. The major advantage of using mice is the ability to study

transgenic animals to further understand the mechanisms associated with either the acceleration or inhibition of calcification.

2.7 Evidence from studies using the electrocautery mouse model to assess mechanisms and treatments of CKD induced VC

Using this model, phosphorus binders, whether calcium-based or non calcium based (calcium carbonate or sevelamer respectively), appear to lower phosphorus levels (range from 27% to 42% reduction) and demonstrate a consistent decrease in aortic calcium content when compared to untreated CKD animals in both LDLR-/- and apoE-/- knockouts[47-49]. Interestingly, the phosphorus binder sevelamer is capable of reducing intimal calcification in apoE-/- CKD mice and therefore may also prevent atherosclerosis progression in this model [47]. It is well-known that sevelamer reduces cholesterol levels in humans suggesting that a non-phosphorus lowering benefit of this drug may exist [50-52]. These findings in the electrocautery mouse model are in correspondence with the 5/6 Nx and adenine models of CKD [24, 53] (table 2,3).

Davies *et al.* [54, 55] have investigated the link between low turnover osteodystrophy and vascular calcification in LDLR-/- mice with CKD receiving a high fat diet. Bone morphogenic protein 7 (BMP-7) is an important regulator of bone remodeling and development. It restores bone anabolic balance by stimulating bone formation and reduces serum phosphorus via increased uptake into bone. Treatment with BMP-7 lowered serum phosphorus and prevented CKD-induced vascular calcification. Whether reduced serum phosphorus is achieved by a phosphate binder (calcium-based or

sevelamer), or agents that stimulate bone formation (BMP-7), the result is reduced vascular calcification in this mouse model of CKD.

2.8 Adenine rat model of CKD

The adenine model has recently gained attention due to its relative ease of design and encouraging outcomes. Within four weeks on a 0.75% adenine diet, calcification of the tunica media of aorta has been reported, and combined with a low protein diet, the calcification outcome is more consistent[26]. This model, unlike the other models, does not require surgery, and has a high survival rate (the poorest survival rate from surgery or anesthesia is the mouse models). As demonstrated in table 4, following 4 weeks of an adenine diet, the creatinine levels are 4.9 to 10.5-fold higher than control animals. Serum phosphorus levels range from 1.2 to 2.6-fold higher than normal with severe secondary hyperparathyroidism despite minimal fluctuations in the serum calcium (table 4).

With dietary phosphorus concentrations ranging from 0.4% to 1.2%, the adenine model produces the highest serum phosphorus levels, likely due, in part, to the severity of kidney damage. With the severity of CKD produced in this model, vascular calcification is consistently generated without additional calcitriol (as in the 5/6th Nx model) or a genetic predisposition to calcification (as in the mouse electrocautery model). However, those studies which report weight loss, rats fed 0.75% adenine lost more than 30% of their initial weight within 4 weeks on the diet [26, 44, 56]. Whether the extensive medial calcification observed is due, in part, to weight loss and volume contraction or the adenine itself remains to be determined.

The adenine model has been used to study the role of phosphate binders (sevelamer and lanthanum) and the bisphosphonate, ibandronate, in modifying the vascular calcification phenotype (table 4). Compared to animals with CKD receiving adenine alone, these treatments resulted in significant reductions in aortic calcium content (99% reduction with ibandronate and 84% reduction with sevelamer).

The major advantage of the adenine model is that it does not require surgery to generate severe CKD. The major disadvantage is the weight loss that ensues when rats receiving a diet containing 0.75% adenine.

2.9 Other models of CKD

To date we have discussed three mild to moderate CKD models that have addressed the abnormal serum biochemistries (Ca, P, PTH and vitamin D) and bone abnormalities (i.e. the LDLR-/- CKD model) that accelerate vascular calcification. However the CKD models discussed thus far occur following an acute injury to the kidney which then develops persistent CKD. A recent report by Moe *et al.*[57] describes spontaneous development of CKD over time in rats with autosomal dominant polcystic kidney disease. Heterozygous Han:SPRD rats (Cy/+) develop azotemia at about 10 weeks of age and progress to uremia by about 40 weeks. These animals develop persistent elevated BUN at 20 weeks as well as increased creatinine, reduced body weight and anemia by week 38. On a normal phosphorus diet, these animals develop progressive hyperphosphatemia, hyperparathyroidism and bone abnormalities however medial calcification is not progressive and is only evident in 60% of the older animals by week

38. This model provides the advantage of spontaneous CKD but at the expense of a long duration of study. This model may, however, prove the most useful for the study of preventive strategies. Also of interest is the report of Persy *et al*. who successfully demonstrated *in vivo* calcification of living rats using high-resolution X-ray microtomography (micro-CT)[58]. Their method is reproducible and allows quantification of calcification without a need for *ex vivo* analysis, which could provide future studies a new perspective in designing interventions (Table 1-3).

2.10 Comparing studies in animal models to clinical trials in humans with CKD

There are few randomized controlled trials studying vascular calcification outcomes in humans with CKD despite the documentation of the extent of the clinical problem. The Treat-to-Goal study [59] compared two dietary phosphate binders (Sevelamar versus calcium-based phosphate binders) in patients with ESKD. Consistent with the findings in the mouse electrocautery model [47, 49] and the adenine model [53], humans with ESKD randomized to sevelamer had a slower rate of accumulation of calcium in the coronary arteries and the aorta after one year of treatment. These results have been further corroborated in a study examining incident dialysis patients (RIND) [23]. In both of these trials, treatment with calcium-based phosphate binders appears to enhance calcification.

The role of vitamin D in either the promotion or prevention of calcification in CKD patients remains controversial. In the 5/6 Nx model, the administration of calcitriol clearly accelerates tissue calcium deposition, a finding which is somewhat ameliorated

with vitamin D analogues. We found that the use of vitamin D was associated with either the absence of or only mild increases in calcium levels, the purported mechanism via which vitamin D may accelerate vascular calcification. Therefore, the results of these animal studies suggest that vitamin D may have a direct effect on the vascular smooth muscle cell itself. Exploring this concept further in animal models is critical to the care of kidney patients given the frequency with which patients with ESKD are prescribed vitamin D preparations.

The role of calcimimetics in modifying coronary artery calcification in humans is currently under randomized-controlled study [60, 61]. To the best of our knowledge, there is no published human study evaluating its role in calcification whilst pre-clinical data demonstrating benefit is only just emerging. As demonstrated in the 5/6 Nx model [43, 44, 62] there may be a role for this calcium-sensing receptor antagonist in ameliorating vascular calcification in humans possibly through its well-described ability to lower serum calcium and phosphate or perhaps through its potential to up-regulate matrix gla protein (MGP), the VSMC protein considered the most important local inhibitor of vascular calcification.

Two animal studies (5/6 Nx and adenine), have demonstrated that bisphosphonates ameliorate calcification[26, 45]. Preliminary evidence in humans with CKD confirms these findings and randomized controlled trials evaluating this outcome are in progress [63]

Summary

In summary, animal models are useful to study CKD-associated vascular calcification and the results obtained in these pre-clinical animal studies appear to translate to the evidence, however limited, that exists in humans with CKD. Transgenic mouse models have proved their usefulness in studying potential mechanisms underlying the biologic process while studies in rats are useful to study the natural history of the process and the treatments that may modify it. By taking observations made in humans in CKD and subjecting those observations to controlled experimentation in animals, we may be able to better understand and control the epidemic of cardiovascular disease in patients with kidney disease.

Table 1 – 5/6 Nephrectomy model (without vitamin D) – Up-arrow indicates fold increase over sham/control

Reference	Animals	Duration Post Nx Wks	Diet	**Groups**	Serum Cr	Serum PTH	Serum P	Serum Ca	Aortic Ca content	Von kossa analysis
1979 Ejerblad *et al*[37]	SD 200-334g (n=366)	12/36	2% Ca 1% P	**Nx -12wks**	↑2.8x	NA	↑1.7x	0.92x	NA	-/+
				Nx -36wks	↑4.0x		0.96x	↑1.0x		++
1980 Haut *et al.* [64]	SD rats (n=54) Partial Nx or Uni-Nx	18	0.5, 1, 2% P	**Uni-Nx+ 2% P (n=6)**	↑2.3x	NA	↑2.6x	NA	↑165x	NA
2003 Cozzolino et al. [24]	6wks old SD rats (n=97+)	24	0.6% Ca 0.9% P	**Nx**	↑2.6x	↑77x	↑2.4x	1.0x	↑5.5x	
2005 Mizobuchi *et al.* [65]	7wks old SD rats (n=52) 5/6Nx	10	0.4% Ca 1.2% P 300IU/kg Vit D	**Nx+high P**	↑2.1x	↑2.4x	↑3.8x	0.99x	NA .	+++

Table 1: List of abbreviations

SD: Sprague-Dawley Nx: nephrectomy
Cr: creatinine Ca: calcium
PTH: parathyroid hormone P: phosphorus

Table 2 – 5/6 Nephrectomy model with vitamin D - Up-arrow indicates fold increase over sham/control and down arrows indicate percent reduction from Nx group

Table 2: List of abbreviations

SD: Sprague-Dawley
Nx: nephrectomy
PTx: parathyroidectomy
Cr: creatinine
PTH: parathyroid hormone
P: phosphorus
Ca: calcium

Reference	Animals	Diet	Groups	Serum Cr	Serum PTH	Serum P	Serum Ca	Aortic Ca content	Von Kossa analysis
1984 Krog *et al.* [66]	225-335g SD rats 3/4Nx 12wks	1.0% Ca 0.6% P 1000IU vit D3	**Nx**	↑5.3x	NA	↑1.4x	1.0x	NA	+
			Nx+1α(OH)D_3 (0.1ug/kg)	↑6.5x		↑1.8x	1.0x		+++
			Nx+PTx+1α(OH)D_3 (0.1ug/kg)	↑3.9x		↑1.5x	0.92x		-
2003 Hirata *et al.* [41]	7wks old SD rats (n=47) 5/6 Nx 12wks	1.18% Ca 1.03% P	**Nx**	NA	↑7.3x	↑1.3x	1.0x	1.1x	NA
			Nx+22-oxacalcitriol (6.25ug/kg)		0.62x	↑1.5x	↑1.2x	↑1.6x	
			Nx+Calcitriol (0.125ug/kg)		↑1.8x	↑1.6x	↑1.2x	↑7.3x	
2005 Henley et al. [44]	SD rats 5/6 Nx (ligation) <4weeks (26 days)	1.17% Ca 1.0% P	**Nx**	↑2x	↑↑↑	↑1.1x	↑1.0x	NA	-
			Nx+Cinecalcet (10mg/kg) n=10	↑2x	↓66%	↑1.3x	0.9x		-
			Nx+Calcium (0.28ug/kg) n=8	↑2.2x	↓69%	↑1.5x	↑1.1x		+++
			Nx+Calcitriol +Cinecalcet n=10	↑2.5x	↓88%	↑1.8x	1.0x		+++
2006 Lopez *et al* [62].	250g Wistar 5/6Nx 8wks	0.6% Ca 0.9% P	**Nx**	↑2.0x	↑3.0x	0.94x	1.0x	1.0x	NA
			Nx+Calcitriol (0.08ug/kg)	↑2.2x	↑1.2x	↑1.5x	1.0x	↑4.2x	
			Nx+R-568 (1.5mg/kg)	↑1.9x	↑1.9x	0.96x	1.0x	1.0x	
2006 Ruth Wu-Wong *et al.* [42]	Male SD rats 5/6Nx ~10wks	0.6%Ca 0.9%P	**Nx**	↑2.8x	↑17x	↑1.8x	0.83x	1.0	NA
			Nx+1α(OH)D_2 (0.17ug/kg)	↑2.5x	↑1.8x	↑1.5x	1.0x	↑28x	
			Nx+Paricalcitol (0.17ug/kg)	↑2.1x	↓95%	↑1.5x	↑1.1x	1.0	
2007 Tamura *et al.* [45]	7.5wks old Wistar-Imamichi - 5/6Nx 5wks	4% Ca 1.8% P	**Nx+Calcitriol (1ug/kg)**	↑1.6x	NA	↑1.1x	1.0x	NA	+++
			Nx+Calcitriol+Etidronate (1ug/kg;10mg/kg)	↑1.4x		1.0x	0.9x		+
2007 Cardús el al. [40]	Male SD 200-225g 5/6Nx 8wks	NA	**Nx**	NA	↑↑↑	6.1	11.0	-	NA
			Nx+Paricalcitol (3ug/kg)		↓64%	1.1x	1.1x	↑6.5x	
			Nx+Calcitriol (1ug/kg)		↓83%	1.1x	1.1x	↑21X	
2008 Mendoza *et al.* [46]	Male 250g Wistar. 5/6Nx 2wks	0.6%Ca 0.9%P	**Nx group**	↑1.7x	↑4.1x	↑1.1x	1.0x	1.0x	NA
			5/6Nx+Calcitriol (0.08ug/kg)	↑1.9x	↑68%	↑1.5x	↑1.1x	↑2.0X	
			5/6Nx+Calcitriol+Acidosis (0.08ug/kg, 0.3%)	↑2.0x	↓96%	↑1.6x	↑1.1x	0.67x	
2008 Lopez *et al.* [43]	Male 250g Wistar – 5/6 Nx 26days	0.6% Ca 1.2% P 500 IU/kg Vitamin D	**Nx**	↑2.0x	↑10.1x	↑2.3x	0.44x	↑1.5x	-
			Nx+Calcitriol (0.08ug/kg IP)	↑3.3x	↑5.6x	↑3.3x	0.89x	↑6.7x	+++
			Nx+Paricalcitol (0.24 ug/kg)	↑3.2x	↑4.3x	↑2.7x	0.78x	↑2.8x	++
			Nx+AMG 641 (1.5mg/kg)	↑1.6x	↑1.9x	↑1.5x	0.80x	1.3x	-

Table 2 – see pg. 26 for details

Table 3 – Mouse Electrocautery model Up-arrow indicates fold increase over sham/control and down arrows indicate percent reduction from CKD group

Reference	Animals	Duration post Sx (wks)	Diet	**Groups**	BUN	PTH	P	Ca	Ca content	Von kossa analysis
Davies *et al*, 2003 [54]	10 wk LDLR (n=59)	18	High Fat diet	**CKD**	↑3.5x	NA	NA	NA	↑2.6x	NA
				CKD BMP-7	↑2.8x				↓70%	
Davies et al, 2005[55]	10 wk Male/female LDLR (n=64)	14	High Fat diet	**CKD**	↑2.33x	↑3.5x	↑1.5x	0.92x	↑6.0x	NA
				CKD + $CaCO_3$ (1%)	↑2.6x	↓97%	↑1.15x	↓32%	↑2.7x	
				CKD + $CaCO_3$ + BMP-7	↑4.1x	↑1.1x	↑1.2x	1.0	↑1.4x	
Massy et al, 2005[67]	8 wk ApoE (n=48)	6	regular chow	**CKD**	↑3.6x	↑3.6x	↑1.1x	↑1.1x	NA	++
Phan et al, 2005[47]	8 wk ApoE (n=48)	10	regular chow	**CKD**	↑3.3x	↑2.6x	↑1.1x	↑1.1x	NA	++
				CKD + Sev (3%)	↑3.6x	↓70%	↓28%	↑1.1x		+
Mathew et al, 2006[49]	12 wk LDLR (n=50)	14	High Fat diet	**Fat CKD**	↑1.6x	↑19.3x	↑1.6x	↑1.2x	↑1.5x	NA
				Fat CKD + Sev (3%)	↑1.5x	↓98%	↓42%	↑1.4x	↓54%	
Phan *et al,* 2008[48]	8wk ApoE (n=70)	8	regular chow	**CKD**	↑3.0x	↑3.2x	1.0	↑1.1x	NA	++
				CKD + CaCO3 (3%)	↑2.8x	↓79%	↓28%	↑1.2x		+
Ivanovski *et al*. 2009[68]	8wks old female ApoE (n=48)	10	NA	**CKD -/-**	↑2.6x	NA	↑1.3x	↑1.2x	NA	++
				CKD -/- + Cin	↑3.3x		↑1.1x	↑1.1x		+
				CKD + Calcitriol	↑3.3x		↑1.5x	↑1.7x		+++
Maizel *et al.* 2009 [69]	8wks old Female ApoE	6-10wks	Standard Chow (0.98% Ca,	**WT CKD – 6 wks**	↑3.4x	NA	1.0	1.1	NA	+
				ApoE-/- CKD – 6wks	↑4.8x		1.0	1.1		+++

Table 3: List of abbreviations

LDLR: low density lipoprotein receptor
ApoE -/- : apolipoprotein E knock-out mouse
C57B WT: C57Black wild-type
CKD: chronic kidney disease
BMP-7: Bone morphogenic protein 7
$CaCO_3$: calcium carbonate
BUN: blood urea nitrogen
PTH: parathyroid hormone
P: phosphorus
Ca: calcium
High Fat Diet: 0.6% Ca, 0.6% P High Fat/High cholesterol (42% fat, 0.15% cholesterol)

Table 4 – Adenine model – Up-arrow (or no arrow) indicates fold increase over sham/control and down arrows indicate percent reduction from Ad group

Reference	Animals	Duration (total wks)	Diet	**Groups**	Cr	PTH	P	Ca	Calcium amount	Von Kossa analysis
2003 Katsumata *et al.* [53]	12wks Wistar (n=36)	8 (4Ad)	0.75% Ad, 0.92% P 1.06% Ca	**Ad n=10**	↑4.9x	↑14.7x	↑1.2x	0.86x	↑20.8x	NA
				Ad+Sev 1% n=10	↑7.0x	↓89%	↑1.4x	↑1.1x	↑2.9x	
				Ad + Sev 2% n=11	↑6.0x	↑1.4x	0.7x	↑1.1x	↑3.3x	
2006 Tamagaki *et al.*[70]	8wks Wister (n=45)	6Ad	0.75% Ad 0.6% P 1.2% Ca	**Adenine n=30**	↑5.4x	↑19.0x	↑2.2x	0.90x	NA	+++
2006 Price *et al.* [26]	13wks SD (n=47)	4Ad	0.75% Ad 0.92% P 1.06% Ca 2.5%/25% Protein	**Ad+25% Protein (n=11)**	↑9.3x	↑27.5x	↑ 1.9x	0.92x	↑5x	++
				Ad+2.5% Protein (n=13)	↑10.5	↑34.3x	↑2.2x	0.77x	↑81x	+++
				Ad+2.5%+Ibandronate (n=11)	↑10.4x	↑39.5x	↑ 2.3x	0.65x	1.0	-
2007 Neven *et al.* [71]	10wks Wistar (n=50)	10 (4Ad)	0.75% Ad 1.03/1.2% P 1.06% Ca	**Adenine n=39**	↑4.9x	↑ 8.9x	↑2.6x	0.59x	↑210x	+++
2009 Henley *et al.* [56]	350-390g SD rats (n=55)	4Ad	0.75% Ad 0.9% P 1.1% Ca	**Adenine**	↑11.7	↑16.1	↑3.2	1.0	NA	++
				Adenine+AMG641	↑9.3	0.37	↑3.0	0.9		-
				Adenine+Calcitriol	↑11.0	↑11.3	↑2.6	1.0		+++
2009 Neven *et al.* [72]	10wks Wistar (n=n/a)	8wks (4Ad)	0.75% Ad 0.92% P 1.0% Ca	**Adenine**	↑3.9	↑10.5	↑1.4	0.6	NA	-
				Adenine+2%Lanthanum	↑4.9	↑18.5	↑1.5	0.6		++
										-/+

Table 4: List of abbreviations

SD: Sprague-Dawley
Ad: dietary adenine
Cr: creatinine
PTH: parathyroid hormone
P: phosphorus
Ca: calcium

Chapter 3

Development of the CKD model

3.1 Introduction

The development of methodologies for CKD models in which VC (see chapter 2) occurs can be separated into two major categories: those which require a surgical procedure, or those which do not. Initially we sought to use the kidney surface electrocautery surgical mouse model, but for very practical reasons the non-surgical dietary adenine rat model proved to be a more robust as well as reproducible. The electrocautery model has been used extensively with transgenic and knockout mice to study VC, although surprisingly it has not been used as often with wild-type mice. It was not clear from the literature why this approach was not more commonly used, although in our own preliminary studies we discovered the main disadvantage of the electrocautery approach in wild-type mice was a high mortality rate and inconsistent calcification. In contrast, as reported by others and confirmed by our lab, the adenine model of CKD in rats develops consistent VC. In fact, following our selection of the adenine model this approach has gained significantly greater attention by investigators, despite the fact that published studies report a substantial weight loss. Previous reports did not explain the cause of the weight loss, although our own preliminary data suggested the main issue was reduced palatability when using a 0.75% adenine containing diet. Following this we sought out to modify this model to produce a more robust CKD model in which VC occurs consistently.

3.2 Methods

i) Electrocautery Mouse Model

Renal failure was induced by a two step procedure using electrocautery (unit: ELMED ESU 30, electrosurgical unit, ELMED Inc, Addison, Il, USA). Initially the cortex of the right kidney was completely electrocauterized followed by left kidney nephrectomy. 20-25g C57B/L male Mice were anaesthetized using ketamine and xylazine (1mg/kg/mouse i.p.) and placed on their left side. After shaving the area of the skin directly above the right kidney, the kidney was approached via a two centimeter lumbar incision. The adrenal was carefully separated from the kidney tissue by blunt dissection, and the right kidney was lifted out of the renal fossa and exposed on the surface with the capsule still intact. The animal was carefully lifted and placed on the electrocautery plate. The surface of the kidney was then completely electrocauterized without injuring the 2mm section around the hilum. The cauterization was performed in a systematic fashion starting from the superior pole down to the inferior pole, until the entire surface had been cauterized. Following this procedure the kidney was placed back into the renal fossa and the incision was closed using 3-0 silk sutures.

The mice were monitored daily and allowed to recover for two weeks before the second procedure was performed. The analgesic, buprenorphine (Temgesic 0.1mg/kg s.c.) was administered daily as needed. After two weeks of recovery the mice were anaesthetized in a similar fashion and the intact healthy left kidney was exposed. The vessels leading into the kidney were tied off using 3-0 silk and then using a surgical blade the left kidney was carefully

removed. The incision was sutured and once again the animals were monitored and allowed to recover.

ii) Adenine diet

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 7:00am). Animals were allowed to acclimatize for at least 1 week before initiation of the experiment and were given Purina Rat Chow and water *ad libitum*.

At the start of the experiment, the normal Purina Rat Chow was exchanged with a specially formulated (but nutritionally balanced) diet (Harlan, Teklad, Madison, WI) on which the animals were maintained until kidney failure was achieved. The specially formulated diets contained adenine at different concentrations; 0% (n=7; control), 0.25% (n=6, diet D), 0.50% (n=5, diet C) or 0.75% (n=9, diet A or B). Initially an adenine concentration of 0.75% was used based on previous study[26] which contained 2.5% protein, 1.06% Ca and 0.92% P but with the addition of artificial flavours to increase palatability (bacon and sugar flavours). In the next studies we used a lower adenine concentration with a few more modifications to increase the incidence of vascular calcification. We used two formulated diets both containing 6% protein, 1% P, 1% Ca and 0.2ppm vitamin K, however at differing adenine concentrations (0.25% and 0.5%). The 6% protein was chosen to be considered a low protein diet similar to Price *et al.* however high enough not to impact the growth of the animals based on a study done by Du *et al.*

[73] The level of vitamin K was chosen to be lower than in normal diet perhaps to reduce vitamin K dependent inhibitory proteins of vascular calcification (i.e. MGP).

Various diets used in the development of the adenine CKD rat model

Diet A:

Company:	TestDiet
Adenine:	0.75%
P:	0.95%
Ca:	1.12%
Vitamin K:	0.29ppm (menadione)
Vitamin D:	1 IU/g
Protein:	2.50%
Other:	APPLESAUCE

Diet B:

Company:	Harlan Teklad
Adenine:	0.75%
P:	0.94%
Ca:	1.20%
Vitamin K:	0.75ppm (phylloquinone)
Vitamin D:	1IU/g
Protein:	2.50%
Other:	Bacon and Sucrose artificial flavours

Diet C:

Company:	Harlan Teklad
Adenine:	0.5%
P:	1%
Ca:	1%
Vitamin K:	0.2ppm
Vitamin D:	1IU/g
Protein:	6%
Other:	None

Diet D:

Company:	Harlan Teklad
Adenine:	0.25%
P:	1%
Ca:	1%
Vitamin K:	0.2ppm
Vitamin D:	1IU/g
Protein:	6%
Other:	None

iii) Weight measurement

Based on previous studies[26] and our own observations, the rats fed a 0.75% adenine diet were expected to lose up to or more than 30% of their initial weight. Therefore, once the animals were given one of the adenine diets their weights and food intake were monitored on a daily basis, and supplemented with normal chow and/or Nutri-Cal if their weight had dropped 10-15%.

iv) Blood collection – parameters measured at KGH

In order to monitor serum creatinine levels during the study, blood was collected at different time points via the saphenous vein (2-4 weeks). Small volume of blood was collected in microcapillary tubes (~70μL) via the saphenous vein using a 23gauge needle while the animal was in restraint. A maximum of 4 tubes of blood were collected from each animal. The tubes were then spun in a microcapillary centrifuge at 1800g for 15 minutes. The plasma from this sample was carefully collected and transferred to a 1 mL eppendorf tube and diluted to a total volume of 300 μL with 0.9% Na saline. The samples were sent to the Clinical Chemistry Core Laboratory (Department of Pathology and Molecular Medicine, Kingston General Hospital, Kingston, Ontario, Canada) for blood creatinine, blood urea, calcium, and phosphate values to be determined. The results were corrected for the dilution factor.

v) Tissue collection

At the end of the studies, blood samples were collected from the inferior vena cava and spun (at 4°C, 4000g, 20 min) using a BHG Hermle Z320K refrigerated centrifuge (Mandel Scientific Company Inc, Gosheim, Germany). The plasma was used to assess the blood uremic

parameters. Levels of creatinine, urea, phosphate and calcium were measured by the Clinical Chemistry Core Laboratory at KGH.

The heart was excised and the right ventricle was separated from the left ventricle and the septum. The left and right ventricles were weighed, and the degree of left ventricle hypertrophy determined from the increase in the ratio of LV to body weight. Thoracic aorta and abdominal aorta and sections of the femoral were cleaned of extraneous connective tissue using scissors under microscope. A 5mm section of thoracic aorta was kept for von Kossa analysis, but all other collected tissues were snap frozen in liquid nitrogen and stored in a -80°C freezer for further analysis.

vi) Calcium Determinations

Previously frozen thoracic aorta tissue was thawed and a 10mg section was used for calcium analysis. Aortic tissues were weighed, and homogenized in 0.6N hydrochloric acid (HCL). Calcium was eluted out of the tissue by HCL for 24 h at 4°C. Then the calcium content in the supernatant was determined colorimetrically using the O-cresolphthalein complexone method (Sigma). 50 µL of supernatant was removed and serial dilutions (1/10, 1/100, 1/1000) were made in a 96 well plate. The pH of each well was adjusted to 10.7 using 150 µL of 2-amino-2-methyl-1-propanol buffer. Then 100 µL of o-cresolphthalein colour reagent is added to enable formation of a purple complex with the available calcium. The absorbance was measured for both standards and the tissue homogenates at 540nM (SynergyHT Microplate Reader, Bio-

Tek Instruments Inc, Winooski, VT, USA). If the results were beyond the linear range of the assay, the sample was diluted to bring it into range.

vii) Von Kossa method of visualizing vascular calcification

The thoracic aorta was fixed in 10x neutral phosphate-buffered saline with 4% paraformaldehyde over night. 2-3mm rings were embedded upright in paraffin blocks, so that every aortic section comprises on average eight to nine cross-sections (minimum six) at different sites along the vessel. Sections (3-4μm) were stained for calcification with Von Kossa's method. In brief, the sections were first deparaffinized and then re-hydrated to distilled water. Then they were placed in 1% silver nitrated and exposed to ultraviolet light (20 min). Afterwards they were placed in 5% sodium thiosulfate (2 min) and finally counterstained with nuclear Fast Red (5 min). Areas of calcification appeared as dark brown regions in the medial wall of the aortas.

viii) Statistical analysis

Urea levels from electrocautery mice were presented as mean ±SD and mean differences were determined by paired student's t-test from urea levels in week 1. All data from the adenine studies were presented as mean ± SEM. The differences between diet groups were analyzed using one-way analysis of variance (ANOVA) followed by Newman-keuls *post hoc* test to compare all pairs of diet groups. P-value <0.05 was considered significant. Analysis was performed using Microsoft Excel (paired t-test) or Graph Pad Prism v.5.

3.3 Results

Development of the CKD model

1) Electrocautery mouse model

Mice which survived the electrocautery procedure had elevated blood urea after 3 weeks of kidney damage (n=9 out of 19; figure 3.1). In contrast, blood creatinine was found to be below detection levels for all mice. These mice maintained their elevated blood urea for the remainder of the study. The study was 12 weeks long, but after 6 weeks of uremia four animals were sacrificed to assess calcification status. No calcification was detected in the aortas with the von Kossa staining technique at either time point.

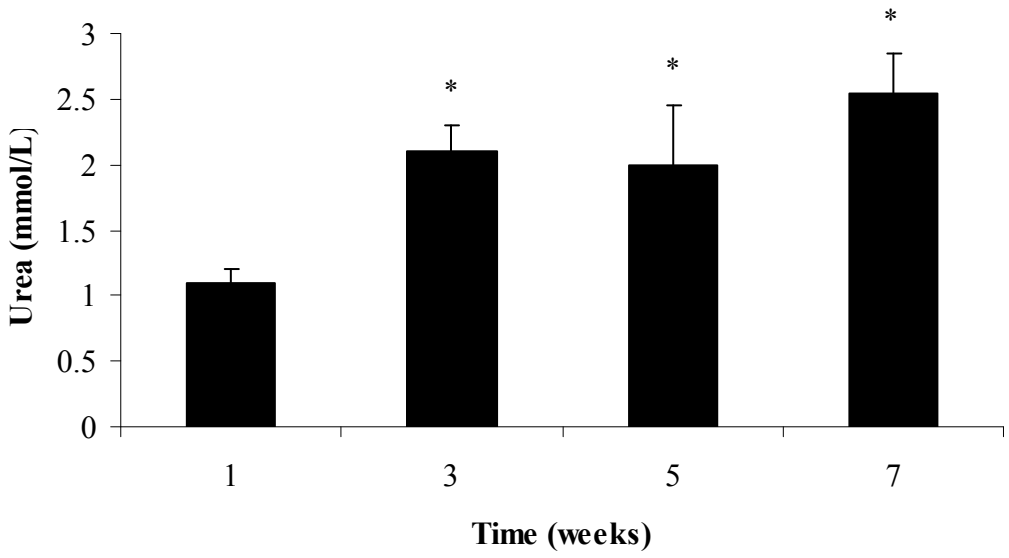


Figure 3.1 – Electrocautery mice develop uremia. Serum urea measured in blood collected via the saphenous vein of mice without the use of anesthesia (n=9). All time points (3,5 and 7) are significantly ($p<0.05$) higher than week 1. Data are presented as mean of serum urea concentration ±SD.

*** -** significantly higher than week 1

2) Adenine rat models

i. Impact of dietary 0.75% adenine (diet A and B) on weight loss and palatability

Rats (n=5) on diet A were supplemented with applesauce (for palatability), normal Purina chow or Nutri-Cal. These rats consumed on average 30.3±4.0 grams of food daily. Of this amount, 24% by weight contained Diet A (7.2±1.0g) and 76% contained supplements (22.9±2.7). The rats gradually lost weight over the course of the adenine diet regimen (Figure 3.2). The starting average weight was 512±21g, and by the end they lost on average 12.9% of their initial weight (final average weight: 446±24g)

Rats (n=9) on diet B were supplemented with normal Purina Chow and/or Nutri-cal when needed. These rats consumed 5.4±1.8g of Diet B daily. Their weight dropped from 369±10g to 296±19g by day 17, but gradually regained weight by day 24 (Figure 3.2). These rats had lost 14.7% of their initial weight on the last day of adenine diet feeding (diet B).

ii. Impact of dietary 0.75% adenine on kidney function and calcification

Rats on diet A as well as those on diet B had significantly elevated blood creatinine and urea concentrations, however only rats receiving diet B developed significant hyperphosphatemia. Serum calcium was not significantly different between the groups (Table 3.1). There was also a small, but statistically significant, elevation in thoracic aorta calcium content in the group which consumed diet B (Figure 3.3); however no calcification was detected via the von Kossa method (data not shown). Rats on diet C and D (0.5% and 0.25% adenine) had tissue calcium content above 25μg/g (see Chapter 4 for details). Regardless of adenine concentration and diet, and with duration of 4-5 weeks, animals eating a total amount of 1.0g or more of adenine developed high blood creatinine and urea, hence CKD (Table 3.1).

iii. Effect of decreased adenine concentration on palatability and bodyweight

Five rats given Diet C (0.5% adenine) consumed 6.9±2.7g per day over a period of six weeks, whereas rats given Diet D (0.25% adenine) consumed more (18.5±2.4g/day) than any of the other diets (0.5% or 0.75% adenine) and maintained their body weight longer (Figure 3.4). The rats which were given Diet C (0.5% adenine) lost 15±6% of their initial body weight, but rats given Diet D (0.25% adenine) lost only 8±6% of their initial body weight. Also with the lower adenine concentration, they still ate the same amount of total adenine as compared to diets which had 0.75% adenine (Table 3.1).

iv) Von Kossa stain of thoracic aorta

In figure 3.5, four thoracic aortas have been stained with von Kossa method. Calcification is stained black/brown. Control animals did not have any calcification, however those animals fed an adenine diet either had low or no calcification (i.e. figure 3.5B), or they demonstrated marked calcification as seen in figure 3.5 C and D.

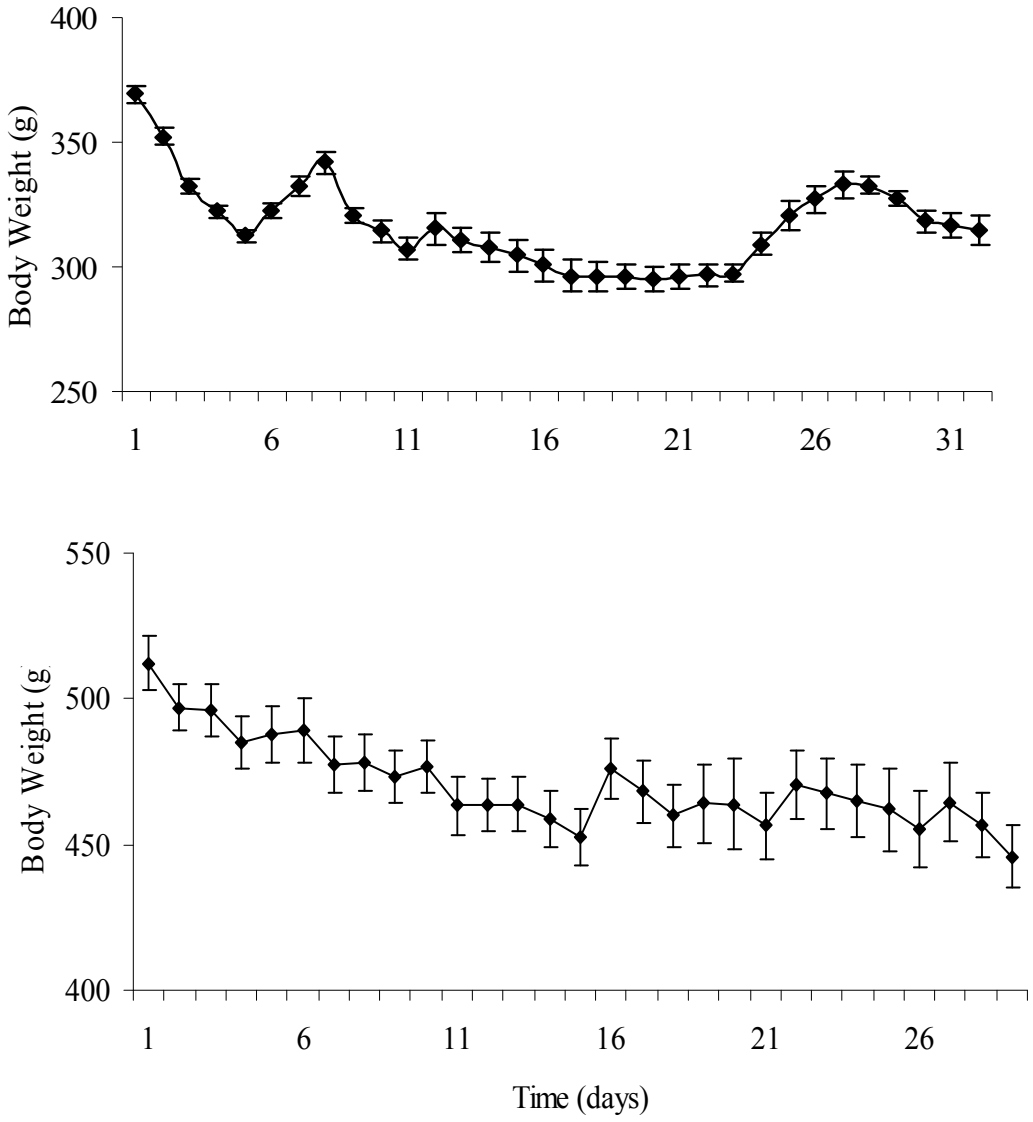


Figure 3.2 – Rats lose body weight with the 0.75% adenine diet. Upper panel: Average daily body weights (n=5) over the course of adenine Diet A trial (0.75% adenine). Lower panel: average daily body weight (n=9) over the course of adenine diet B trial (0.75% adenine). Data are represented as mean ± SEM.

Table 3.1 – Adenine duration and amount plus serum biochemical measurements

	Control (n=10)	Diet A (n=5)	Diet B (n=9)	Diet C (n=5)	Diet D (n=6)
Duration on adenine (wks)	7	4	4	5	5
Total adenine eaten (g)	-	1.4 ±0.2	1.0±0.3	1.1±0.3	1.4±0.2
Creatinine (µmol/l)	33.7±3.2	91.8±47.4*	80.9±29.1*	181.8 ±55.6*	170.3± 41.0*
Urea (mmol/l)	6.1±0.1	14.9±8.5	14.0±5.4	19.5 ±16.3	9.0±11.1
Serum Phosphorus (mmol/l)	2.4±0.21	2.6±0.35	3.1±0.92*	2.5±0.3	3.1±0.5
Serum Calcium (mmol/l)	2.4±0.06	2.4±0.04	2.2±0.32	2.3±0.1	2.2±0.3

* $p<0.05$ from control

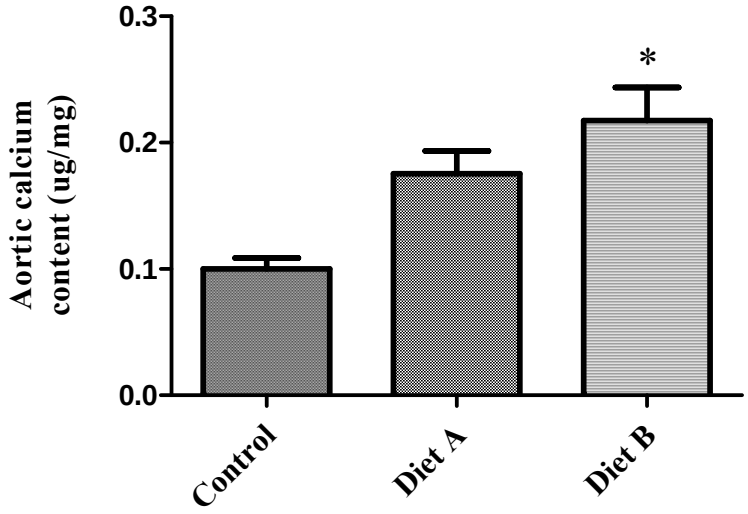


Figure 3.3 – CKD increases thoracic aorta content. Thoracic aorta calcium content, as measured by o-cresolphthalein complexone method, in control (n=12), Diet A rats (n=5) and Diet B rats (n=9). Data are represented as mean ± SEM. * $p<0.05$ from controls.

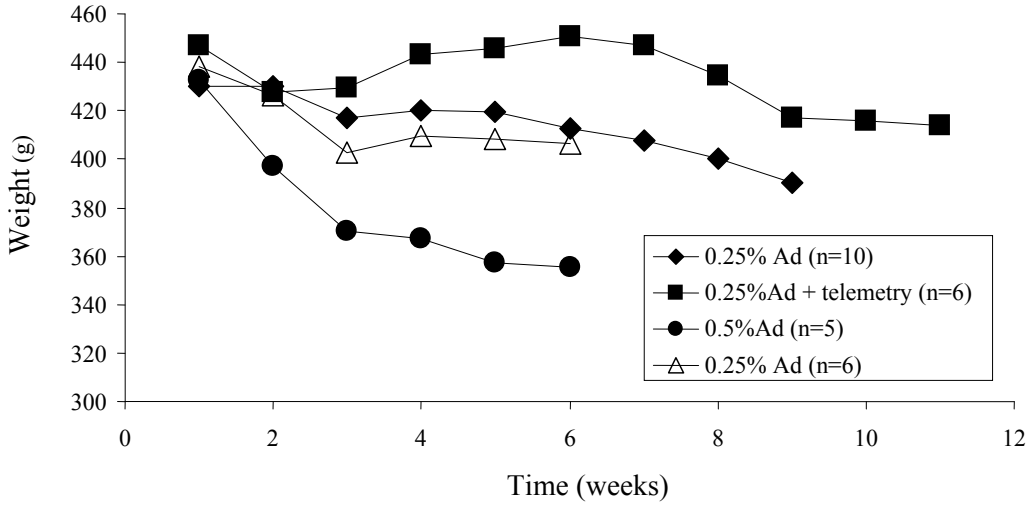


Figure 3.4 – Rats with do not lose more than 15% with the 0.5 or 0.25% diets. Average body weights of rats in four different studies conducted using either 0.25% or 0.5% adenine diet C. The rats which were given Diet C (0.5% adenine) lost 15±6% of their initial body weight, but rats given Diet D (0.25% adenine) lost only 8±6% of their initial body weight.

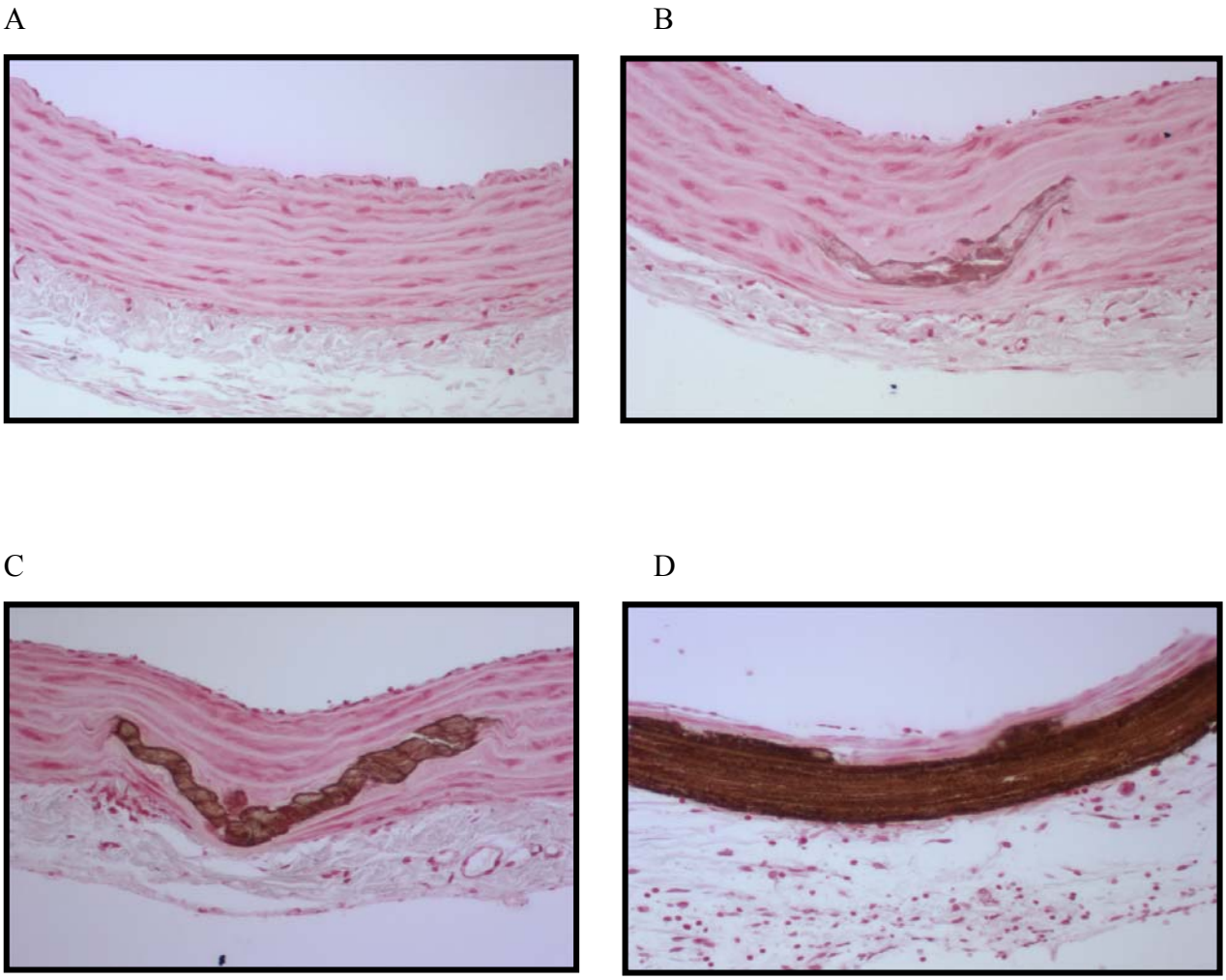


Figure 3.5 – CKD rats develop vascular calcification as detected by von Kossa staining. Control rat; A (100x). Adenine fed rats: panel B was given diet A, panels C and D were given diet C or D(100x). The tissue calcium content of panel C and D were above 50µg/mg, but panel A and B had tissue calcium content below 1µg/mg.

3.4 Discussion - Model Development

In the process of developing a robust rodent CKD model, the adenine CKD model was found to be more advantageous over other available rodent models of CKD such as the renal electrocautery model. In particular, the adenine CKD model was found to produce elevated serum creatinine which was representative of moderate to severe CKD. Compared to the other CKD models (electrocautery, 5/6 Nx), the adenine model has the clear advantage of not requiring a surgical procedure as well as a higher survival rate [48, 74]. In the adenine model both kidneys remain intact during the CKD progression yet the severity of kidney damage (as determined by serum creatinine level) is higher than the surgical models that involve uninephrectomy. The modifications that were done to the adenine model generated an approach that models moderate to severe CKD more effectively.

It appears the marked hyperphosphatemia, which causes secondary hyperparathyroidism[26, 53, 70, 70] in this model (although PTH was not measured), is predisposing the animals to vascular calcification (the mechanism as to how hyperphosphatemia is causing calcification is discussed in Chapter 4). This cause is consistent with clinical outcomes, that is, hyperphosphatemia and hyperparathyroidism are associated with CKD patients with calcification[22, 59, 75]. Most other rodent models of CKD developed to date are resistant to vascular calcification. That is, calcification in other models has been reported when supraphysiological doses of vitamin D (i.e. 5/6Nx model) are concomitantly administered, or when knockout mice were used (i.e. electrocautery model), in which case these modifications might not represent clinical settings closely.

Initially, the electrocautery method of inducing CKD in mice was considered to be a good approach to use in studies of CKD induced calcification, however, the failure of the model to produce calcification 12 weeks after the kidney damage was induced suggested otherwise. This result is likely because the level of kidney failure induced in the mice using this method was not as high as compared to adenine rat model. Most importantly, this technique involved a surgical procedure which had a very high mortality rate (~40-50%). The low recovery rate from the electrocauterization process could have been attributed to difficulties with anesthesia or the variability in kidney damage induced. This occurred despite that a systematic approach was followed during the electrocautery procedure. In the end, the surviving mice had variable kidney damage, but none developed severe kidney failure similar to the adenine rat model. To the best of our knowledge only one study has reported vascular calcification in wild-type mice (C54B/L). In that report the urea levels were similar to the ones in the present study[69]. Unlike that investigation, the aortas did not show any vascular calcification using the von Kossa method. Other groups, who utilize this model, have been successful in generating calcification only in mice which have a genetic predisposition to accelerated calcification (see Chapter 2). That is, models such as the LDLR or apoE knockout CKD mice have been found useful since they develop metabolic disorder as well as CKD. Complicating the interpretation is that the knockout mice still develop vascular calcification even without the induction of CKD. Although the presence of CKD further amplifies the VC the occurrence of a combination of disorders makes assessment of the etiology of the calcification process more difficult, i.e. is it the CKD or the metabolic disorder that has the greater impact on calcification.

In contrast, the adenine model, regardless of adenine concentration in diet, produced moderate to severe CKD with both elevated serum urea and creatinine, and the accompanying pathological manifestations (e.g. VC) appear to be a direct consequence of this not just a contributing factors to the process. The 5/6Nx model although consistent, only produces CKD with lower creatinine levels compared to adenine, and calcification is not usually present unless it is accelerated with the use of high-dose vitamin D (see Chapter 2 – Table 2). The results suggest that the adenine model is more robust with regard to development of VC. Further, the findings indicate that the severity of CKD can be manipulated both by duration and dose of adenine throughout the course of the study. The main limitation of the adenine model appears to be the severe weight loss; an attribute we were able to minimize in the modified approach used herein. .

In our preliminary studies, 0.75% adenine diet mixed with applesauce did not sufficiently enhance palatability in order to prevent weight loss. Lower adenine concentrations (0.5% and 0.25%) were more palatable, but all animals on the adenine diet did not gain weight at the same rate as control animals. This was expected in part, as previous reports have shown that dietary adenine can blunt growth in rats, an effect which can be reversed with a normal diet.[76] The main issue was that previous studies using dietary adenine in rats reported final body weight loss of 30% or higher[26, 27]; a severity which was deemed unacceptable for both scientific and ethical reasons. With the final modification, the method was improved such that the rats did not exceed 15% weight loss, and the rats still ate the same amount of total adenine if not more by the end of

the studies. That is, a lower dietary adenine concentration over a longer study period generated severe kidney disease similar to those in the study done by Price *et al.* with minimal weight loss.

Calcification in our pilot study was not severe; as only 2 of 14 animals given the adenine had visible calcification (using von Kossa staining method). However, tissue calcium content was elevated in adenine fed animals even if calcification was not detected histologically. Although serum calcium levels between all adenine fed and control animals were similar, serum phosphorous was elevated and thereby followed a similar trend as tissue calcium content. This suggests there is a mechanistic link between elevated serum phosphorous and elevated tissue calcium concentration (discussed in Chapter 4).

Other limitations of this model include a lack of full understanding regarding adenine toxicity in all cardiovascular tissues, even though evidence suggests adenine toxicity primarily impacts the kidneys. For example, Lindblad *et al.* (1972)[77] characterized adenine toxicity in dogs. They analyzed numerous tissues (such as myocardium, lung, brain and striated muscle) and they found adenine toxicity in the form of crystals only within the kidneys. Furthermore, Yokozawa *et al.* were able to characterize the toxic impact of dietary adenine on rat kidneys[39, 78-80]. Their studies revealed that after 30 days of dietary adenine, the kidney tissue had accumulated crystals within the lumen of the tubules, as well as the interstitium of cortex and medulla. They did not observe these crystal structures in the heart, brain, or liver tissue. They found the crystals resulted from accumulation of 2,8-hydroxyadenine, an oxidized metabolite of adenine. It was proposed that adenine might be oxidized by the enzyme xanthine oxidase (found in the kidneys

and liver). However, Story *et al.*, found that whenthe xanthine oxidase inhibitor, allopurinol, was co-administered with adenine, the prevalence of 2,8-dihydroxyadenine precipitation in kidney tissue was not reduced[76]. Furthermore, they did not find 2,8-dihydroxyadenine accumulated in the liver, a tissue which is known to have high levels of xanthine oxidase. Similarly, they did not find plasma contained significant levels of the 2,8-dihydroxyadenine. Although evidence is lacking but based on their finding, Story *et al.* proposed that it is the mitochondrial oxidases within the proximal tubules of the kidney that are responsible for the majority of the oxidation of adenine to 2,8-hydroxyadenine. Supporting these concepts, similar to Yokozawa's findings, all adenine diet studies to date have only reported toxicity within the kidney. [39, 76-81]

Taken together, it does not appear that adenine is directly involved in the initiating vascular calcification in the vascular media, but rather facilitates the process by generating a CKD milieu within the affected circulations. Furthermore, the adenine concentration is not a determining factor in generation of severe CKD. That is, the more palatable diet which has the lowest adenine concentration is more appealing to the animals, and creates a model of CKD with VC within 5 (or more) weeks. This could be due to increased food consumption, which also impacts the total amount of adenine converted to 2,8-dihydroxyadenine by the kidneys, resulting in increased kidney failure.

In conclusion, I have produced a model of CKD which can be used to study the pathophysiology of CKD induced VC. This model is robust and develops a range of CKD, which

allows me to analyze different stages of kidney disease and the development of vascular calcification. Furthermore, I learned that not the concentration of adenine, but the total amount of adenine consumed by the animals leads to kidney damage and uremia. Therefore, by reducing adenine concentration to fix the issue with palatability and weight loss, I can increase the duration of adenine feeding, which would perhaps advance the animals to a stage where vascular calcification is a more prominent feature. The other advantage is the ease of this model which requires no surgery, and the outcomes which represent clinical CKD closely. These animals develop consistent hyperphosphatemia which is a key criterion for studying vascular calcification. To study VC, the following experiments in Chapter 4 were conducted using diet containing adenine concentration of 0.25% (5 animals were given 0.5% diet, but were switched to 0.25% later on) for a duration of 6 or more weeks.

Chapter 4

Mechanisms of VC in CKD and its impact on the cardiovascular system

4.1 Introduction

The dietary adenine model, unlike the other models, does not require a surgical procedure to induce CKD. Furthermore, kidney damage can be manipulated by dietary intake, and as a result the stimulus can be controlled (i.e. increase adenine exposure to increase kidney damage) such that these rats are predisposed to vascular calcification. The pathophysiology of vascular calcification in this model has not been completely characterized. The link between calcification and changes in circulation (pulse pressure, diastolic and systolic changes) has not been established just as the association between calcification and LVH also needs to be better characterized. Therefore in this chapter, using the modified adenine model (see Chapter 3), the pathophysiology of vascular calcification is characterized. We investigate the impact of severity of CKD on vascular calcification. As mentioned earlier, serum creatinine levels are used as index of kidney function. However, serum metabolites might also be association with the pathogenesis of vascular calcification[23, 49, 59], therefore we investigate the association of serum calcium and phosphorous with VC. Furthermore, the longitudinal blood pressure profile of CKD with VC using radiotelemetry is assessed. Finally the association of vascular calcification and cardiac changes are studied.

Previous reports have shown that hyperphosphatemia is a common feature of this model which may lead to secondary hyperparathyroidism (Chapter 2, Table 3). In the current studies, although we did not measure PTH we investigated the association between hyperphosphatemia and vascular calcification. Regulation of phosphorous is primarily mediated by renal excretion in

healthy individuals. Phosphorous reservoirs in the body include 70% intracellular, 29% skeletal mineralization front and less than 1% in the blood[82]. Exit from the reservoirs is either by excretion through the kidneys, intestine secretion, or deposition in bone. In normal healthy adults, bone resorption equals bone deposition; however in CKD bone resorption might be higher. Therefore, it has been theorized that during CKD excess phosphate from bone, combined with impaired kidneys to excrete excess phosphate, result in high serum phosphorous leading to soft tissue calcium and phosphate deposition[55, 82]. In our studies, CKD animals developed hyperphosphatemia, and when we compared longitudinal serum phosphate levels, VC was associated with groups that demonstrated the highest intermediate phosphate levels. The current adenine model is a robust CKD model that can be used to investigate the pathology of CKD induced vascular calcification.

Overall the objectives in this Chapter were to investigate the impact of adenine induced CKD on vascular calcification, the link between circulatory changes and vascular calcification, the link between left ventricular hypertrophy, VC and CKD and the role of serum phosphate in the pathogenesis of VC.

4.2 Methods

(Additional methods which were not covered in Chapter 3 (i.e. other methods are described previously in Chapter 3 p30-p33)

i) Adenine diet

Based on previous experiments (see Chapter 3), only adenine diets C and D (pg. 39; 0.25% and 0.5% respectively) were used in the studies carried out in this Chapter. Only 5 animals were given adenine diet C (0.5%), and if weight loss was approaching 15%, they were switched to adenine diet D (0.25%). All other CKD animals (n=22) were given diet D which contained the lowest adenine concentration (0.25%). Although they were given lower adenine, the duration was extended (6-10 weeks), and the criteria for CKD were based on elevated serum creatinine. The animals were given the adenine diet for duration of 6 to 10 weeks. Blood was taken at intermediate time points, and level of creatinine was determined. The intermediate data presented in the Results section refers to blood taken 15 to 20 days before the animals were sacrificed (whether at 6 weeks or 10 weeks). The lower adenine was chosen as the main CKD inducing diet in this Chapter due to increased palatability and reduced weight loss.

ii) Telemetry

Under isoflurane anesthesia (dosed to effect by inhalation), SD rats were implanted with a radiotelemetric pressure transducer (model TA11PA-C40, Data Sciences

Inc). Following a midline section, the abdominal aorta was isolated. A fluid filled telemetric catheter was introduced into the lower abdominal aorta, such that it was positioned approximately 1 cm. below the left renal artery. The body of the transducer was sutured to the

muscular layer of the abdominal wall to prevent device movement. All animals were allowed to recover for 7-10 days before recording arterial pressure. Blood pressure profile and activity was determined from data collected every 4 minutes (30 seconds, 150 Hz) by a digital radio signal generated by each abdominal transducer and received by units under each cage (model RA1010, RA1020, or RPC-1; Data Sciences). Data was then transferred by a consolidation matrix (BCM100, Data Sciences) to the data acquisition system (Dataquest LabPRO or Dataquest ART, Data Sciences).

ii) Pulse wave velocity

Pulse wave velocity was assessed using the foot-to-foot method[83]. In order to do this, two catheters at the superior and inferior ends of the aorta were used to measure blood pressure simultaneously. Rats were anesthetized with ketamine (30mg/kg body wt i.p.; Rogar/STB, London, ON) and Inactin (thiobarbital sodium; 100mg/kg body wt i.p.; Sigma Chemical Co.) and additional ketamine and Inactin were given during surgery if necessary. Body temperature was monitored and maintained using a thermistor (model 402; Yellow Springs Instruments, Yellow Springs, OH) coupled with a temperature controller (model 73A; Yellow Springs Instruments), maintained at 37±0.5°C using a a heating pad and a lamp.

An incision at the cervical region was made to access the common carotid arteries. Once the right carotid artery was isolated by blunt dissection, it was cannulated using a PE-50 heparanized saline filled (50 IU/ml) cannula (ID 0.58mm, OD 0.965mm; Becton Dickson and Company, Sparks, MD). The cannula was advanced to the arch of the aorta and the free end was

connected to a pressure transducer (Cobe, Lakewood, CA). A bridge amplifier (ETH-400; CB Sciences, Milford, MA) connected to MacLab hardware (MacLab/8s; AD Instruments, NSW, Australia) was used to amplify the electrical signal from the pressure transducer. The transducer was calibrated between 40 and 160 mmHg using a sphygmomanometer. Blood pressure was recorded as a pulsatile waveform at a frequency of 1000Hz. A second midline incision was made and the common iliac arteries as well as the right femoral artery were isolated. A second identical catheter attached to another transducer but recording simultaneously using the same equipment as the first catheter was advanced into the right femoral artery until the tip reached the iliac bifurcation. Once we had obtained a stabilized pressure recording from both catheters (~20minutes), the animals were euthanized by drawing blood out of the inferior vena cava. At the end of the pressure recording, the distance from the tip of the superior catheter to the inferior catheter was measured using a 1-0 silk stretched between the two points. Pulse wave velocity was measured as the speed it takes for waveforms to travel from the carotid cannula down to the femoral cannula. It was calculated using the formula: PWV = propagation distance ÷ propagation time (m/s) as described previously[83]. At least 10 normal consecutive waveforms were individually analyzed and averaged. Systolic blood pressure, diastolic blood pressure as well as mean arterial pressure were calculated in Chart program using the Carotid catheter.

iii) Tissue collection

Following PWV measurements, blood samples collected from the inferior vena cava each were spun (at 4°C, 4000g, 20 min) using a BHG Hermle Z320K refrigerated centrifuge (Mandel Scientific Company Inc, Gosheim, Germany) and the plasma used to assess the blood uremic parameters at the end of study. Levels of creatinine, urea, phosphate and calcium were measured by the Clinical Chemistry Core Laboratory at KGH.

The heart was excised and the right ventricle was separated from the left ventricle and the septum. The left and right ventricles were weighed, and the degree of left ventricle hypertrophy determined from the increase in the ratio of LV to body weight. Thoracic aorta and abdominal aorta and sections of the femoral were cleaned of extraneous connective tissue using scissors under microscope. All collected tissues were snap frozen in liquid nitrogen and stored in a -80°C freezer for further analysis (see methods Chapter 3).

v) Statistical analysis

To analyze correlation between parameters, linear regression analysis was performed with creatinine and the following parameters: urea, phosphorous, calcium, tissue calcium, heart weight and pulse pressure. Tissue phosphate and tissue calcium linear regression analysis were also performed. As well, pulse pressure linear regression analysis was performed with tissue calcium, and cardiac mass (left ventricle and right ventricle). All data from control, CKD and CKD+VC groups were presented as mean ± SEM. The differences between the groups were analyzed using one-way analysis of variance (ANOVA) followed by Newman-Keuls post hoc test to compare differences between diet groups. An unpaired student's t-test was performed to

determine the difference between phosphorous of calcified (VC group) and none calcified (No VC group) animals. A paired student's t-test was performed to determine the difference between intermediate phosphate and end-study phosphate from the same group. P-values less than 0.05 were considered statistically significant. Analysis was performed using Microsoft Excel (paired t-test) or Graph Pad Prism v.5 (linear regression and ANOVA).

4.3 Results

Determination of CKD groups

The groups in this Chapter were chosen based on whether the animals were control or adenine fed, and whether those with adenine diet developed vascular calcification or not. Figure 4.i shows the distribution of tissue calcium of all animals. From this figure, I noticed two populations; those which had low tissue calcium (bins 1-7, ~0.3µg/mg calcium, black bard, Figure 4.i) and those which had a much higher tissue calcium (bins 13-16, ~63µg/mg calcium, Figure 4.i). Based on this figure, Figure 4.ii was generated for the tissue calcium distribution from control animals (Figure 4.ii a), adenine fed animals with low tissue calcium (Figure 4.ii b) and those with high tissue calcium (Figure 4.ii c). The adenine fed animals with low tissue calcium were not significantly different from control, but those with high tissue calcium had a 95% confidence interval between 46.5-65.5µg/mg, and were significantly higher than other groups. Therefore the groups were chosen as control (Figure 4.i a), adenine fed animal (CKD; Figure 4.ii b) and adenine fed animals with calcification (CKD+VC; Figure 4.ii c).

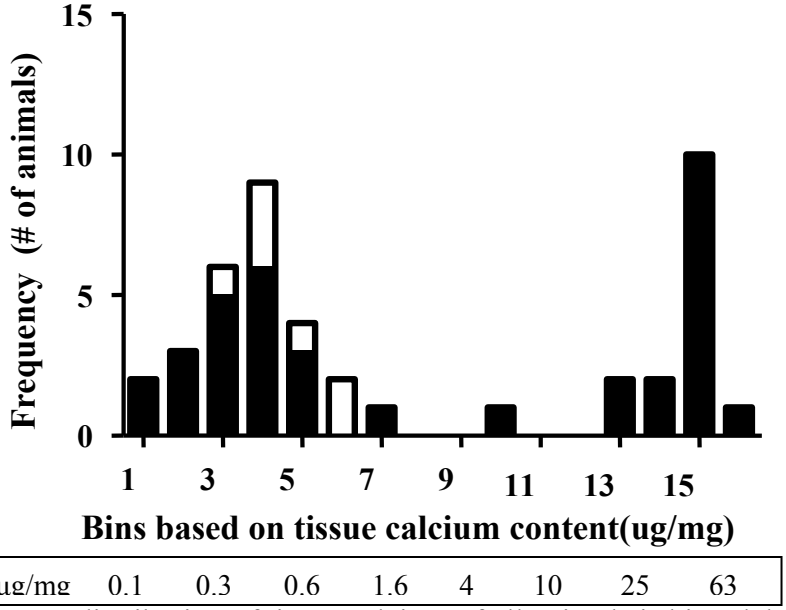


Figure 4.i – Frequency distribution of tissue calcium of all animals is bimodal. Black bars are adenine fed, white are control, and then 16 bins are based on log of tissue calcium content. There appears to be two distinct populations, those that have low tissue calcium, bins 1-7,10 on the graph (below 5µg/mg), and those that have high tissue calcium, bins 13-16 (above 25 µg/mg).

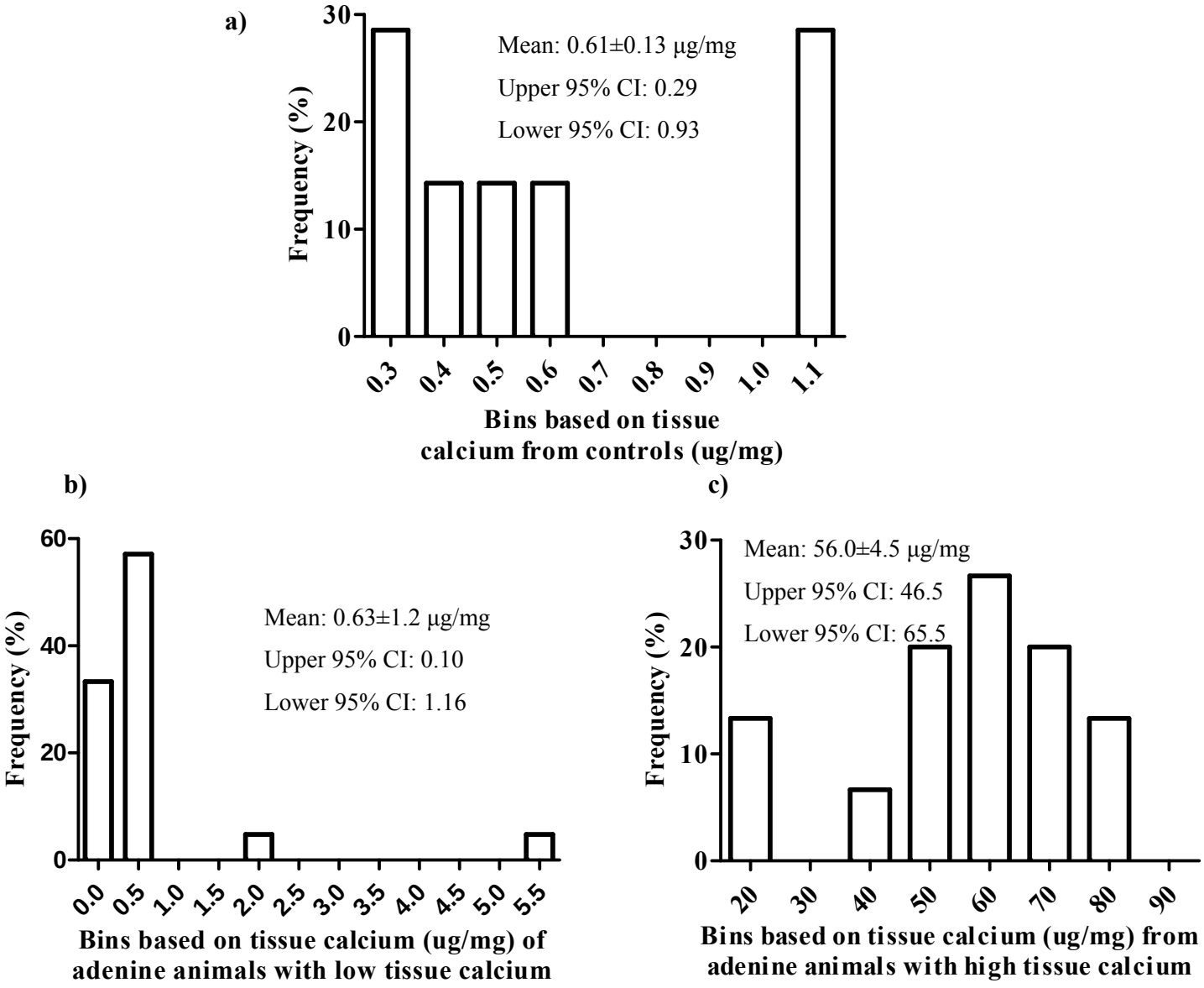


Figure 4.ii – Adenine animals have normal or high tissue calcium. Groups were chosen based on frequency distribution of tissue calcium and confidence intervals (CI). Frequency distribution, mean and CI from control animals (a) and adenine fed (b and c) animals. Based on the distribution from figure 4.i, graphs b and c were generated to compare the distribution, mean and 95% confidence interval of those with low (b) and high (c) tissue calcium. There is no significant difference between the mean from b and controls (a). Therefore groups were chosen as controls (a), adenine fed (CKD; b) and adenine fed with calcification (CKD+VC; c).

Outcomes of the CKD model

i. Overall relationship between CKD and serum biochemistry, vascular calcification, hemodynamics and cardiac weight

Serum creatinine from animals given diet C/D (0.5% n=5 or 0.25% n=22) or control (n=7) was positively correlated with serum urea levels (r^2=0.64) (Figure 4.1A). In addition, there was a significant positive correlation between creatinine and serum phosphorous (Figure 4.1B), but not with serum calcium which remained consistent across all severities of kidney dysfunction (Figure 4.1C). There was also a significant positive correlation between creatinine (and therefore severity of kidney dysfunction) and tissue calcium (Figure 4.1D).

Hemodynamic changes were measured under anesthesia (see methods). There was no correlation between creatinine and pulse wave velocity (r^2 = 0.05). On the other hand, there was a significant negative correlation between serum creatinine and diastolic blood pressure (r^2 = 0.26), a positive correlation with systolic blood pressure (r^2 = 0.29), but no correlation with mean arterial pressure (r^2 = 0.1). As demonstrated in Figure 4.2A, there was a strong correlation between creatinine and pulse pressure (Figure 4.2A), as well as with left ventricular weight, but not right ventricular weight (Figure 4.2B)

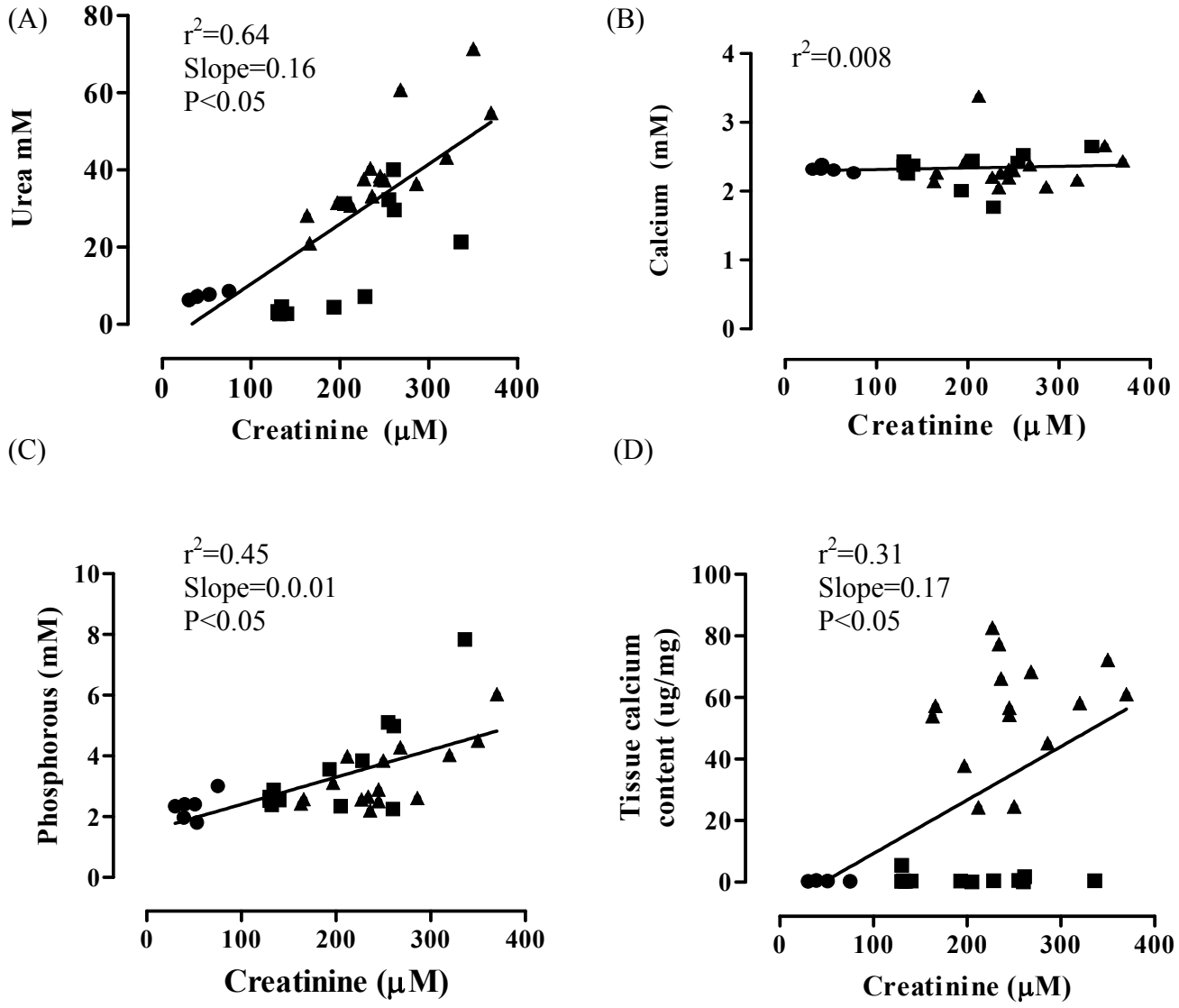
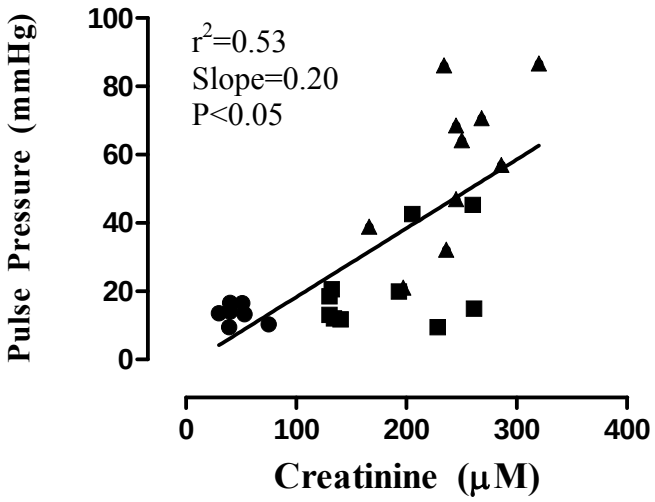


Figure 4.1 – CKD impacts serum phosphorous, calcium and tissue calcium. Relationship between serum creatinine with (A) serum urea, (B) serum calcium, (C) serum phosphorous and (D) aortic calcium content. Circles represent control (n=7), squares are CKD (n=12) animals and triangles are CKD with VC (n=15) animals. There is a positive non-zero ($p<0.05$) linear correlation with urea, phosphorous and aortic calcium, but not with serum calcium. N=34 for all graphs

(A)



(B)

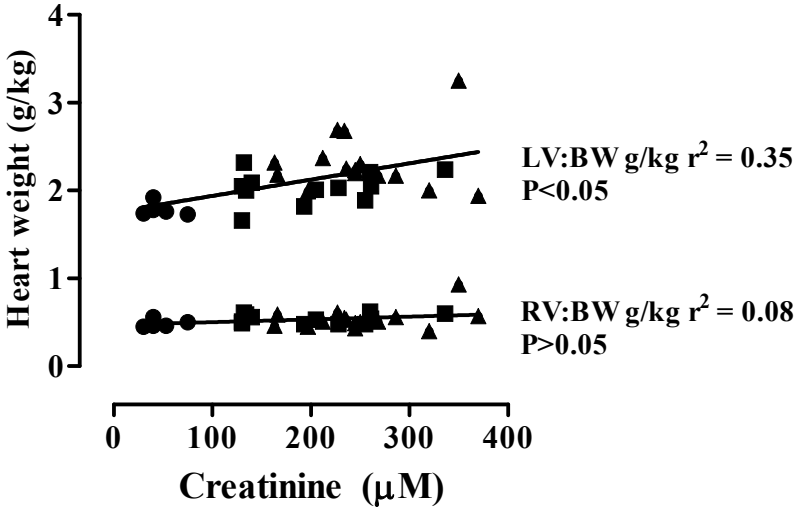
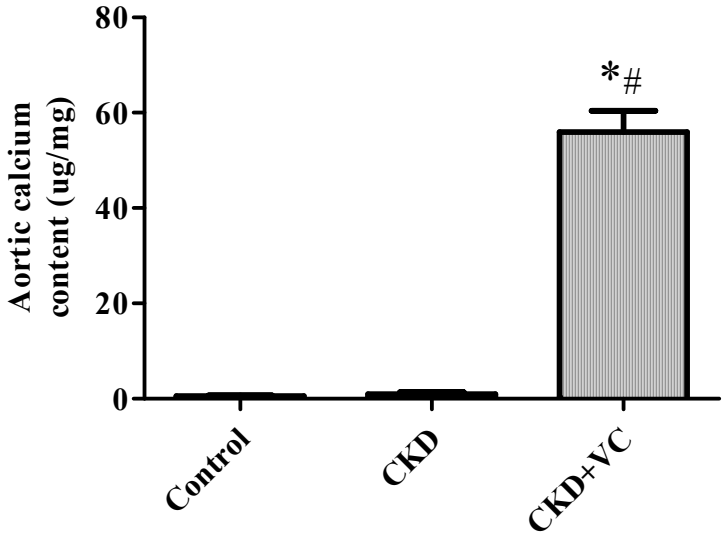


Figure 4.2 – CKD impacts the heart. Relationship between serum creatinine and (A) pulse pressure and (B) cardiac weight. (n=34). Circles represent control (n=7), squares are CKD (n=12) animals and triangles are CKD with VC (n=15) animals. Serum creatinine had a positive significant ($p<0.05$) relationship with pulse pressure (circulatory outcome) and left ventricular weight but not right ventricular weight (cardiac outcome).

ii. Overall outcomes of CKD with vascular calcification

Animals given Diet C or D (0.5% or 0.25% adenine) developed graded CKD as shown in Figure 4.1A. However, as demonstrated in Figure 4.1D, not all CKD animals developed vascular calcification. Figures 4.3 represents control animals (n=8; tissue calcium 0.56±0.34μg/mg), CKD animals which did not develop VC (n=12; tissue calcium 0.94±1.5μg/mg) and CKD animals which did develop VC (n=15; tissue calcium 56.0±17.2μg/mg). Although there was greater than a four-fold increase in serum creatinine in the CKD animals without VC over control animals (mean creatinine 200±69 versus 47±15 μM), CKD animals with VC had worse kidney function than CKD animals without VC (mean creatinine 200±69 versus 251±60; $p<0.005$). Serum phosphorous was measured at an intermediate time point during the study and at the end of the study. There was a 1.5 fold increase in serum phosphorous at the study end in both the CKD without VC group and the CKD with VC group over control, however this increase was not significant (Figure 4.4A). However, at the intermediate time point, serum phosphorous was significantly higher in all CKD groups over control and highest in the CKD with VC group (Figure 4.4B). All groups had similar calcium levels at the intermediate time point and at the end of the study (data not shown) and did not differ significantly from each other (Figure 4.5). As demonstrated in Figure 4.6, there was a very strong correlation between tissue phosphate concentration and tissue calcium concentration ($r^2=0.97$; Figure 4.6).

(A)



(B)

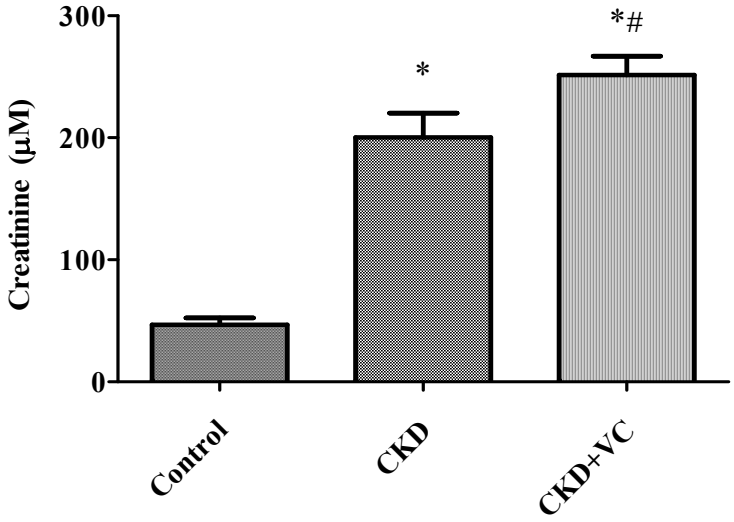
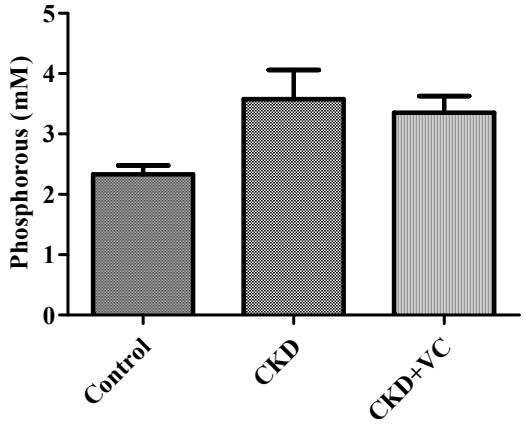


Figure 4.3 – Kidney failure is worse in calcified animals. Thoracic aorta calcium content from control (n=7), CKD (n=12), and CKD with VC (n=15); A. Serum creatinine from the same groups; B. The CKD group which developed VC also had higher serum creatinine levels than CKD or control groups. Data are presented as mean ± SEM
*p< 0.0001 vs control
#p< 0.0001 vs CKD

(A)



(B)

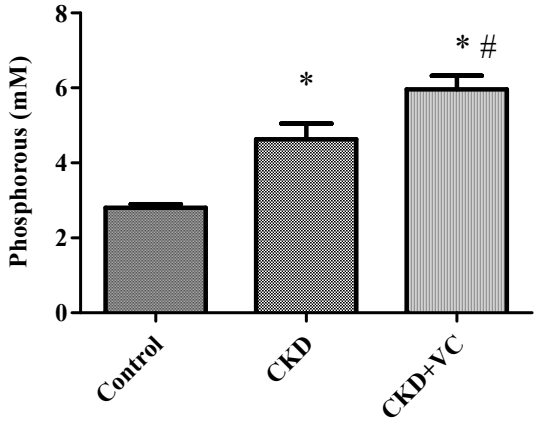


Figure 4.4 – Calcified CKD animals have high early serum phosphorous. Serum phosphorous measured from control (n=7), CKD (n=12), and CKD with vascular calcification (n=15) at the end of the studies (A) and at an intermediate time point during the studies (B). Serum phosphorous was higher in CKD groups at intermediate time point, and highest in CKD+VC group. Data are presented as mean ± SEM.
*$p < 0.0005$ from control
#$p < 0.0005$ from CKD

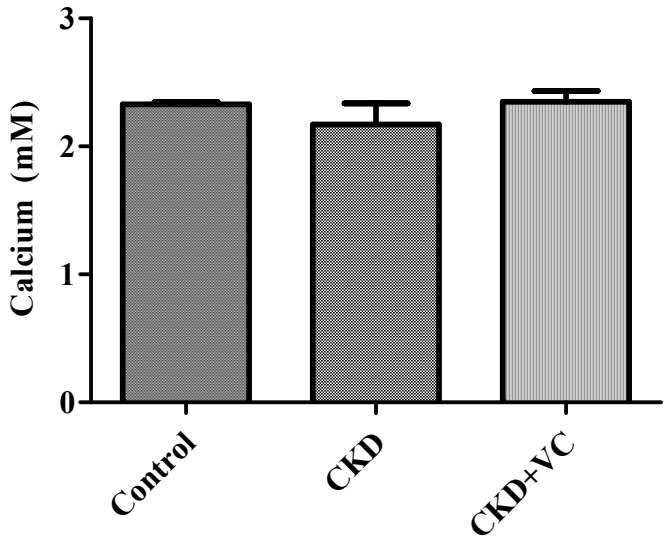


Figure 4.5 – Serum calcium is not different in all animals. Serum calcium measured from control (n=7), CKD (n=12), and CKD with VC (n=15) at the end of the studies. There was no difference between the groups. Data are presented as mean ± SEM.

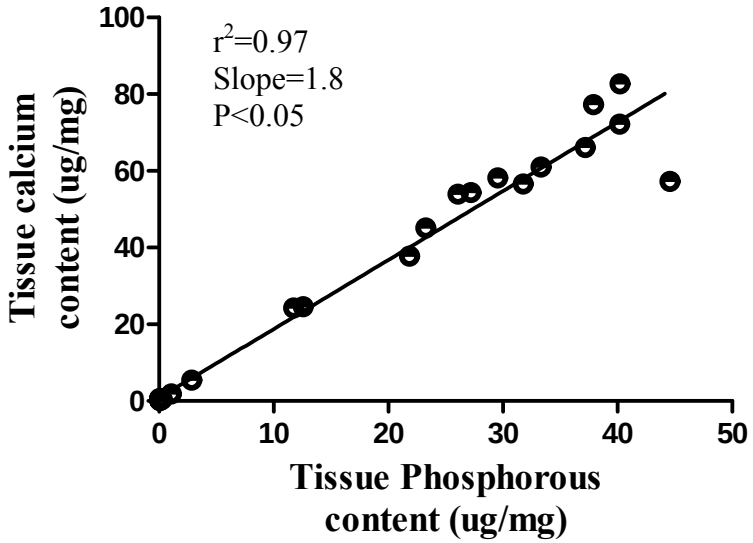


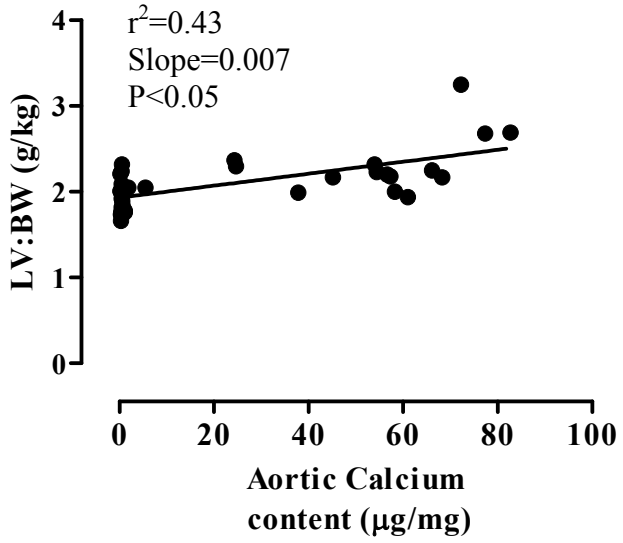
Figure 4.6 – Calcium and phosphorous deposit together in aortic tissue. Relationship between tissue calcium and tissue phosphorous. As shown here, tissue calcium and tissue phosphorous had a high correlation indicating the co-precipitation of both metabolites perhaps in the form of hydorxyapatite. The slope is 1.8 which is close to the Ca:P ratio (by mass) of hydroxyapatite at 2.15. (n=32)

iii. Impact of CKD and vascular calcification on the cardiovascular system.

There was a significant relationship between vascular calcification and left ventricle cardiac weight (Figure 4.7A). Although all CKD animals exhibited significantly increased left ventricle weight over control animals, those CKD animals with vascular calcification had the highest LV weight (Figure 4.7B). Only the CKD group with calcification had significantly higher pulse pressure, lower diastolic BP and higher systolic BP over control animals. (Figure 4.8). The CKD with VC animals also demonstrated elevated PWV (Figure 4.9).

When examining the data by severity of calcification, there was an overall positive relationship between aortic calcium content and pulse pressure (Figure 4.10). Pulse pressure in turn correlated positively with mean arterial pressure (r^2=0.24), systolic blood pressure (r^2= 0.92) and had a negative correlation with diastolic blood pressure (r^2=0.58) (Figures not shown). There was a significant correlation between pulse pressure and left ventricle weight but not with right ventricle weight (Figure 4.11 A&B). Left ventricular weight was associated with higher systolic BP (r^2=0.40), lower diastolic BP (r^2=0.44) but not with mean arterial pressure (r^2=0.04) (Figures not shown).

(A)



(B)

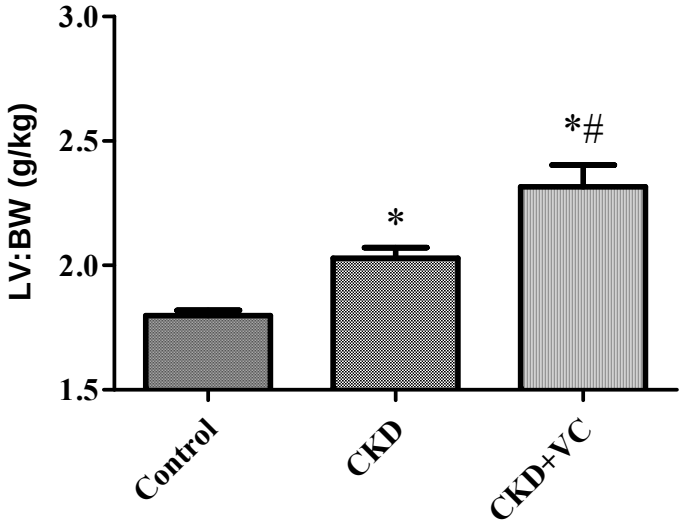
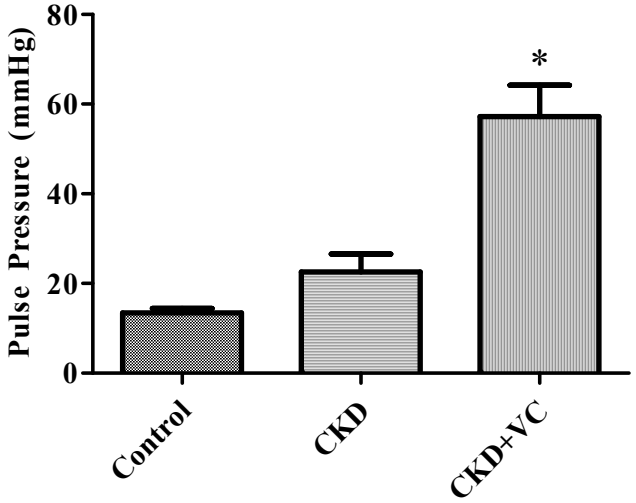
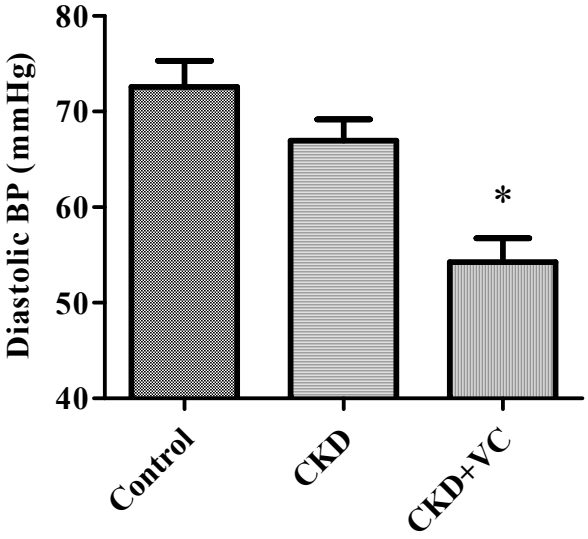


Figure 4.7 – Left ventricular hypertrophy in CKD. There was a positive overall relationship between left ventricle to body weight ratio (LV:BW) and calcification; (A). There was a significant increase in LV weight from CKD (n=12) vs control (n=7), but CKD with VC (n=15) had a significantly elevated LV weight over control and CKD; (B). Data are presented as mean ± SEM.
*p< 0.0001 from control
#p< 0.0001 from CKD

(A)



(B)



(C)

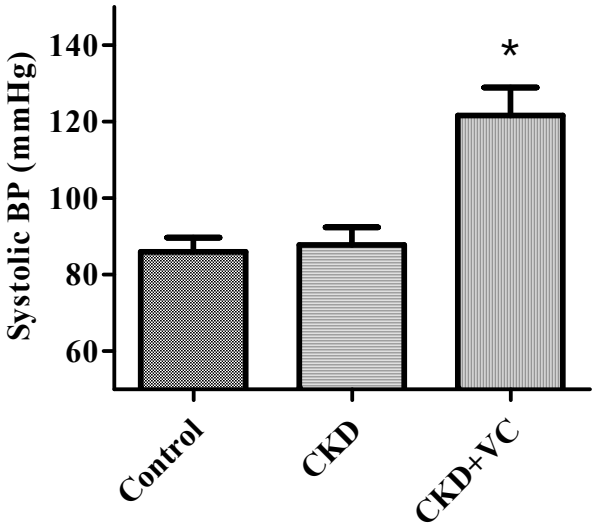


Figure 4.8 – Pulse pressure is widened in calcified CKD animals. Pulse pressure (A), diastolic BP (B) and systolic BP (C) from control (n=7), CKD (n=12), and CKD with VC (n=15). Only the Severe CKD+VC group developed significantly elevated pulse pressure compared to other groups *p< 0.0005 from CKD and control. Data are presented as mean ± SEM.

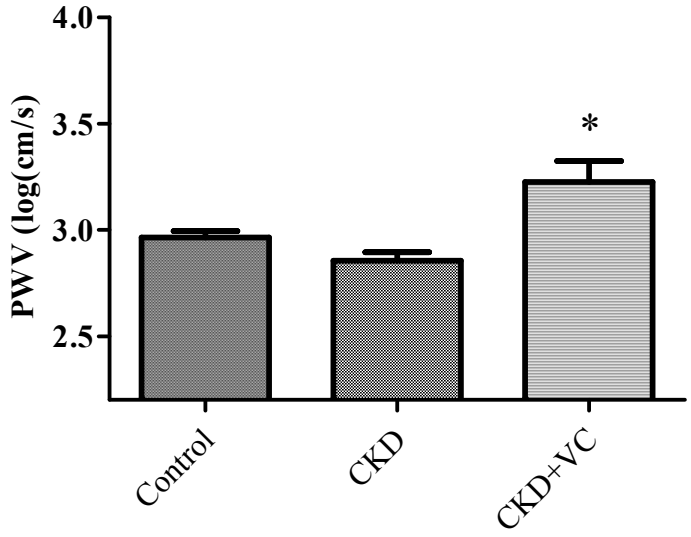


Figure 4.9 – PWV is increased in calcified CKD animals. Log of PWV from control (n=7), CKD (n=12), and CKD with VC (n=15). There was a significant increase in CKD+VC group compared to both CKD and control (*p< 0.0001) indicating vascular stiffness. Data are presented as mean ± SEM.

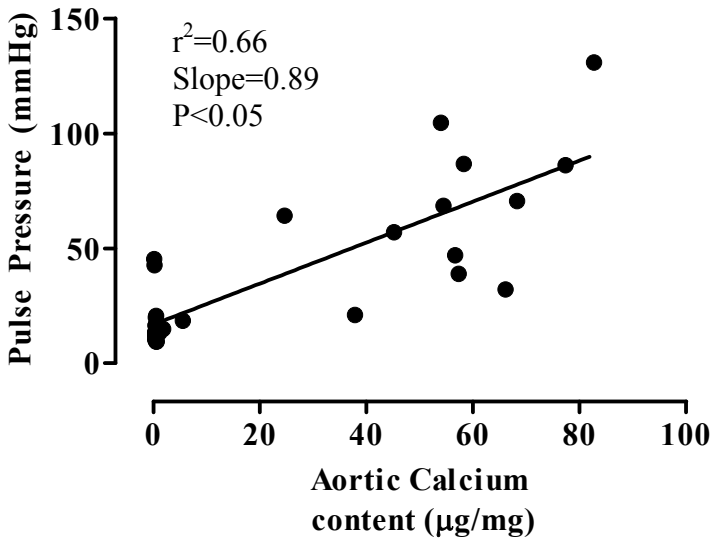
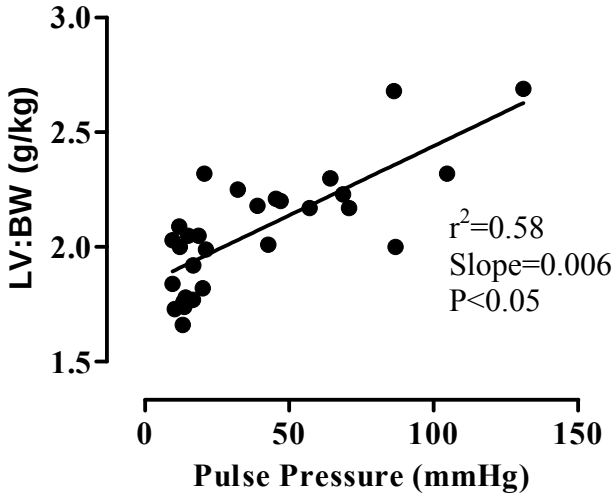


Figure 4.10 – Pulse pressure increases with calcification. Overall relationship between aortic calcium and pulse pressure. There was a positive significant correlation indicating calcification might alter pulse pressure ($p<0.05$)

(A)



(B)

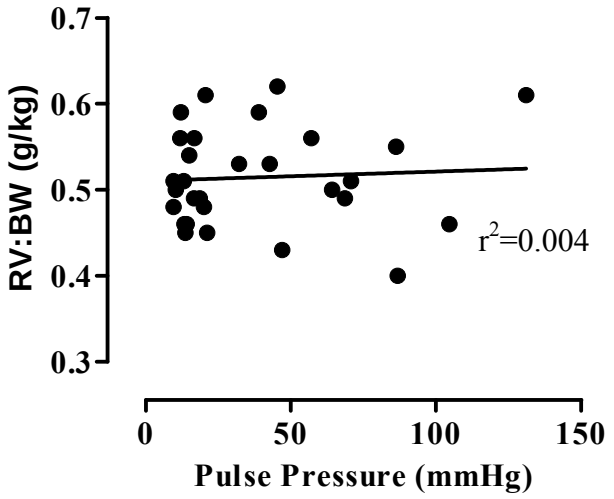


Figure 4.11 – PP correlates with left ventricular weight but not right. Relationship between pulse pressure and heart weight; the left ventricle (A) and right ventricle (B). There was a significant correlation with LV ($p<0.05$), but no correlation with RV weight.

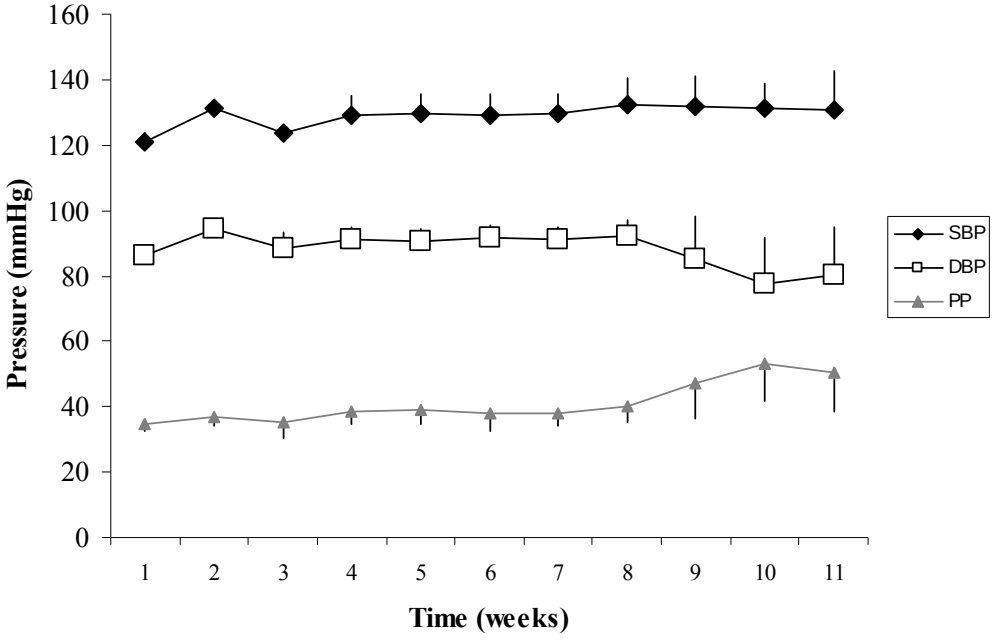
iv. The role of phosphorus in the development of vascular calcification

CKD rats instrumented for radiotelemetry demonstrated increased pulse pressure over time which was caused by a decrease in diastolic blood pressure (Figure 4.12A). As shown in Figure 4.13, these changes were more pronounced in CKD animals with VC and occurred after 8 weeks of CKD. At eleven weeks, once the rats were euthanized, 4 out of 6 rats had extensive aortic calcification (67.1± 15μg/mg versus 0.16± 0.03μg/mg). Figure 4.13 shows individual blood pressure profiles of the 6 animals studied with radiotelemetry. Compared to the 2 non-calcified animals (lower panel), the 4 animals with VC had the most substantial alteration in hemodynamics, which included higher SBP in some, lower diastolic although all of the calcified animals demonstrated increased pulse pressure. The relationship between these hemodynamic changes and serum phosphorus (intermediate time point and study-end) was examined. Prior to an increase in pulse pressure at week 8, serum phosphorus was higher compared to the end of study phosphorus levels at which point PP (i.e.VC) was significantly elevated. (Figure 4.12B)

The relationship between the intermediate time point and end of study measurement of serum phosphorus and VC was examined and these results are demonstrated in Figure 4.14A and B. Figure 4.14A considers CKD animals with and without VC who had end-of-study phosphorus concentrations that were not elevated (Phosphorous ≤ 2.6 mM) nor were they different from control (lightly shaded bars). Of this group only those animals with significantly elevated intermediate time point phosphorus concentrations (black bars) had calcification at the end of the study. Similarly, in those animals with and without calcification which had high end of study phosphate values (≥4 mM), only those animals with elevated intermediate time point phosphorus values calcified. In all groups regardless of end high phosphorous or low phosphorous, only animals which had serum phosphorous concentrations above 5.5mM at the intermediate time

point developed vascular calcification. These animals also had higher serum creatinine values (Figure 4.14).

(A)



(B)

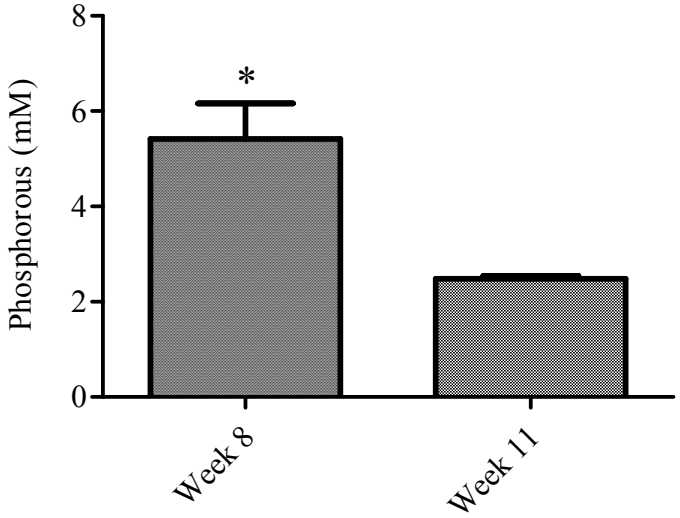


Figure 4.12 – Radiotelemetry indicates an increase in PP due to diastolic drop. Radiotelemetry recordings of systolic, diastolic and pulse pressure of rats given diet D (0.25% adenine) for 11 weeks (A; n=6). Phosphorous levels of the same rats at week 8 were significantly higher than week 11 (B)(*$p<0.0001$). Data are presented as mean ± SEM

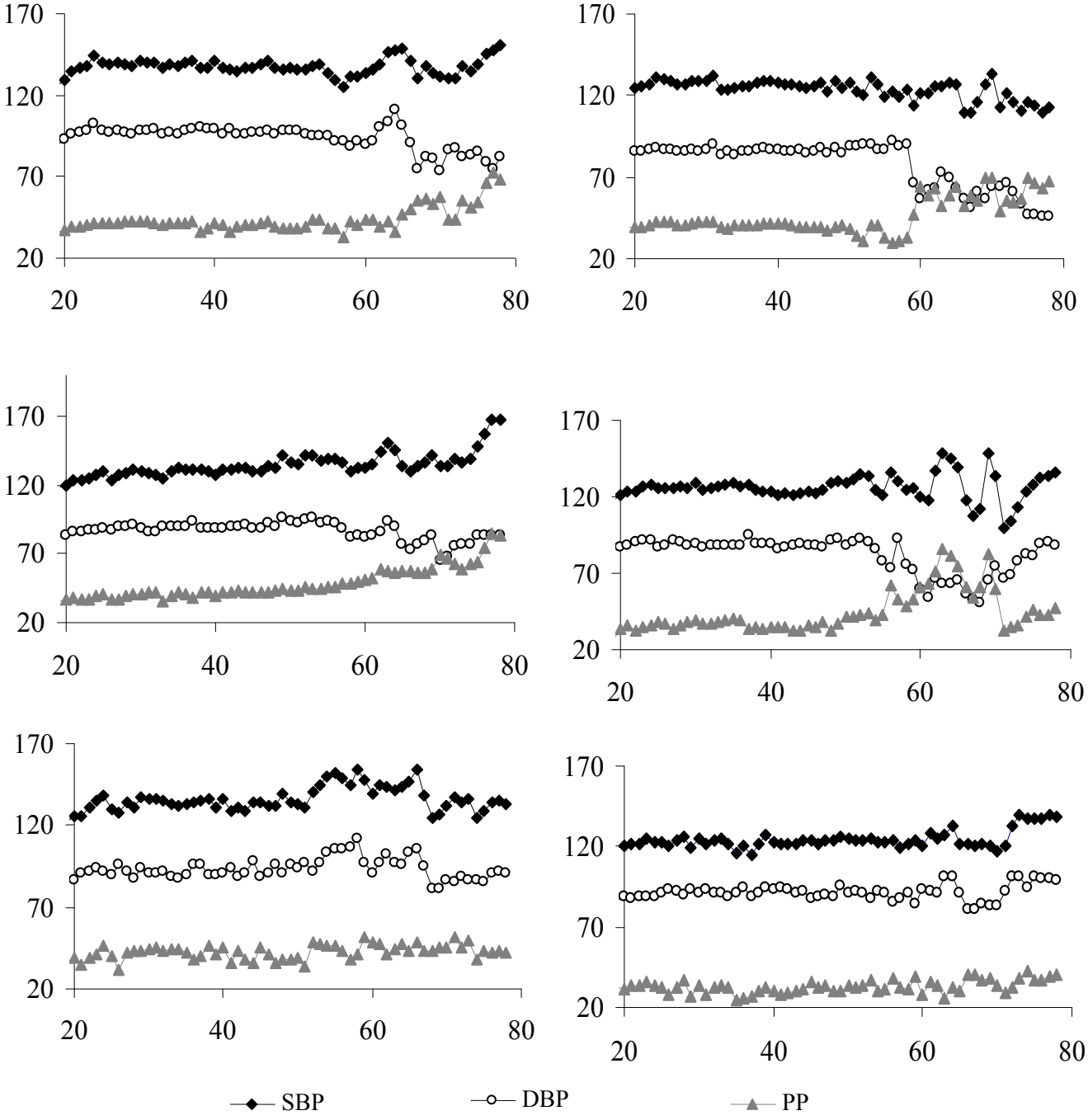


Figure 4.13 – Individual radiotelemetry outcomes correlate with VC. Comparison of individual blood pressure profiles of 6 rats with CKD. The two panels at the bottom did not have any calcification (or elevated PP), but the upper four panels had significant calcification (and elevated PP).

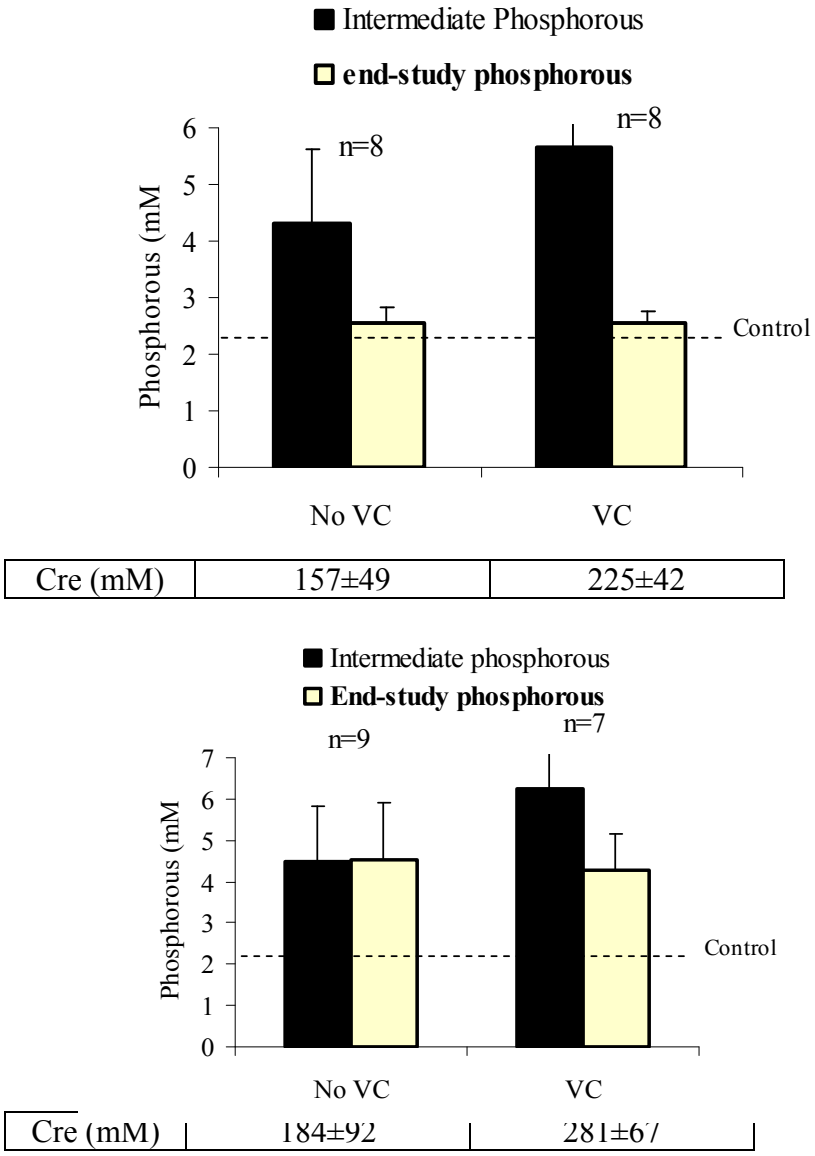


Figure 4.14 – Intermediate but not end serum phosphorous predicts VC. Phosphorous levels divided based on end phosphorous values. Bars side by side are from the same animals, black bar indicates serum phosphorous at an intermediate time and light bars indicated serum phosphorous from same animals at study end. Low phosphorous =< 2.6 mM (based on control values) and high phosphorous >= 4.0 mM. Intermediate phosphorous measured 15 or 20 days before death. Only animals which had significantly higher serum phosphorous at an intermediate time developed calcification ($p<0.05$, VC vs No-VC black bars). In other words, regardless of high or low end serum phosphorous, high intermediate phosphorous predisposed animals to VC.

4.4 Discussion

i) Overall CKD outcomes

The major finding in this study was that chronic kidney disease predisposed SD rats to vascular calcification. SD rats receiving a 0.25% adenine diet generated a variable severity of CKD; some animals developed mild-moderate CKD (creatinine concentrations of 150-250mM) whilst others developed severe CKD (creatinine concentration greater than 300mM). Although there was a linear relationship between the severity of CKD and tissue calcium content, we have also identified that not all CKD animals manifest tissue calcification. CKD animals with elevated tissue calcium content also exhibited histological evidence of vascular calcification within the media of the vessel wall. In the present studies, there was a significant correlation between tissue calcium content and tissue phosphorous content, indicating that calcification was occurring in the form of hydroxyapatite, the main mineral form in bone. Despite the strong association between elevated tissue calcium and elevated tissue phosphorous, only serum phosphorous concentrations (and not calcium) showed an association with severity of CKD suggesting that deregulated phosphorous metabolism may be a key signaling molecule in the extra-osseous calcification process.

Individuals with CKD have markedly increased vascular calcification compared to the general population [84-88]. It is unclear at which point during the time course of CKD that individuals develop vascular calcification. In general terms, it has been suggested that the degree and duration of CKD predisposes patients to VC, despite that it is recognized not all patients (CKD or ESKD) develop VC. We have also found that some animals, despite severe kidney dysfunction, did not exhibit calcification and, in this regard, our model appears to be consistent

with what is observed in humans. Other commonly used CKD models however have not identified that some animals may be relatively protected. For instance, 5/6Nx rats with CKD are reported to demonstrate consistent calcification when additional vitamin D is administered with high dietary phosphorous regardless of severity of CKD [44]. In transgenic mice, genetic predisposition to calcification increases VC six weeks after onset of any degree of kidney damage[67]. Although the etiology of calcification might be multifaceted, these other CKD models, to date have required a dietary or drug manipulation that aids in accelerating VC. The present studies are the first to examine the impact of CKD alone on progression of calcification and these data suggest, similar to what is observed in the human condition, that the severity of CKD is not the only determining factor in VC. In this investigation, most animals with high serum creatinine concentrations demonstrated VC, but there were a few animals that were resistant to vascular calcification even with severe CKD. Seven out of twenty-two rats receiving the adenine diet developed moderate to severe CKD but did not develop VC. This same vascular protective phenomenon is observed in the clinical setting where it is being increasingly recognized that not all dialysis patients develop VC [75, 84, 88]. Indeed there has been calcification inhibitory proteins identified, such as MGP, BMP-7 and fetuin, which prevent the formation of hydroxyapatite in blood vessels and other soft tissues; however, their regulation during the course of CKD is not well understood. On the other hand, promoters of vascular calcification have been identified and their regulation may also be altered during the development of CKD. For instance, deregulated phosphorous balance in CKD and the resulting hyperphosphatemia has been regarded as the main signaling molecule that might initiate the cascade of events leading to vascular calcification. In the present studies, it is not completely clear as to why some animals

with CKD and elevated serum phosphorous did not develop concurrent VC. It is likely that a single measure of serum phosphorus does not reflect the overall phosphorus handling of the kidney. Future studies should therefore be designed to determine phosphorus kinetics at the various stages of CKD and should consider the status of up-regulation of the various VC inhibitors.

ii) Cardiovascular outcomes

The presence of calcification in SD rats was associated with the presence of left ventricular hypertrophy. LVH is the most common cardiac abnormality identified in incident hemodialysis patients.[17, 89, 90]. In our studies, significant predictors of LVH included severity of CKD, tissue calcium content, SBP, DBP and PP.

There are a number of potential mechanisms that could be implicated in the development of LVH in these CKD rats. We identified that the severity of tissue calcification was associated with left ventricular weight. Furthermore, LVH in CKD rats with VC was significantly greater than in control and non-calcifying CKD rats. LVH, therefore, could be an adaptive response to the stiffened aortic vessel which then provides the necessary mechanical adjustment in an attempt to maintain left ventricular function against altered hemodynamics. In 2004, Nitta *et al* reported that LVH in dialysis patients had a strong correlation with VC, PWV and SBP[91]. However, this association has not been demonstrated in animal models of CKD. In the current studies, there were also strong correlations between both LVH and extent of calcification and pulse pressure, SBP as well as DBP. In addition, elevated pulse wave velocity in calcified animals indicated that vascular calcification altered the pulsatile dynamics of blood pressure

presumably via arterial stiffening. These circulatory changes defined by increased SBP and decreased DBP (hence increased PP), may have contributed therefore to increased left ventricular after load causing LVH.

Serum phosphorous levels were elevated in CKD rats with LVH, suggesting that CKD-associated mineral imbalance may play a role. Indeed even within the general population there is emerging evidence that phosphorous, even within the normal range, represents a cardiovascular risk factor. In 2005, Tonelli *et al.* reported that elevated serum phosphorous increased the rate of cardiovascular events in people with coronary disease[92]. More recently, Foley *et al.* (2009) reported that elevated serum phosphorous is a risk factor for LVH in otherwise healthy young adults[93]. The underlying mechanism by which serum phosphorous might cause cardiovascular events in CKD, specifically LVH, is not clearly understood, however it is conceivable that the link between phosphorous metabolism and vascular calcification plays a role. However, there is evidence from our studies that elevated phosphorous might cause LVH via mechanisms which are independent of vascular calcification or increased PWV. For instance, the non-calcifying CKD animals with concurrent hyperphosphatemia had LVH, but they retained relatively normal hemodynamics. Recently, there has been accumulating evidence that a phosphorous-regulating hormone, fibroblast growth factor 23 (FGF-23), is independently associated with LVH[94]. Although we did not measure FGF-23 in the current studies, the CKD rats with hyperphosphatemia had LVH, and those with hyperphosphatemia and concurrent VC had further increase in left ventricular mass. It is possible that the initial increase in LV was mediated via FGF-23, and LV mass increased further with cardiovascular disease and altered hemodynamics. Future studies will be performed in which we measure serum as well as cardiac FGF-23 levels.

Another mechanism which may have caused LVH via phosphorous imbalance is by inducing cardiac valve calcification, leading to a form of heart failure [95]. The mechanism through which phosphorous causes valve calcification in CKD is considered to be similar to VSMC calcification; however we did not measure valve calcification in the CKD rats. Adeney *et al.* (2009) reported in patients with moderate CKD, that an increase of 1 unit in serum phosphorous increased the prevalence of aortic valve calcification by 25% and mitral valve calcification by 62%[22]. The prevalence of cardiac valve calcification in ESKD patients is reported to be quite high. Braun *et al.* reported a valve calcification prevalence of 50% in ESKD patients[85]. Huting found mitral and aortic valve calcification in 44% and 34%, respectively[96]. More recently, Ribeiro *et al.* described a similar prevalence of 44.5% and 52% of mitral and aortic valve calcification, respectively, in dialysed patients[95]. To the best of our knowledge, there is only one publication in which the adenine CKD model was used to assess the development of aortic valve calcification following a 7 week 0.75% adenine diet[97]. In the present studies, the telemetry data supports the suggestion that aortic valve calcification might have been involved, since the increase in pulse pressure appeared to be the result of a drop in diastolic pressure, rather than an increase in systolic pressure. With aortic valve dysfunction, regurgitation of blood during diastole might have caused decreased diastolic blood pressure, and an increase in left ventricular volume overload leading to LVH [98].

Based on the current studies, it is evident that LVH might have multiple etiologies, but whether these act independently or synergistically in CKD still needs to be investigated. Still, the data show that the most calcified animals had the highest levels of LVH, even if it was caused by

multiple mechanisms originating from increased after load, cardiac failure (i.e. valve calcification) or the phosphorous regulating hormone FGF-23.

iii) Mechanisms of VC

There is a growing body of evidence that suggests phosphorous is a prominent signaling molecule in the development of calcification[25, 99]. In the setting of CKD, as GFR falls, phosphorous homeostasis is lost and positive phosphorous balance occurs. Randomized controlled studies in humans with ESKD have shown that reducing serum phosphorous levels with phosphate binders ameliorates the progression of coronary artery calcification when taken over a one-year period[59]. In vitro studies have demonstrated that excess serum phosphorous enters vascular smooth muscle cells via a sodium-phosphorous co-transporter and initiates a cascade of signals that lead to calcification[25]. Although this may be true in vivo as well, not all CKD individuals with elevated serum phosphorous develop vascular calcification and the fate of excess phosphorous in the CKD setting requires further investigation.

In the current studies, longitudinal phosphorous measurements revealed that serum phosphorous measured at mid-study was a better predictor of calcification than the serum phosphorous measured at study end. In the CKD rats, there was a strong relationship between the severity of CKD and serum phosphorous. However aortic calcium content was not associated with serum phosphorus measures at study-end. In other words, final serum phosphorus measures did not identify which animals demonstrated calcification. When we examined the relationship between calcification and phosphorus levels measured at a mid-study time point there were some interesting associations. Calcifying animals with similar degree of kidney failure to non-

calcifying animals demonstrated higher serum phosphorus values earlier on. Regardless of whether end-study phosphorous values were high or normal (compared to control) only the animals with elevated mid-study phosphorous concentrations developed calcification.

Previous CKD rodent models with calcification have reported hyperphosphatemia with elevated phosphorous levels anywhere from 1.2-2.6 folds higher than control to be critical for the development of calcification (see Chapter 2). In the present studies, the calcifying animals had a range of 1.1-2.7 fold elevation in phosphorous (as compared to controls) depending on when the measurement was taken. Given the discordance in serum phosphorous levels and severity of calcification in previous animal models, our results do not conflict with these previous reports and might fill the missing link. In the 5/6 Nx model, only Mizobuchi *et al.* showed remarkably high study-end serum phosphorous (i.e. above 5.5mM) with extensive calcification without the use of vitamin D[65]. In the adenine CKD model, most investigators report hyperphosphatemia. Using the adenine model, Price *et al.*[26] showed that CKD animals with final high serum phosphorous developed calcification. In the current studies, the CKD animals that developed calcification had higher serum phosphorous levels at an earlier time point (similar to those animals from Mizobuchi and Price). The serum phosphorous levels from these same animals at the end of the study were lower but consistent with phosphorus values reported by other studies [46, 53]. Whether this drop reflects the excess phosphorous being driven into soft tissues or whether it reflects lower dietary caloric intake at the time of death is unknown. Our results suggest that early hyperphosphatemia demonstrating a lack of phosphorous handling at an early stage of CKD predisposes animals to soft tissue calcification later on. With this finding, we believe it is important to know the fate of serum phosphorous longitudinally in the course of kidney disease,

and whether a phosphorous threshold exists and is critical for the development of subsequent calcification.

4.5 Conclusions

In the present studies, the variables showing the strongest associations with vascular calcification were serum creatinine, serum urea, tissue calcium content, tissue phosphorous content, PP and LVH. VC measured by tissue calcium content or von Kossa). The presence of tissue calcium and tissue phosphorous confirmed that VC was present whilst the cardiovascular parameters such as widened pulse pressure and LVH suggest a mechanistic link between VC and blood vessel/cardiac function. The current adenine model appears to be a useful representation of many aspects of clinical CKD, with similar co-morbidities. To the best of our knowledge, we are the first to utilize a lower dietary adenine concentration over a longer time period to achieve CKD with calcification, while also maintaining the health of the animal. This modified adenine model with its duration of longer than 4 weeks may represent chronic kidney damage and its associated mineral imbalance more closely [26]. Although VC is a feature of this model, further research is required to elucidate why some animals with high serum creatinine do not develop VC.

The current CKD model also provides a foundation for studying the cardiovascular system in the setting of CKD. Future studies should be designed to elucidate the longitudinal regulation of phosphorous in CKD, with special attention to its impact on the cardiovascular system. Whether normalizing phosphorous in different target tissues in CKD rats will ameliorate cardiovascular disease requires further investigation. Studies will also have to take into

consideration the fate of dietary phosphorous, excess phosphorous released from bones as well as the role of phosphorous regulating hormones such as FGF-23 and parathyroid hormone.

Reference List

(1) Coresh J, Selvin E, Stevens LA et al. Prevalence of chronic kidney disease in the United States. *JAMA* 2007 November 7;298(17):2038-47.

(2) Alebiosu CO, Ayodele OE. The global burden of chronic kidney disease and the way forward. *Ethn Dis* 2005;15(3):418-23.

(3) Mann JF, Gerstein HC, Pogue J, Bosch J, Yusuf S. Renal insufficiency as a predictor of cardiovascular outcomes and the impact of ramipril: the HOPE randomized trial. *Ann Intern Med* 2001 April 17;134(8):629-36.

(4) Sarnak MJ, Levey AS, Schoolwerth AC et al. Kidney disease as a risk factor for development of cardiovascular disease: a statement from the American Heart Association Councils on Kidney in Cardiovascular Disease, High Blood Pressure Research, Clinical Cardiology, and Epidemiology and Prevention. *Circulation* 2003 October 28;108(17):2154-69.

(5) Go AS, Chertow GM, Fan D, McCulloch CE, Hsu CY. Chronic kidney disease and the risks of death, cardiovascular events, and hospitalization. *N Engl J Med* 2004 September 23;351(13):1296-305.

(6) K/DOQI clinical practice guidelines for chronic kidney disease: evaluation, classification, and stratification. *Am J Kidney Dis* 2002 February;39(2 Suppl 1):S1-266.

(7) Sigrist MK, Taal MW, Bungay P, McIntyre CW. Progressive vascular calcification over 2 years is associated with arterial stiffening and increased mortality in patients with stages 4 and 5 chronic kidney disease. *Clin J Am Soc Nephrol* 2007 November;2(6):1241-8.

(8) Raggi P, Kleerekoper M. Contribution of bone and mineral abnormalities to cardiovascular disease in patients with chronic kidney disease. *Clin J Am Soc Nephrol* 2008 May;3(3):836-43.

(9) Ganesh SK, Stack AG, Levin NW, Hulbert-Shearon T, Port FK. Association of elevated serum PO(4), Ca x PO(4) product, and parathyroid hormone with cardiac mortality risk in chronic hemodialysis patients. *J Am Soc Nephrol* 2001 October;12(10):2131-8.

(10) Persy VP, Behets GJ, Bervoets AR, De Broe ME, D'Haese PC. Lanthanum: a safe phosphate binder. *Semin Dial* 2006 May;19(3):195-9.

(11) Moe SM, Drueke T, Lameire N, Eknoyan G. Chronic kidney disease-mineral-bone disorder: a new paradigm. *Adv Chronic Kidney Dis* 2007 January;14(1):3-12.

(12) Foley RN, Parfrey PS, Sarnak MJ. Clinical epidemiology of cardiovascular disease in chronic renal disease. *Am J Kidney Dis* 1998 November;32(5 Suppl 3):S112-S119.

(13) Blacher J, Guerin AP, Pannier B, Marchais SJ, Safar ME, London GM. Impact of aortic stiffness on survival in end-stage renal disease. *Circulation* 1999 May 11;99(18):2434-9.

(14) Blacher J, Guerin AP, Pannier B, Marchais SJ, London GM. Arterial calcifications, arterial stiffness, and cardiovascular risk in end-stage renal disease. *Hypertension* 2001 October;38(4):938-42.

(15) Blacher J, Guerin AP, Pannier B, Marchais SJ, Safar ME, London GM. Impact of aortic stiffness on survival in end-stage renal disease. *Circulation* 1999 May 11;99(18):2434-9.

(16) Darne B, Girerd X, Safar M, Cambien F, Guize L. Pulsatile versus steady component of blood pressure: a cross-sectional analysis and a prospective analysis on cardiovascular mortality. *Hypertension* 1989 April;13(4):392-400.

(17) Silberberg JS, Barre PE, Prichard SS, Sniderman AD. Impact of left ventricular hypertrophy on survival in end-stage renal disease. *Kidney Int* 1989 August;36(2):286-90.

(18) Parfrey PS, Foley RN, Harnett JD, Kent GM, Murray D, Barre PE. Outcome and risk factors of ischemic heart disease in chronic uremia. *Kidney Int* 1996 May;49(5):1428-34.

(19) Blacher J, Pannier B, Guerin AP, Marchais SJ, Safar ME, London GM. Carotid arterial stiffness as a predictor of cardiovascular and all-cause mortality in end-stage renal disease. *Hypertension* 1998 September;32(3):570-4.

(20) Blacher J, Safar ME, Guerin AP, Pannier B, Marchais SJ, London GM. Aortic pulse wave velocity index and mortality in end-stage renal disease. *Kidney Int* 2003 May;63(5):1852-60.

(21) London GM, Marchais SJ, Guerin AP, Metivier F. Arteriosclerosis, vascular calcifications and cardiovascular disease in uremia. *Curr Opin Nephrol Hypertens* 2005 November;14(6):525-31.

(22) Adeney KL, Siscovick DS, Ix JH et al. Association of serum phosphate with vascular and valvular calcification in moderate CKD. *J Am Soc Nephrol* 2009 February;20(2):381-7.

(23) Block GA, Spiegel DM, Ehrlich J et al. Effects of sevelamer and calcium on coronary artery calcification in patients new to hemodialysis. *Kidney Int* 2005 October;68(4):1815-24.

(24) Cozzolino M, Staniforth ME, Liapis H et al. Sevelamer hydrochloride attenuates kidney and cardiovascular calcifications in long-term experimental uremia. *Kidney Int* 2003 November;64(5):1653-61.

(25) Li X, Yang HY, Giachelli CM. Role of the sodium-dependent phosphate cotransporter, Pit-1, in vascular smooth muscle cell calcification. *Circ Res* 2006 April 14;98(7):905-12.

(26) Price PA, Roublick AM, Williamson MK. Artery calcification in uremic rats is increased by a low protein diet and prevented by treatment with ibandronate. *Kidney Int* 2006 November;70(9):1577-83.

(27) Nagano N, Miyata S, Abe M et al. Effect of manipulating serum phosphorus with phosphate binder on circulating PTH and FGF23 in renal failure rats. *Kidney Int* 2006 February;69(3):531-7.

(28) Hujairi NM, Afzali B, Goldsmith DJ. Cardiac calcification in renal patients: what we do and don't know. *Am J Kidney Dis* 2004 February;43(2):234-43.

(29) Proudfoot D, Skepper JN, Hegyi L, Bennett MR, Shanahan CM, Weissberg PL. Apoptosis regulates human vascular calcification in vitro: evidence for initiation of vascular calcification by apoptotic bodies. *Circ Res* 2000 November 24;87(11):1055-62.

(30) Reynolds JL, Joannides AJ, Skepper JN et al. Human vascular smooth muscle cells undergo vesicle-mediated calcification in response to changes in extracellular calcium and phosphate concentrations: a potential mechanism for accelerated vascular calcification in ESRD. *J Am Soc Nephrol* 2004 November;15(11):2857-67.

(31) Shanahan CM, Proudfoot D, Tyson KL, Cary NR, Edmonds M, Weissberg PL. Expression of mineralisation-regulating proteins in association with human vascular calcification. *Z Kardiol* 2000;89 Suppl 2:63-8.

(32) Shroff RC, Shanahan CM. The vascular biology of calcification. *Semin Dial* 2007 March;20(2):103-9.

(33) Bucay N, Sarosi I, Dunstan CR et al. osteoprotegerin-deficient mice develop early onset osteoporosis and arterial calcification. *Genes Dev* 1998 May 1;12(9):1260-8.

(34) Galvin KM, Donovan MJ, Lynch CA et al. A role for smad6 in development and homeostasis of the cardiovascular system. *Nat Genet* 2000 February;24(2):171-4.

(35) Luo G, Ducy P, McKee MD et al. Spontaneous calcification of arteries and cartilage in mice lacking matrix GLA protein. *Nature* 1997 March 6;386(6620):78-81.

(36) Chauntin A, Ferris EB. Experimental renal insufficiency produced by partial nephrectomy. Arch.Int.Med. 49, 767-787. 1932.
Ref Type: Generic

(37) Ejerblad S, Eriksson I, Johansson H. Uraemic arterial disease. An experimental study with special reference to the effect of parathyroidectomy. *Scand J Urol Nephrol* 1979;13(2):161-9.

(38) Gagnon RF, Gallimore B. Characterization of a mouse model of chronic uremia. *Urol Res* 1988;16(2):119-26.

(39) Yokozawa T, Oura H, Okada T. Metabolic effects of dietary purine in rats. *J Nutr Sci Vitaminol (Tokyo)* 1982 October;28(5):519-26.

(40) Cardus A, Panizo S, Parisi E, Fernandez E, Valdivielso JM. Differential effects of vitamin D analogs on vascular calcification. *J Bone Miner Res* 2007 June;22(6):860-6.

(41) Hirata M, Katsumata K, Endo K, Fukushima N, Ohkawa H, Fukagawa M. In subtotally nephrectomized rats 22-oxacalcitriol suppresses parathyroid hormone with less risk of cardiovascular calcification or deterioration of residual renal function than 1,25(OH)2 vitamin D3. *Nephrol Dial Transplant* 2003 September;18(9):1770-6.

(42) Wu-Wong JR, Noonan W, Ma J et al. Role of phosphorus and vitamin D analogs in the pathogenesis of vascular calcification. *J Pharmacol Exp Ther* 2006 July;318(1):90-8.

(43) Lopez I, Mendoza FJ, guilera-Tejero E et al. The effect of calcitriol, paricalcitol, and a calcimimetic on extraosseous calcifications in uremic rats. *Kidney Int* 2008 February;73(3):300-7.

(44) Henley C, Colloton M, Cattley RC et al. 1,25-Dihydroxyvitamin D3 but not cinacalcet HCl (Sensipar/Mimpara) treatment mediates aortic calcification in a rat model of secondary hyperparathyroidism. *Nephrol Dial Transplant* 2005 July;20(7):1370-7.

(45) Tamura K, Suzuki Y, Matsushita M et al. Prevention of aortic calcification by etidronate in the renal failure rat model. *Eur J Pharmacol* 2007 March 8;558(1-3):159-66.

(46) Mendoza FJ, Lopez I, Montes de OA, Perez J, Rodriguez M, guilera-Tejero E. Metabolic acidosis inhibits soft tissue calcification in uremic rats. *Kidney Int* 2008 February;73(4):407-14.

(47) Phan O, Ivanovski O, Nguyen-Khoa T et al. Sevelamer prevents uremia-enhanced atherosclerosis progression in apolipoprotein E-deficient mice. *Circulation* 2005 November 1;112(18):2875-82.

(48) Phan O, Ivanovski O, Nikolov IG et al. Effect of oral calcium carbonate on aortic calcification in apolipoprotein E-deficient (apoE-/-) mice with chronic renal failure. *Nephrol Dial Transplant* 2008 January;23(1):82-90.

(49) Mathew S, Lund RJ, Strebeck F, Tustison KS, Geurs T, Hruska KA. Reversal of the adynamic bone disorder and decreased vascular calcification in chronic kidney disease by sevelamer carbonate therapy. *J Am Soc Nephrol* 2007 January;18(1):122-30.

(50) Chertow GM, Burke SK, Dillon MA, Slatopolsky E. Long-term effects of sevelamer hydrochloride on the calcium x phosphate product and lipid profile of haemodialysis patients. *Nephrol Dial Transplant* 1999 December;14(12):2907-14.

(51) Sadek T, Mazouz H, Bahloul H et al. Sevelamer hydrochloride with or without alphacalcidol or higher dialysate calcium vs calcium carbonate in dialysis patients: an open-label, randomized study. *Nephrol Dial Transplant* 2003 March;18(3):582-8.

(52) Ferramosca E, Burke S, Chasan-Taber S, Ratti C, Chertow GM, Raggi P. Potential antiatherogenic and anti-inflammatory properties of sevelamer in maintenance hemodialysis patients. *Am Heart J* 2005 May;149(5):820-5.

(53) Katsumata K, Kusano K, Hirata M et al. Sevelamer hydrochloride prevents ectopic calcification and renal osteodystrophy in chronic renal failure rats. *Kidney Int* 2003 August;64(2):441-50.

(54) Davies MR, Lund RJ, Hruska KA. BMP-7 is an efficacious treatment of vascular calcification in a murine model of atherosclerosis and chronic renal failure. *J Am Soc Nephrol* 2003 June;14(6):1559-67.

(55) Davies MR, Lund RJ, Mathew S, Hruska KA. Low turnover osteodystrophy and vascular calcification are amenable to skeletal anabolism in an animal model of chronic kidney disease and the metabolic syndrome. *J Am Soc Nephrol* 2005 April;16(4):917-28.

(56) Henley C, Davis J, Miller G et al. The calcimimetic AMG 641 abrogates parathyroid hyperplasia, bone and vascular calcification abnormalities in uremic rats. *Eur J Pharmacol* 2009 August 15;616(1-3):306-13.

(57) Moe SM, Chen NX, Seifert MF et al. A rat model of chronic kidney disease-mineral bone disorder. *Kidney Int* 2009 January;75(2):176-84.

(58) Persy V, Postnov A, Neven E et al. High-resolution X-ray microtomography is a sensitive method to detect vascular calcification in living rats with chronic renal failure. *Arterioscler Thromb Vasc Biol* 2006 September;26(9):2110-6.

(59) Chertow GM, Burke SK, Raggi P. Sevelamer attenuates the progression of coronary and aortic calcification in hemodialysis patients. *Kidney Int* 2002 July;62(1):245-52.

(60) Chertow GM, Pupim LB, Block GA et al. Evaluation of Cinacalcet Therapy to Lower Cardiovascular Events (EVOLVE): rationale and design overview. *Clin J Am Soc Nephrol* 2007 September;2(5):898-905.

(61) E.V.O.L.V.E. Trial: EValuation Of Cinacalcet HCl Therapy to Lower CardioVascular Events. 2009.
Ref Type: Unpublished Work

(62) Lopez I, guilera-Tejero E, Mendoza FJ et al. Calcimimetic R-568 decreases extraosseous calcifications in uremic rats treated with calcitriol. *J Am Soc Nephrol* 2006 March;17(3):795-804.

(63) Hill JA, Goldin JG, Gjertson D et al. Progression of coronary artery calcification in patients taking alendronate for osteoporosis. *Acad Radiol* 2002 October;9(10):1148-52.

(64) Haut LL, Alfrey AC, Guggenheim S, Buddington B, Schrier N. Renal toxicity of phosphate in rats. *Kidney Int* 1980 June;17(6):722-31.

(65) Mizobuchi M, Ogata H, Hatamura I et al. Up-regulation of Cbfa1 and Pit-1 in calcified artery of uraemic rats with severe hyperphosphataemia and secondary hyperparathyroidism. *Nephrol Dial Transplant* 2006 April;21(4):911-6.

(66) Krog M, Ejerblad S, Eriksson I, Johansson H. Arterial calcifications in uraemic rats treated with 1-alpha-hydroxycholecalciferol and parathyroidectomy. *Scand J Urol Nephrol* 1984;18(3):227-39.

(67) Massy ZA, Ivanovski O, Nguyen-Khoa T et al. Uremia accelerates both atherosclerosis and arterial calcification in apolipoprotein E knockout mice. *J Am Soc Nephrol* 2005 January;16(1):109-16.

(68) Ivanovski O, Nikolov IG, Joki N et al. The calcimimetic R-568 retards uremia-enhanced vascular calcification and atherosclerosis in apolipoprotein E deficient (apoE-/-) mice. *Atherosclerosis* 2009 July;205(1):55-62.

(69) Maizel J, Six I, Slama M et al. Mechanisms of aortic and cardiac dysfunction in uremic mice with aortic calcification. *Circulation* 2009 January 20;119(2):306-13.

(70) Tamagaki K, Yuan Q, Ohkawa H et al. Severe hyperparathyroidism with bone abnormalities and metastatic calcification in rats with adenine-induced uraemia. *Nephrol Dial Transplant* 2006 March;21(3):651-9.

(71) Neven E, Dauwe S, De Broe ME, D'Haese PC, Persy V. Endochondral bone formation is involved in media calcification in rats and in men. *Kidney Int* 2007 September;72(5):574-81.

(72) Neven E, Dams G, Postnov A et al. Adequate phosphate binding with lanthanum carbonate attenuates arterial calcification in chronic renal failure rats. *Nephrol Dial Transplant* 2009 June;24(6):1790-9.

(73) Du F, Higginbotham DA, White BD. Food intake, energy balance and serum leptin concentrations in rats fed low-protein diets. *J Nutr* 2000 March;130(3):514-21.

(74) Ivanovski O, Nikolov IG, Joki N et al. The calcimimetic R-568 retards uremia-enhanced vascular calcification and atherosclerosis in apolipoprotein E deficient (apoE(-/-)) mice. *Atherosclerosis* 2008 November 18.

(75) Raggi P, Boulay A, Chasan-Taber S et al. Cardiac calcification in adult hemodialysis patients. A link between end-stage renal disease and cardiovascular disease? *J Am Coll Cardiol* 2002 February 20;39(4):695-701.

(76) Story DL, Shrader R.E., Theriault L.L et al. Effect of dietary protein, adenine, and allopurinol on growth and metabolism of rats. *The Journal of Nutrition* 1976 August 28;1044-52.

(77) Lindblad G., Jonsson G., Fan D. Adenine toxicity: a three week intravenous study in dogs. *Acta pharmacol et toxicol* 1972 August 17;32:246-56.

(78) Yokozawa T, Zheng PD, Oura H, Koizumi F. Animal model of adenine-induced chronic renal failure in rats. *Nephron* 1986;44(3):230-4.

(79) Yokozawa T, Oura H, Koizumi F. 2,8-Dihydroxyadenine urolithiasis induced by dietary adenine in rats. *Nippon Jinzo Gakkai Shi* 1985 March;27(3):371-8.

(80) Yokozawa T, Zheng PD, Oura H. Biochemical features induced by adenine feeding in rats. Polyuria, electrolyte disorders, and 2,8-dihydroxyadenine deposits. *J Nutr Sci Vitaminol (Tokyo)* 1984 June;30(3):245-54.

(81) Koeda T, Wakaki K, Koizumi F, Yokozawa T, Oura H. Early changes of proximal tubules in the kidney of adenine-ingesting rats, with special reference to biochemical and electron microscopic studies. *Nippon Jinzo Gakkai Shi* 1988 March;30(3):239-46.

(82) Hruska KA, Mathew S, Lund R, Qiu P, Pratt R. Hyperphosphatemia of chronic kidney disease. *Kidney Int* 2008 July;74(2):148-57.

(83) Essalihi R, Dao HH, Yamaguchi N, Moreau P. A new model of isolated systolic hypertension induced by chronic warfarin and vitamin K1 treatment. *Am J Hypertens* 2003 February;16(2):103-10.

(84) Kuzela DC, Huffer WE, Conger JD, Winter SD, Hammond WS. Soft tissue calcification in chronic dialysis patients. *Am J Pathol* 1977 February;86(2):403-24.

(85) Braun J, Oldendorf M, Moshage W, Heidler R, Zeitler E, Luft FC. Electron beam computed tomography in the evaluation of cardiac calcification in chronic dialysis patients. *Am J Kidney Dis* 1996 March;27(3):394-401.

(86) Maher ER, Young G, Smyth-Walsh B, Pugh S, Curtis JR. Aortic and mitral valve calcification in patients with end-stage renal disease. *Lancet* 1987 October 17;2(8564):875-7.

(87) Mazzaferro S, Coen G, Bandini S et al. Role of ageing, chronic renal failure and dialysis in the calcification of mitral annulus. *Nephrol Dial Transplant* 1993;8(4):335-40.

(88) Goodman WG, Goldin J, Kuizon BD et al. Coronary-artery calcification in young adults with end-stage renal disease who are undergoing dialysis. *N Engl J Med* 2000 May 18;342(20):1478-83.

(89) Levin A, Singer J, Thompson CR, Ross H, Lewis M. Prevalent left ventricular hypertrophy in the predialysis population: identifying opportunities for intervention. *Am J Kidney Dis* 1996 March;27(3):347-54.

(90) Middleton RJ, Parfrey PS, Foley RN. Left ventricular hypertrophy in the renal patient. *J Am Soc Nephrol* 2001 May;12(5):1079-84.

(91) Nitta K, Akiba T, Uchida K et al. Left ventricular hypertrophy is associated with arterial stiffness and vascular calcification in hemodialysis patients. *Hypertens Res* 2004 January;27(1):47-52.

(92) Tonelli M, Sacks F, Pfeffer M, Gao Z, Curhan G. Relation between serum phosphate level and cardiovascular event rate in people with coronary disease. *Circulation* 2005 October 25;112(17):2627-33.

(93) Foley RN, Collins AJ, Herzog CA, Ishani A, Kalra PA. Serum phosphate and left ventricular hypertrophy in young adults: the coronary artery risk development in young adults study. *Kidney Blood Press Res* 2009;32(1):37-44.

(94) Gutierrez OM, Januzzi JL, Isakova T et al. Fibroblast growth factor 23 and left ventricular hypertrophy in chronic kidney disease. *Circulation* 2009 May 19;119(19):2545-52.

(95) Ribeiro S, Ramos A, Brandao A et al. Cardiac valve calcification in haemodialysis patients: role of calcium-phosphate metabolism. *Nephrol Dial Transplant* 1998 August;13(8):2037-40.

(96) Huting J. Mitral valve calcification as an index of left ventricular dysfunction in patients with end-stage renal disease on peritoneal dialysis. *Chest* 1994 February;105(2):383-8.

(97) Shuvy M, Abedat S, Beeri R et al. Uraemic hyperparathyroidism causes a reversible inflammatory process of aortic valve calcification in rats. *Cardiovasc Res* 2008 August 1;79(3):492-9.

(98) O'Rourke RA, Crawford MH. Mitral valve regurgitation. *Curr Probl Cardiol* 1984 May;9(2):1-52.

(99) Moe SM, Chen NX. Mechanisms of vascular calcification in chronic kidney disease. *J Am Soc Nephrol* 2008 February;19(2):213-6.